AUTOPHAGY

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CANCER, OTHER PATHOLOGIES, INFLAMMATION, IMMUNITY, INFECTION, AND AGING

VOLUME 6

Edited by

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Dedication

To:

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The men and women involved in the odyssey of deciphering the molecular mechanisms underlying the complexity of the autophagy process that governs our lives.

Mitophagy and Biogenesis

mTOR and nutrient sensors control
Autophagy processes in all of our cells;
Dozens of proteins must play each their role
To enable engulfment of bad organelles.

Those who are young may mistakenly think one
Is safe and immune to the dangers of aging
But if you are lacking in proper PINK1
Mitochondrial fires are already raging.

For insight and knowledge some turn to the fly;
Drosophila's genes can help us discover
The causes of aggregates seen in the eye,
And even find drugs to help us recover.

Ubiquitin's role in degeneration
Is to set out red flags on relevant cargo
Marking the junk that needs degradation
At a pace that is presto rather than largo.

Mitochondria fear Parkin known as PARK2
Whose ubiquitin tags on two mitofusins
Determine the fate of one or a slew,
For a lonely short life of network exclusion.

Their fate is ensured by sequestosome 1
Who recruits membranes rich with LC3-II
Autophagosome to lysosome a perfect home run
Cellular housekeeping momentarily through.

But the work isn't over and the job isn't done
Unless Paris is tagged with ubiquitin too
Then repression is lifted from PGC1
So biogenesis starts and mitos renew!

Roberta A. Gottlieb

Life in the Balance, Longevity the Goal
Self-eating, recycling, cash-for-your clunkers:
Trade up to the mitochondrial equivalent Prius.
The road to rejuvenation is paved with destruction
For clearing the rubble precedes reconstruction
But remember that life's circular dance
Depends on opposite forces in balance
Excess destruction, too much biogenesis,
Brings heart failure, cancer or neurodegeneries.

Roberta A. Gottlieb

Autophagy and Cancer

When speaking of cancer, autophagy's good
By culling mitochondria and clearing deadwood
Autophagy limits the radical chain
That breaks DNA and mutates a gene
That makes a cell double, so careless and mean
In order for cells to malignant transform
They lose mitochondria except for a few
Using glycolysis as the source of their fuel
How they achieve mitochondrial decimation
Is nothing more than autophagic elimination

Then one cell is many, an ominous mass
Demanding more glucose, hungry and crass,
Directing formation of artery and vein
'Til capsular fibers give way under strain
Then cancer cells spread so far and so wide
They demand blood vessels the body provide
But until those are patent the tumor cells strive
To rely on autophagy to neatly survive
The hurdles required for metastasis
Until blood flow's established for cancerous bliss.

Blocking autophagy sends them over the brink
And how chloroquine works, we think
But tumors are slowed by statin's effects
Which induce autophagy and tumor cell death
Autophagy's good, autophagy's bad
The confusion's enough to drive us all mad
So study we must, and learn ever more
'Til enlightenment finally opens the door
Oncologists must heed the tumor's agenda
And decide whether autophagy is a friend or foe.

Roberta A. Gottlieb

Foreword

It is with great pleasure that I introduce Volume 6 of the impressive seven-volume series on autophagy edited by M.A. (Eric) Hayat. This volume addresses a number of mechanistic advances in our understanding of the regulation of autophagy, particularly the importance of nutrient availability. Regulatory mechanisms through microRNAs and cross-talk with other protein degradation pathways are presented. Several chapters cover the expanding role of autophagy in host immunity and the ways in which various intracellular pathogens repurpose the pathway for their own benefit. Finally, this volume addresses selective autophagy for degradation of mitochondria and endocytosed gap junctions.

The importance of autophagy in host defense represents an exciting emerging field. Autophagy facilitates antigen presentation, participates in thymic development, and shares many regulatory nodes with innate immunity, including cross-talk with Toll-like receptors, reflecting its important role in regulating the immune response. Autophagy is also a participant in the dynamic struggle between intracellular pathogens and the host. While cells often use autophagy to eliminate intracellular pathogens and to activate innate and adaptive immunity, bacterial and viral pathogens have evolved defensive mechanisms, enabling them to subvert autophagy for their own purposes. As mitochondria can be viewed as domesticated intracellular bacteria, it is not surprising that autophagy plays a significant role in their removal.

The state of current knowledge on these important topics is summarized in the chapters of Volume 6, with contributions from experts from around the world. Researchers in immunology and infectious disease will find this volume to be particularly valuable, as well as those interested in selective autophagy and its regulation.

Roberta A. Gottlieb M.D.
Cedars-Sinai Heart Institute

Preface

It is becoming clear that cancer is an exceedingly complex molecular network, consisting of tumor cells at different stages of differentiation and noncancerous cells from the tumor microenvironment, both of which play a role in sustaining cancer progression. The latter cells maintain a proinflammatory environment conducive to cancer progression through induction of angiogenesis and evasion of the innate immune system. Although induction of cancer cell death by apoptosis, autophagy and necroptosis has been the main system exploited as anticancer strategies, an understanding of the role of the alterations in cellular metabolism is necessary for the development of new, more effective anticancer therapies. For example, it is known that cancer cells switch towards aerobic glycolysis from mitochondrial oxidative phosphorylation.

Autophagy, on the other hand, also possesses mechanisms that can promote cancer cell survival and growth of established tumors. Regarding cell survival, tumor cells themselves activate autophagy in response to cellular stress and/or increased metabolic demands related to rapid cell proliferation. Autophagy-related stress tolerance can enable cell survival by maintaining energy production that can lead to tumor growth and therapeutic resistance. Tumors are often subjected to metabolic stress due to insufficient vascularization. Under these circumstances, autophagy is induced and localized to these hypoxic regions where it supports survival of tumors. Aggressive tumors have increased metabolic demands because of their rapid proliferation and growth. Thus, such tumors show augmented dependency on autophagy for their survival.

Defective autophagy causes abnormal mitochondria accumulation and reduced mitochondrial function in starvation, which is associated with reduced energy output. Because mitochondrial function is required for survival during starvation, autophagy supports cell survival. The recycling of intracellular constituents as a result of their degradation serves as an alternative energy source for tumor survival, especially during periods of metabolic stress. In this context, in tumor cells with defective apoptosis, autophagy allows prolonged survival of tumor cells. However, paradoxically, as mentioned above, autophagy is also associated with antitumorigenesis. Autophagy induced by cancer therapy can also be utilized by cancer cells to obtain nutrients for their growth and proliferation. Therefore, such treatments are counterproductive to therapeutic efficacy.

This is the sixth volume of the seven-volume series, *Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection and Aging*. This series discusses in detail almost all aspects of the autophagy machinery in the context of cancer and certain other pathologies. Emphasis is placed on maintaining homeostasis during starvation or stress conditions by balancing the synthesis of cellular components and their degradation by autophagy.

Both autophagy and ubiquitin-proteasome systems degrade damaged and superfluous proteins. Degradation of intracellular

components through these catabolic pathways results in the liberation of basic building blocks required to maintain cellular energy and homeostasis. However, less than or more than optimal protein degradation can result in human pathologies. An attempt is made in this volume to include information on the extent to which various protein degradation pathways interact, collaborate or antagonize one another.

It is known that conditions resulting in cellular stress (e.g., hypoxia, starvation, pathogen entry) activate autophagy, but dysregulation of autophagy at this stage might result in pathological states including cancer. MicroRNAs are non-protein-coding small RNAs that control levels of transcripts and proteins through post-transcriptional mechanisms. Current knowledge of microRNA regulation of autophagy is presented in this volume.

Autophagy (macroautophagy) is strictly regulated and the second messenger Ca^{+2} regulates starvation-induced autophagy. Withdrawal of essential amino acids increases intracellular Ca^{+2}, leading to the activation of adenosine monophosphate-activated protein kinase and the inhibition of the mTORC1, which eventually results in the enhanced formation of autophagosomes. The importance of this signaling pathway and other pathways (AMPK, AKT) within the autophagy signaling network is emphasized in this volume.

Recent discoveries of autophagic receptors that recognize specific cellular cargo have opened a new chapter in the autophagy field. Receptors are indispensable for the initiation and finalization of specific cargo removal by autophagy. For example, BNIP3L/NIX mediates mitochondrial clearance, which is discussed in this volume. It is pointed out that, in the absence of such clearance, accumulation of ROS can severely damage the mitochondrial population within the neuron and ultimately cause apoptosis of the affected neurons. Mitochondrial dysfunction is implicated in Parkinson's disease. Toll-like receptors (TLRs) play critical roles in host defense by recognizing specific molecular patterns from a wide variety of pathogens. In macrophages, TLR signaling induces autophagy, limiting the replication of intracellular pathogens. How TLRs activate autophagosome formation in macrophages and enhance immunity is discussed in this volume.

Autophagy plays an important role during viral and bacterial infection. Autophagy can act either as a part of the immune defense system or as a pro-viral or pro-bacterial mechanism. In other words, although autophagy suppresses the replication of some viruses, it enhances the replication of others. Several examples of the latter viruses are discussed in this volume. For example, *Herpes viridae* family members encode autophagy-regulating proteins, which contribute to the host antiviral defenses, either by enhancing innate immunity or by helping antigen presentation. Herpes viruses have also evolved proteins that are able to inhibit this cellular mechanism. Positive or negative impact of autophagy on viral infection is explained in this volume.

Another example of the role of a virus in inducing autophagy is varicella-zoster virus (VZV); this human herpes virus causes chickenpox. Infected cells show a large number of autophagosomes and an enlarged endoplasmic reticulum (ER) indicating its stress, which is a precursor to autophagy through the inositol requiring enzyme-1 pathway and PERK pathway. Hepatocellular β virus (HBV) also activates the autophagic pathway while avoiding lysosomal, protein degradation.

As in the case of VZV, ER stress also plays a positive role in HBV replication.

The possible effect of autophagy on HBV-induced hepatocarcinogenesis is also included in this volume. *Staphylococcus aureus* pathogen not only induces an autophagic response in the host cell (localizing in LC3 decorated components), but also benefits from that state.

Although inflammatory responses are essential for eradicating intracellular pathogens and tissue repair, they can be detrimental to the host when uncontrolled. Therefore, inflammation needs to be tightly controlled to prevent excessive inflammation and collateral damage. Cytokine IL-1β (produced by microglia in the CNS) is one of the pro-inflammatory mediators. The pivotal role of autophagy in regulating the production and secretion of the IL-1 family members is explained in this volume. Atg6L1, an essential component of autophagy, suppresses pro-inflammatory signaling. Better understanding of the role of the autophagy-lysosomal pathway in the maturation and secretion of IL-1 should provide a new strategy for targeting inflammation in various pathological conditions.

Excess adiposity contributes to the development of obesity-associated metabolic disturbances such as insulin resistance, type 2 diabetes, or metabolic syndrome. It is pointed out that imbalance between ghrelin (a gut-derived hormone) and tumor necrosis factor in states of insulin resistance may contribute to altered apoptosis and autophagy found in the adipose tissue of patients with type 2 diabetes.

By bringing together a large number of experts (oncologists, physicians, medical research scientists and pathologists) in the field of autophagy, it is my hope that substantial progress will be made against terrible diseases that inflict humans. It is difficult for a single author to discuss effectively and comprehensively various aspects of an exceedingly complex process such as autophagy. Another advantage of involving more than one author is to present different points of view on various controversial aspects of the role of autophagy in health and disease. I hope these goals will be fulfilled in this and future volumes of this series.

This volume was written by 46 contributors representing 11 countries. I am grateful to them for their promptness in accepting my suggestions. Their practical experience highlights the very high quality of their writings, which should build and further the endeavors of the readers in this important medical field. I respect and appreciate the hard work and exceptional insight into the role of autophagy in disease provided by these contributors.

It is my hope that subsequent volumes of this series will join this volume in assisting in the more complete understanding of the complex process of autophagy and eventually in the development of therapeutic applications. There exists a tremendous urgent demand by the public and the scientific community to develop better treatments for major diseases. In the light of the human impact of these untreated diseases, government funding must give priority to researching cures over global military superiority.

I am grateful to Dr. Dawood Farahi and Phillip Connelly for recognizing the importance of medical research and publishing through an institution of higher education. I am thankful to my students for their contributions to the final preparation of this volume.

M. A. Hayat
July 2014

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Abbreviations and Glossary

1AP	inhibitor of apoptosis protein
3-MA	3-methyladenine, an autophagy inhibitor
3-methyladenine	an autophagic inhibitor
5-Fu	5 fluorouracil
AAP	protein that mediates selective autophagy
ACF	aberrant crypt foci
aggrephagy	degradation of ubiquitinated protein aggregates
aggresome	inclusion body where misfolded proteins are confined and degraded by autophagy
AIF	apoptosis-inducing factor
AIM	Atg8-family interacting motif
Akt	protein kinase B regulates autophagy
Alfy	autophagy-linked FYVE protein
ALIS	aggresome-like induced structures
ALR	autophagic lysosome reformation
AMBRA-1	activating molecule in Beclin 1-regulated autophagy
AMP	adenosine monophosphate
amphisome	intermediate compartment formed by fusing an autophagosome with an endosome
AMPK	adenosine monophosphate-activated protein kinase
aPKC	atypical protein kinase C
APMA	autophagic macrophage activation
apoptosis	programmed cell death type 1
ARD1	arrest-defective protein 1
ASK	apoptosis signal regulating kinase
AT1	Atg8-interacting protein
ATF5	activating transcription factor 5
ATF6	activating transcription factor 6
Atg	autophagy-related gene or protein
Atg1	serine/threonine protein 1 kinase
Atg2	protein that functions along with Atg18
Atg3	ubiqitin conjugating enzyme analogue
Atg4	cysteine protease
Atg5	protein containing ubiquitin folds
Atg6	component of the class III PtdIns 3-kinase complex
Atg7	ubiquitin activating enzyme homologue
Atg8	ubiquitin-like protein
Atg9	transmembrane protein

Atg10	ubiquitin conjugating enzyme analogue
Atg11	fungal scaffold protein
Atg12	ubiquitin-like protein
Atg13	component of the Atg1 complex
Atg14	component of the class III PtdIns 3-kinase complex
Atg15	vacuolar protein
Atg16	component of the Atg12-Atg5-Atg16 complex
Atg17	yeast protein
Atg18	protein that binds to PtdIns
Atg19	receptor for the Cvt pathway
Atg20	PtdIns P binding protein
Atg21	PtdIns P binding protein
Atg22	vacuolar amino acid permease
Atg23	yeast protein
Atg24	PtdIns binding protein
Atg25	coiled-coil protein
Atg26	sterol glucosyltransferase
Atg27	integral membrane protein
Atg28	coiled-coil protein
Atg29	protein in fungi
Atg30	protein required for recognizing peroxisomes
Atg31	protein in fungi
Atg32	mitochondrial outer membrane protein
Atg33	mitochondrial outer membrane protein
Atg101	Atg13-binding protein
ATM	ataxia-telangiectasia mutated protein
autolysosome protein	lysosomal associated membrane protein 2
autolysosome	formed by fusion of the autophagosome and lysosome, degrading the engulfed cell components
autophagic body	the inner membrane-bound structure of the autophagosome
autophagic flux	the rate of cargo delivery to lysosomes through autophagy
autophagosome	double-membrane vesicle that engulfs cytoplasmic contents for delivery to the lysosome
autophagosome maturations	events occurring post-autophagosome closure followed by delivery of the cargo to lysosomes
autophagy	programmed cell death type 2
AV	autophagic vacuole
axonopathy	degradation of axons in neurodegeneration
BAD	Bcl-2 associated death promoter protein
Bafilomycin	inhibitor of the vacular-type ATPase
Bafilomycin A1(BAF-A1)	an autophagy inhibitor
BAG	Bcl-2-associated athanogene
BAG3	Bcl-2-associated athanogene 3
BAK	Bcl-2 antagonist/killer
Barkor	Beclin 1-associated autophagy-related key regulator

BATS	Barkor/Atg14(L) autophagosome targeting sequence
BAX	Bcl-2-associated X protein
Bcl-2	B cell lymphoma-2
Beclin 1	mammalian homologue of yeast Atg6, activating macroautophagy
Beclin 1	Bcl-2-interacting protein 1
BH3	Bcl-2 homology domain-3
BH3-only proteins	induce macroautophagy
BHMT	betaine homocysteine methyltransferase protein found in the mammalian autophagosome (metabolic enzyme)
BID	BH3-interacting domain death agonist
Bif-1 protein	interacts with Beclin 1, required for macroautophagy
Bim	Bcl-2 interacting mediator
BNIP	pro-apoptotic protein
BNIP3 protein	required for the HIF-1-dependent induction of macroautophagy
bortezomib	selective proteasome inhibitor
CaMKKβ protein	activates AMPK at increased cytosolic calcium concentration
CaMK	calcium/calmodulin-dependent protein kinase
CASA	chaperone-assisted selective autophagy
caspase	cysteine aspartic acid specific protease
CCI-779	rapamycin ester that induces macroautophagy
CD46 glycoprotein	mediates an immune response to invasive pathogens
chloroquine	an autophagy inhibitor which inhibits fusion between autophagosomes and lysosomes
c-Jun	mammalian transcription factor that inhibits starvation-induced macroautophagy
Clg 1	a yeast cyclin-like protein that induces macroautophagy
CMA	chaperone-mediated autophagy
COG	functions in the fusion of vesicles within the Golgi complex
COP1	coat protein complex1
CP	20S core particle
CRD	cysteine-rich domain
CSC	cancer stem cell
CTGF	connective tissue growth factor
Cvt	cytoplasm-to-vacuole targeting
DAMP	damage-associated molecular pattern molecule/danger-associated molecular pattern molecule
DAP1	death-associated protein 1
DAPK	death-associated protein kinase
DAPK1	death-associated protein kinase 1
DDR	DNA damage response
DEPTOR	DEP domain containing mTOR-interacting protein
DFCP1	a PtdIns (3) P-binding protein
DISC	death-inducing signaling complex

DMV double-membrane vesicle
DOR diabetes- and obesity-regulated gene
DRAM damage-regulated autophagy modulator
DRAM-1 damage-regulated autophagy modulator 1 induces autophagy in a p53-dependent manner.
DRC desmin-related cardiomyopathy
DRiP defective ribosomal protein
DRP1 dynamin-related protein 1
DUB deubiquitinases that accumulate proteins into aggresomes
E2F1 a mammalian transcription factor
efferocytosis phagocytosis of apoptotic cells
EGFR epidermal growth factor receptor
EIF2α eukaryotic initiation factor 2 alpha kinase
endosomes early compartments fuse with autophagosomes to generate amphisomes
ERAA endoplasmic reticulum-activated autophagy
ERAD endoplasmic reticulum-associated degradation pathway
ERK extracellular signal regulated kinase
ERK1/2 extracellular signal regulated kinase 1/2
ERT enzyme replacement therapy
ESCRT endosomal sorting complex required for transport
everolimus mTOR inhibitor
FADD Fas-associated death domain
FKBP12 FK506-binding protein 12
FoxO3 Forkhead box O transcription factor 3
FYCO1 FYVE and coiled domain containing 1
GAA acid α-glucosidase
GABARAP gamma-aminobutyric acid receptor-associated protein
GAS group A streptococcus
GATE-16 Golgi-associated ATPase enhancer of 16 kDa
GFP green fluorescent protein
glycophagy degradation of glycogen particles
GPCR G protein-coupled receptor
GSK-3β glycogen synthase kinase 3 beta regulates macroautophagy
GST-BHMT BHMT fusion protein used to assay macroautophagy in mammalian cells
HAV heavy autophagic vacuole
HCV hepatitis C virus
HDAC histone deacetylase
HDAC6 histone deacetylase 6
HIF hypoxia-inducible factor
HIF1 hypoxia-inducible factor 1
HMGB1 high mobility group box 1
HR-PCD hypersensitive response programmed cell death
Hsc70 heat shock cognate protein

HSP	heat shock protein
Hsp90	heat shock protein 90
HspB8	heat shock cognate protein beta-8
Htraz	high temperature requirement factor Az is a pro-apoptotic protein
I13P	phosphatidylinositol
IAP	inhibitor of apoptosis protein
IKK	inhibitor of nuclear factor κB
IL3	interleukin-3
IM	isolation membrane
inflammasome	an intracellular protein complex that activates caspase-1
IRF	interferon regulatory factor
IRGM	immunity-associated GTPase family M
IRS	insulin receptor substrate
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
KRAS	an oncogene that induces autophagy in cancer cells
LAMP	lysosome-associated membrane protein
LAMP1	lysosome marker, lysosome-associated membrane protein 1
LAMP2	lysosomal-associated membrane protein 2
LAMP-2A	lysosomal-associated membrane protein 2A
LAP	LC3-associated phagocytosis
LAV	light autophagic vacole
LC3 (MAP1LC3B)	autophagosome marker microtubule-associated protein 1 light chain 3B
LC3	microtubule-associated protein light chain 3
LET	linear energy transfer
lipophagy	selective delivery of lipid droplets for lysosomal degradation
LIR	LC3 interacting region
LKB	liver kinase B
LSD	lysosomal storage disorder
lysosomotropic agent	compound that accumulates preferentially in lysosomes
macroautophagy	autophagy
macrolipophagy	regulation of lipid metabolism by autophagy
MALS	macroautophagy–lysosome system
MAPK	mitogen-activated protein kinase
MARF	mitofusion mitochondrial assembly regulatory factor
MCU	mitochondrial calcium uptake uniporter pore
MDC	monodansylcadaverine to measure autophagic flux *in vivo*
MEF	mouse embryonic fibroblast
MFN2	mitofusin 2, a mitochondrial outer membrane protein involved in fusion/fission to promote mitochondrial segregation and elimination
MHC	major histocompatibility complex
MHC-II	major histocompatibility complex class II
MiCa	mitochondrial inner membrane calcium channel

micropexophagy or macropexophagy	peroxisome degradation by autophagic machinery
MIPA	micropexophagy-specific membrane apparatus
mitofusion	mitochondrial fusion-promoting factor
mitophagy	degradation of dysfunctional mitochondria
MOM	mitochondrial outer membrane
MPS	mucopolysaccharide
MPT	mitochondrial permeability transition
mPTP	mitochondrial permeability transition pore
MSD	multiple sulfatase deficiency
MTCO2	mitochondrial marker
MTOC	microtubule organizing center
mTOR	mammalian target of rapamycin, which inhibits autophagy and functions as a sensor for cellular energy and amino acid levels
mTORc1	mammalian target of rapamycin complex 1
MTP	mitochondrial transmembrane potential
MTS	mitochondrial targeting sequence
MVB	multivesicular body
NBR1	neighbor of BRCA1 gene 1
NDP52	nuclear dot protein 52 kDa
NEC-1	necrostatin-1
necroptosis	a form of programmed cell death by activating autophagy-dependent necrosis
Nix	a member of the Bcl-2 family required for mitophagy
NLR	NOD-like receptor
NOD	nucleotide-binding oligomerization domain
NOS	nitric oxide synthase
NOX	NADPH oxidase
Nrf2	nuclear factor 2
OCR	oxygen consumption rate
omegasome	PI(3)P-enriched subdomain of the ER involved in autophagosome formation
OMM	outer mitochondrial membrane
OPA1	mitafusin 1 is required to promote mitochondrial fusion
Ox-LDL	oxidized low density lipoprotein is a major inducer of ROS, inflammation, and injury to endothelial cells
p62	an autophagy substrate
p62/SQSTM1	sequestosome 1
PAMP	pathogen-associated molecular pattern molecule
PAS	pre-autophagosomal structure
PB1 domain	Phox and Bem1 domain
PCD	programmed cell death
PDI	protein disulfide isomerase
PE	phosphatidyl ethanolamine

PERK	protein kinase-like endoplasmic reticulum kinase
PFI	proteasome functional insufficiency
phagophore	a cup-shaped, double membraned autophagic precursor structure
PI(3)K-PKB-FOXO	a growth factor that inhibits autophagy and increases apoptosis by regulating glutamine metabolism
PI3K	phosphatidylinositol 3-kinase
PI3KC3	phosphatidylinositol-3-kinase class III
PINK1	PTEN (phosphatase and tensin homologue deleted on chromosome 10)-induced putative kinase 1
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
polyQ	polyglutamine
PQC	protein quality control
prion disease	transmissible spongiform encephalopathy
PRR	pathogen recognition receptor
PS	phosphatidyl serine
PSMB5	proteasome subunit beta type-5
PtdIns	phosphatidylinositol
PTGS	post-transcriptional gene silencing
PUMA	p53 upregulated modulator of apoptosis
R1G	retrograde signaling pathway
Rag	GTPase that activates TORC1 in response to amino acids
RAGE	receptor for advanced glycation end product
rapamycin	a well-known autophagy inducer by suppressing mTOR
RAPTOR	regulatory-associated of mTOR
RE	recycling endosome
residual body	lysosome containing undegraded material
reticulophagy	degradation of endoplasmic reticulum
ribophagy	degradation of ribosomes
RIP	receptor-interacting protein
RISC	RNA-induced silencing complex
RLS	reactive lipid species
RNAi	RNA interference
RNS	reactive nitrogen species
ROS	reactive oxygen species
ROT	rottlerin used as a protein kinase C-delta inhibitor
RP	19S regulatory particle
Rubicon	RUN domain and cysteine-rich domain-containing Beclin 1-interacting protein
selective autophagy	selective recruitment of substrates for autophagy
sequestosome 1	an autophagy substrate
sequestosome 1 (p62/SQSTM1)	a multifunctional adapter protein implicated in tumorigenesis

sequestosome (SQSTMI)1	p62 protein, a ubiquitin-binding scaffold protein
SESN2	sestrin-2
shRNA	small/short hairpin RNA
siRNA	small interference RNA
sirt 1	sirtuin 1 class III histone deacetylase, prevents Alzheimer's disease
SMIR	small molecule inhibitor of rapamycin
SNARE	soluble N-ethylmaleimide-sensitive factor attachment receptor
SNP	single nucleotide polymorphism
SQSTM1	sequestosome 1
Syt1	synaptotagmin 1
T1DM	type 1 diabetes mellitus
TAKA	transport of Atg9 after knocking-out Atg1
TASCC	TOR-autophagy spatial coupling compartment
TCN	trans-Golgi network
TCR	T cell receptor
TECPR1	tectonin beta-propeller repeat containing 1
tensirolimus	mTOR inhibitor
TFEB	transcript factor EB
TGFβ	transforming growth factor β that activates autophagy
TGN	trans-Golgi network
TIGR	TP53 (tumor protein 53)-induced glycolysis and apoptosis regulator
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TLR	Toll-like receptor
TMD	transmembrane domain
TMEM166	transmembrane protein 166 that induces autophagy
TNF	tumor necrosis factor
TNF-α	tumor necrosis factor alpha
Torin1	ATP-competitive mTOR inhibitor
TRAIL	tumor necrosis factor-regulated apoptosis-inducing ligand
TSC	tuberous sclerosis complex
TSC2	tuberous sclerosis complex 2
TSP	thrombospondin
UBA domain	ubiquitin-associated domain
UBAN	ubiquitin-binding domain
ubiquitin	a small protein that functions in intracellular protein breakdown and histone modification
ubiquitination	a well-established signal for inducing autophagy of protein aggregates
Ubl	ubiquitin-like
ULK	Unc-51-like kinase complex
ULK1	putative mammalian homologue of Atg1p
UPR	unfolded protein response

UPS	ubiquitin–proteasome system
UVRAG	UV-irradiation resistance-associated gene
VAchT	vesicular acetylcholine transporter
VAMP	vesicle-associated membrane protein
VCP/p97	valosin-containing protein involved in endosomal trafficking and autophagy
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VMP1	vacuole membrane protein 1, promotes formation of autophagosomes
VPS15	vacuolar protein sorting 15 homologue
VTA	vascular targeting agent
VTC	vacuolar transporter chaperone
wortmannin	an autophagic inhibitor
XBP1	a component of the ER stress response that activates macroautophagy
xenophagy	degradation of invading bacteria, viruses and parasites
YFP	yellow fluorescent protein
zymophagy	lysosomal degradation of zymogen granules (digestive enzymes)

See also Klionsky, D. J., Codogno, P., Cuervo, A. M. *et al*. (2010). A comprehensive glossary of autophagy-related molecules and processes. *Autophagy* 6, 438–448.

Autophagy: Volume 1 – Contributions

Autophagy: Volume 2 – Contributions

Autophagy: Volume 3 – Contributions

Autophagy: Volume 4 – Contributions

Autophagy: Volume 5 – Contributions

CHAPTER

1

Introduction to Autophagy: Cancer, Other Pathologies, Inflammation, Immunity, Infection, and Aging, Volume 6

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OUTLINE

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Abstract

Autophagy plays a direct or indirect role in health and disease. A simplified definition of autophagy is that it is an exceedingly complex process which degrades modified, superfluous (surplus) or damaged cellular macromolecules and whole organelles using hydrolytic enzymes in the lysosomes. It consists of sequential steps of induction of autophagy, formation of autophagosome precursor, formation of autophagosomes, fusion between autophagosome and lysosome, degradation of cargo contents, efflux transportation of degraded products to the cytoplasm, and lysosome reformation.

This chapter discusses specific functions of autophagy, the process of autophagy, major types of autophagy, influences on autophagy, and the role of autophagy in disease, immunity, and defense.

INTRODUCTION

Aging has so permeated our lives that it cannot be stopped, but it can be delayed. Under the circumstances, time is our only friend. Because the aging process is accompanied by disability and disease (for example, Alzheimer's and Parkinson's conditions) and cannot be prevented, it seems that slow aging is the only way to have a healthy longer life. In general, aging can be slowed down by not smoking or chewing tobacco, by preventing or minimizing perpetual stress (anger, competition), by abstinence from alcoholic beverages, by regular exercise, and by having a healthy diet. There is no doubt that regular physical activity is associated with a reduced risk of mortality and contributes to the primary and secondary prevention of many types of diseases. Discipline is required to attain this goal.

Regarding the role of a healthy diet, a caloric restriction induces autophagy that counteracts the development of age-related diseases and aging itself. On the other hand, autophagy is inhibited by high glucose and insulin-induced P13K signaling via Akt and mTOR. Based on its fundamental roles in these and other disease processes' prevention and therapy, autophagy has emerged as a potential target for disease.

Unfortunately, inevitable death rules our lives, and a group of abnormal cells plays a part in it. Safe disposal of cellular debris is crucial to keep us alive and healthy. Our body uses autophagy and apoptosis as clearing mechanisms to eliminate malfunctioning, aged, damaged, excessive, and/or pathogen-infected cell debris that might otherwise be harmful/autoimmunogenic. However, if such a clearing process becomes uncontrollable, it can instead be deleterious. For example, deficits in protein clearance in brain cells because of dysfunctional autophagy may lead to dementia. Autophagy can also promote cell death through excessive self-digestion and degradation of essential cellular constituents.

Humans and other mammals with long lifespans unfortunately have to face the problem of the accumulation of somatic mutations over time. Although most of the mutations are benign and only some lead to disease, there are too many of them. Cancer is one of these major diseases, and is caused by a combination of somatic genetic alterations in a single cell, followed by uncontrolled cell growth and proliferation. Even a single germline deletion of or mutation in a tumor suppressor gene (e.g., *p53*) predisposes an individual to cancer. It is apparent that nature tries to ensure the longevity of the individual by providing tumor suppressor genes and other protective mechanisms. Autophagy (*Beclin 1* gene) is one of these mechanisms that plays an important role in influencing the aging process.

Autophagy research is in an explosive phase, driven by a relatively new awareness of the enormously significant role it plays in health and disease, including cancer, other pathologies, inflammation, immunity, infection, and aging. The term autophagy (*auto phagin*, from the Greek meaning self-eating) refers to a phenomenon in which cytoplasmic components are delivered to the lysosomes for bulk or selective degradation under the lysosomes' distinct intracellular and extracellular milieu. This term was first coined by de Duve over 46 years ago (Deter and de Duve, 1967), based on the observed degradation of mitochondria and other intracellular structures within lysosomes of rat liver perfused with the pancreatic hormone glucagon.

Over the past two decades an astonishing advance has been made in the understanding of the molecular mechanisms involved in the degradation of intracellular proteins in yeast vacuoles and the lysosomal compartment in mammalian cells. Advances in genome-scale approaches and computational tools have presented opportunities to explore the broader context in which autophagy is regulated at the systems level.

A simplified definition of autophagy is that it is an exceedingly complex process which degrades modified, superfluous (surplus), or damaged cellular macromolecules and whole organelles using hydrolytic enzymes in the lysosomes. Autophagy can be defined in more detail as a regulated process of degradation and recycling of cellular constituents participating in organelle turnover, resulting in the bioenergetic management of starvation. This definition, however, still represents only some of the numerous roles played by the autophagic machinery in mammals; most of the autophagic functions are listed later in this chapter.

Autophagy plays a constitutive and basally active role in the quality control of proteins and organelles, and is associated with either cell survival or cell death. Stress-responsive autophagy can enable adaptation and promote cell survival, whereas in certain models,

autophagy has also been associated with cell death, representing either a failed attempt at survival or a mechanism that supports cell and tissue degradation. Autophagy prevents the accumulation of random molecular damage in long-lived structures, particularly mitochondria, and more generally provides a means to reallocate cellular resources from one biochemical pathway to another. Consequently, it is upregulated in conditions where a cell is responding to stress signals, such as starvation, oxidative stress, and exercise-induced adaptation. The balance between protein and lipid biosynthesis, and their eventual degradation and resynthesis, is one critical component of cellular health.

Degradation and recycling of macromolecules via autophagy provides a source of building blocks (amino acids, fatty acids, sugars) that allow temporal adaptation of cells to adverse conditions. In addition to recycling, autophagy is required for the degradation of damaged or toxic material that can be generated as a result of reactive oxygen species (ROS) accumulation during oxidative stress. The mitochondrial electron transport chain and the peroxisomes are primary sources of ROS production in most eukaryotes.

SPECIFIC FUNCTIONS OF AUTOPHAGY (A SUMMARY)

Autophagy plays a direct or indirect role in health and disease, including, among others, control of embryonic and early postnatal development; tissue homeostasis (protein and cell organelle turnover); mitochondrial quality control; protection of cells from stresses; survival response to nutrient deprivation; cellular survival or physiological cell death during development; involvement in cell death upon treatment with chemotherapy and radiotherapy; tissue remodeling during differentiation and development, including regulation of number of cells and cell size, endocytosed gap junctions, villous trophoblasts, cellular house-cleaning, protein, glucose, and lipid metabolism; supply of energy; anti-aging; human malignancy, tumorigenesis, tumor maintenance, inflammation, cancer (pro and anti), ovarian cancer, nasopharyngeal carcinoma, melanoma, colon cancer, and neutrophil differentiation of acute promyelocytic leukemia; lysosomal storage diseases; metabolic disorders; osteoarthritis; cardiovascular diseases; alcoholic cardiomyopathy, and steatosis in alcoholics (fatty degeneration of the heart); neurodegenerative diseases (Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis, and prion disease); muscular dystrophy; skeletal myopathy; atherosclerosis; diabetes; obesity; lipid degradation in the liver; alcoholic liver disease; pancreatitis; cellular quality control; protection of the genome; innate and adoptive immune responses to infection by microbial pathogens; defense against intracellular bacterial, parasitic, and viral infections; protection of intracellular pathogens; epileptogenesis; Pompe disease; nephropathy; reduction of liver damage during ischemia–reperfusion; regression of the corpus luteum; protection of stem cells from apoptosis during stress; and cross-talk with apoptosis, among other functions. Neonates also adapt to transitive starvation by inducing autophagy.

AUTOPHAGY IN NORMAL MAMMALIAN CELLS

Although autophagy mediates cell adaptation to a range of stress conditions, including starvation, this stress is not a problem that a normal cell of a multicellular organism

would face on a regular basis. The basal level of autophagy (the so-called basal or quality control autophagy) is found in most cells, and is required for the normal clearance of potentially deleterious protein aggregates that can cause cellular dysfunction. Thus, mammalian autophagy is primarily required for intracellular cleaning of misfolded proteins and damaged/old organelles. In the absence of such cleaning, neoplastic transformation is likely.

As alluded to above, starvation is uncommon in mammalian cells under normal nutritional conditions. Therefore, it is important to know the mechanism responsible for regulating autophagy under normal nutritional conditions. In mammalian cells, mTOR kinase, the target of rapamycin, mediates a major inhibitory signal that represses autophagy under nutrient-rich conditions. Calpain 1 keeps autophagy under tight control by downregulating the levels of Atg12–Atg5 conjugate. Atg5 and Atg12–Atg5 conjugate are key signaling molecules for increasing the levels of autophagy (Xia *et al*., 2010). It is also known that intracellular Ca^{2+} regulates autophagy. Inhibition of Ca^{2+} influx results in the induction of autophagy. Reduction in intracellular Ca^{2+} prevents the cleavage of Atg5, which in turn increases the levels of full-length Atg5 and Atg12–Atg5 conjugate. The Atg12–Atg5 signaling molecule is regulated by calpain 1 in controlling the levels of autophagy in mammalian cells under nutrient-rich conditions. It is known that inhibition of calpains induces autophagy, and reduces the accumulation of misfolded proteins. It is further known that increased levels of LC3-II in fluspirilene-treated cells promote autophagy by increasing the levels of Atg5 and Atg12–Atg5 conjugate; fluspirilene is one of the autophagy inducers. Although autophagy is maintained at very low levels in normal mammalian cells, it can be rapidly induced within minutes upon starvation or invasion by intracellular pathogens.

ENDOPLASMIC RETICULUM STRESS AND AUTOPHAGY

All eukaryotic cells contain an endoplasmic reticulum (ER), and its highly convoluted single membrane typically constitutes more than half of the total membrane system of the cell. Ribosomes are attached to the surface of the rough ER membranes, but ribosomes are also found free in the cytosol. These two types of ribosomes are the site of synthesis of different classes of proteins.

ER plays a central role in cell biosynthesis. The synthesis of transmembrane proteins and lipids of the ER, Golgi complex, lysosomes, and plasma membrane begins in association with the ER membrane. Most of the lipids that constitute the membranes of mitochondria and peroxisomes are also contributed by the ER. In addition, all of the newly-synthesized unfolded proteins are first delivered to the ER lumen for refolding before becoming part of the Golgi complex and lysosomes. Disulfide isomerase and chaperone Hsc70 proteins catalyze the refolding. ER is also involved in the synthesis of secreted proteins and formation of the extracellular matrix. Indeed, ER is the center of chaperone proteins that are responsible for correct folding of secreted proteins. In this system, lectin-binding proteins (calreticulin and calnexin) facilitate glycoprotein folding; glucose regulated protein complex is also involved in this system (McLaughlin and Vandenbroeck, 2011).

Another important function of ER, as indicated above, is in the biogenesis of autophagosomes by providing the site for omegasome formation and the source of membrane used.

Double FYVE domain-containing protein 1 (DFCP1) is also located at ER and Golgi membranes instead of endosomes, and is involved in the formation of autophagosomes. This protein contains two FYVE domains, explaining its PI(3)P binding. Ave *et al.* (2008) have exploited the localization and movement of DFCP1 during amino acid starvation for identifying a PI(3)P-enriched compartment dynamically connected to the ER. It was further demonstrated that PI(3)P compartment was formed near the VPS34-containing vesicles that provide a membrane platform for the accumulation of autophagosomal proteins, expansion of autophagosomal membranes, and fully formed autophagosomes.

ER stress can be caused by physiological or pathological processes that disturb protein folding in the ER. Eukaryotic cells are exposed to a large variety of cellular stresses, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein accumulation, and damaged cell organelles. These cells must also adapt to functions in parameters such as temperature, ultraviolet light, ion concentrations, pH, oxygen tension, redox potentials, hormones, cytokines, and neurotransmitters (Kroemer *et al.*, 2010).

The initial and rapid response of cells to the ER stress is the activation of a set of pro-survival signaling pathways called the unfolded protein response (UPR) (Doyle *et al.*, 2011). UPR regulates the protein folding capacity of the ER by sensing the presence of unfolded proteins in the ER lumen, transmitting the information to the cell nucleus, where it drives a transcriptional program focused to reestablish homeostasis (Bernales *et al.*, 2006b). Bernales *et al.* demonstrated that the ER volume increased under UPR-inducing conditions in the yeast. The ER expansion was accompanied by the formation of autophagosomes that packed membranes derived from the UPR-expanded ER. The ER-specific autophagy utilizes autophagy genes. Such genes are activated by the UPR and are essential for the survival of cells exposed to ER stress. Such selective ER sequestration maintains a steady-state level of ER abundance during continuously accumulating unfolded proteins (Bernales *et al.*, 2006b).

UPR also blocks protein synthesis and activates mechanisms that prepare the cell to cope with the aggregated unfolded proteins. One such mechanism involves the enhancement of the protein folding capacity of the ER by increasing the expression of ER chaperone proteins and upregulating the degradation of misfolded proteins (Doyle *et al.*, 2011). However, prolonged or excess ER stress may activate apoptosis. Pro-apoptotic factors (including cytochrome c) are released via the UPR by opening the mitochondrial permeability transmembrane pores. In conjunction with apoptotic protease activating factor 1, pro-caspase 9 and cytochrome c form the apoptosome (Olson and Kornbluth, 2001). The apoptosome is a complex consisting of adaptor proteins that mediate the activation of initiator caspases at the onset of apoptosis.

In conclusion, the development of the UPR protects cells from the deleterious effects of the ER stress. When the ER stress is not removed, it can be lethal or harmful to cells, causing neurodegenerative and cardiovascular diseases, cancer, and diabetes. Overexpression of Bcl-2 also protects cells from ER stress-induced death. Conditions that induce ER stress also induce autophagy. It is well established that autophagy constitutes a major protective mechanism that allows cells to survive in response to multiple stressors, and it helps organisms to defend against degenerative, inflammatory, infectious, and neoplastic disorders. It needs to be noted that ER stress itself is capable of activating autophagy, while impaired autophagy can promote ER stress.

MAJOR TYPES OF AUTOPHAGIES

Based on the type of cargo delivery, there are three types of autophagy systems in mammals – macroautophagy (autophagy), microautophagy, and chaperone-mediated autophagy – each of which is discussed below. Although significant advances (some of which are included here) have been made in our understanding of different types of autophagies, many unanswered questions remain. A further understanding of the exact functions of the three types of autophagy is necessary before we can manipulate these pathways to treat human diseases.

Macroautophagy (Autophagy)

Whole regions of the cytosol are sequestered and delivered to lysosomes for degradation. Cargo sequestration occurs in the autophagosome, a double-membrane vesicle that forms through the elongation and sealing of a *de novo* generated membrane (Ohsumi and Mizushima, 2004). This limiting membrane originates from a tightly controlled series of interactions between more than 10 different proteins which resemble the conjugation steps that mediate protein ubiquitination (Cuervo, 2009). Formation of the limiting membrane also requires the interaction between a protein and a specific lipid molecule, regulated by conjugating enzymes.

Microautophagy

Microautophagy is the direct uptake of soluble or particulate cellular constituents into lysosomes. It translocates cytoplasmic substances into the lysosomes for degradation via direct invagination, protrusion, or septation of the lysosomal limiting membrane. In other words, microautophagy involves direct invagination and fusion of the vacuolar/lysosomal membrane under nutrient limitation. The limiting/sequestering membrane is the lysosomal membrane, which invaginates to form tubules that pinch off into the lysosomal lumen.

Microautophagy of soluble components, as in macroautophagy (autophagy), is induced by nitrogen starvation and rapamycin. Microautophagy is controlled by the TOR and EGO signaling complexes, resulting in direct uptake and degradation of the vacuolar boundary membrane (Uttenweiler *et al.*, 2007). Hence, this process could compensate for the enormous influx of membrane caused by autophagy.

It seems that microautophagy is required for the maintenance of organelle size and membrane composition rather than for cell survival under nutrient restriction. Uttenweiler *et al.* (2007) have identified the vacuolar transporter chaperone, VTC complex, required for microautophagy. This complex is present on the endoplasmic reticulum and vacuoles, and at the cell periphery. Deletion of the VTC complex blocks microautophagic uptake into vacuoles.

Chaperone-Mediated Autophagy

Chaperone-mediated autophagy (CMA) is a generalized form of autophagy present in almost all cell and tissue types. It has been characterized in higher eukaryotes but not in yeast.

Because of the particular characteristics of this type of delivery, explained below, only soluble proteins, but not whole organelles, can be degraded through CMA (Cuervo, 2009). CMA is dependent on the constitutively expressed heat shock cognate 70 (Hsc70), shares 80% homology with the heat shock protein 70 (Hsp70), and identifies peptide sequences of cytoplasmic substrates; thus, it is more selective than autophagy in its degradation (Hoffman *et al.*, 2012). CMA serves to balance dysregulated energy, and is maximally activated by nutrient/metabolic and oxidative/nitrostative stresses. Cross-talk between CMA and autophagy is likely. CMA differs from the other two types of autophagies with respect to the mechanism for cargo selection and delivery to the lysosomal lumen for degradation. In other words, CMA is involved in the delivery of cargo, which does not require the formation of intermediate vesicles, membrane fusion, or membrane deformity of any type. Instead, the substrates are translocated from the cytosol directly into the lysosomal lumen across the membrane in a process mediated by a translocation protein complex that requires the substrate unfolding.

A chaperone protein binds first to its cytosolic target substrate, followed by a receptor on the lysosomal membrane at the site of protein unfolding. This protein is subsequently translocated into the lysosome for its degradation. In this system the substrate proteins are selectively targeted one-by-one to the lysosomes, and are then translocated across the lysosomal membrane. This selectivity and direct lysosomal translocation have thus become trademarks of CMA.

All the CMA substrate proteins are soluble cystolic proteins. An essential requirement for a protein to become a CMA substrate is the presence of a pentapeptide motif, biochemically related to KFERQ in its amino acid sequence (Dice, 1990). The motif present in ~30% of the proteins in the cytosol, is recognized by a cytosolic chaperone, the heat shock cognate protein of 73 kDa (cyt-Hsc70). The interaction with chaperone, modulated by the Hsc70 co-chaperones, targets the substrate to the lysosomal membrane, where it interacts with the lysosomal membrane protein (LAMP) type 2a (Cuervo and Dice, 1996). During CMS, proteins are directly imported into lysosomes via the LAMP-2a transporter assisted by the cytosolic and lysosomal HSC70 chaperone that recognizes the KFERG-like motif. Substrates of CMA carry signal peptides for sorting into lysosomes, similarly to other protein-transport mechanisms across membranes. Substrates are required to be unfolded before translocation into the lysosomal lumen. Several cytosolic chaperones associated with the lysosomal membrane have been proposed, which assist in the unfolding (Aggarraberes and Dice, 2001). Translocation of the substrate requires the presence of a variant of Hsc70, lys-Hsc70, in the lysosomal lumen. This is followed by the rapid proteolysis of the substrate by residual lysosomal proteases (half-life of 5–10 minutes in the lysosomal lumen).

AUTOPHAGOSOME FORMATION

Autophagy is a highly complex process consisting of sequential steps of induction of autophagy, formation of autophagosome precursor, formation of autophagosomes, fusion between autophagosome and lysosome, degradation of cargo contents, efflux transportation of degraded products to the cytoplasm, and lysosome reformation.

In mammalian cells autophagosome formation begins with a nucleation step, where isolation membranes of varied origins form phagophores which then expand and fuse to form

a completed double-membrane vesicle called an autophagosome (Luo and Rubinsztein, 2010). Autophagosomes are formed at random sites in the cytoplasm. They move along microtubules in a dynein-dependent fashion toward the microtubule-organizing center, where they encounter lysosomes. After fusion with lysosomes the cargo is degraded with hydrolases, followed by the reformation of lysosomes primarily by the Golgi complex.

The isolation membranes may be generated from multiple sources that include endoplasmic reticulum (ER), Golgi complex, outer mitochondrial membrane, and plasma membrane; however, the ER source is more feasible because it, along with its ribosomes, is involved in protein synthesis. The presence of many Atg proteins near the ER also suggests that ER plays an important role as a membrane source for autophagosome formation. The formation of isolation membrane is initiated by class III phosphatidylinositol 3-kinase (PI3KC)/Beclin 1-containing complexes. Elongation of the isolation membrane involves two ubiquitin-like conjugation systems. In one of them, Atg12 associates with Atg5 to form Atg12–Atg5–Atg16L1 molecular complexes that bind the outer membrane of the isolation membrane. In the second, lipidated microtubule-associated light chain 3 (LC3) is conjugated to phosphatidylethanolamine to generate a lipidated LC3-II form, which is integrated in both the outer and inner membranes of the autophagosome (Fujita *et al.*, 2008). Recently, it was reported that human Atg2 homologues Atg2A and AtgB are also essential for autophagosome formation, presumably at a late stage (Velikkakath *et al.*, 2012).

Autophagosome membrane formation requires autophagy-related proteins (Atgs) along with the insertion of lipidated microtubule-associated light chain 3 (LC3) or gamma-aminobutyric acid A receptor-associated protein (GABARAP) subfamily members. Various components in the autophagosomal compartment can be recognized by the presence of specific autophagy molecules. Atg16L1 and Atg5 are mainly present in the phagophore, while LC3 labels isolation membranes, matured autophagosomes, and autolysosomes (Gao *et al.*, 2010). This evidence suggests that different Atg molecules participate in autophagosome biogenesis at various stages. Autophagosome substrate selectivity can be conferred by interactions between LC3 and specific cargo receptors, including sequestosome-1 (SQSTM1 #p62) and a neighbor of BRCA1 (NBR1). During this process of autophagy, both lipidated LC3 (LC3-II) and the cargo receptors are degraded (Hocking *et al.*, 2012).

In yeast, the Atg5–Atg12/Atg16 complex is essential for autophagosome formation (Romanov *et al.*, 2012). This complex directly binds membranes. Membrane binding is mediated by Atg5, inhibited by Atg12, and activated by Atg16. All components of this complex are required for efficient promotion of Atg8 conjugation to phosphatidylethanolamine. However, this complex is able to tether (fasten) membranes independently of Atg8.

AUTOPHAGIC LYSOSOME REFORMATION

Following degradation of engulfed substrates with lysosomal hydrolytic enzymes and release of the resulting molecules (amino acids, fatty acids, monosaccharides, nucleotides), autophagic lysosome reformation (ALR) occurs. Although a great deal is known regarding the molecular mechanisms involved in the formation of autophagosomes and autolysosomes, the available information on post-degradation events, including ALR, is inadequate. The importance of such information becomes apparent considering that autophagosomes can

fuse with multiple lysosomes. Thus, post-degradation of substrates might result in the depletion of free lysosomes within a cell unless free lysosomes are rapidly reformed. A cellular mechanism is required for maintaining lysosome homeostasis during and after autophagy.

Some information is available at the molecular level regarding the process of ALR. The ALR process can be divided into six steps (Chen and Yu, 2012): phospholipid conversion, cargo sorting, autophagosomal membrane budding, tubule extension, budding and fusion of vesicles, and protolysosome maturation. Initially, LAMP1-positive tubular structures extend from the autolysosomes; these appear empty, without detectable luminal contents from the autolysosomes. Lysosomal membrane proteins (LAMP1, LAMP2) only are located on these tubules; autophagosomal membrane proteins (LC3) are absent.

The role of mTOR is also relevant in ALR. It has been found that the starvation-induced autophagy process is transient. During starvation, intracellular mTOR is inhibited before autophagy can occur, but it is reactivated after prolonged starvation, and the timing of this reactivation is correlated with the initiation of ALR and termination of autophagy (Chen and Yu, 2012). Thus, mTOR reactivation is required for ALR. ALR is blocked when mTOR is inhibited, and mTOR reactivation is linked to lysosomal degradation.

The lysosomal efflux transporter spinster is also required to trigger ALR (Rong *et al.*, 2011); these transporters are lysosomal membrane proteins that export lysosomal degradation products. Sugar transporter activity of spinster is essential for ALR. Inhibition of spinster results in the accumulation of a large amount of undigested cytosol in enlarged autolysosomes, seen in the transmission electron microscope, as a result of over-acidification of autolysosomes (Rong *et al.*, 2011).

Clathrin is also essential for ALR. It is known that clathrin proteins play an important role in vesicular trafficking (Brodsky, 1988). Clathrin mediates budding in various membrane systems. A clathrin-PI (4,5) P2-centered pathway regulates ALR. This protein is present on autolysosomes, with exclusive enrichment on buds. Clathrin itself cannot directly anchor to membranes; instead, various adapter proteins (AP2) link clathrin to membranes. Additional studies are needed to fully understand the terminal stage of autophagy, and how this process ends in the reformation of free lysosomes.

AUTOPHAGIC PROTEINS

Cells assure the renewal of their constituent proteins through a continuous process of synthesis and degradation that also allows for rapid modulation of the levels of specific proteins to accommodate the changing extracellular environment. Intracellular protein degradation is also essential for cellular quality control to eliminate damaged or altered proteins, thus preventing the toxicity associated with their accumulation inside cells.

Autophagy essential proteins are the molecular basis of protective or destructive autophagy machinery. Some information is available regarding the signaling mechanisms governing these proteins and the opposing consequences of autophagy in mammals. Genes responsible for the synthesis of these proteins are summarized here.

Autophagy was first genetically defined in yeast, where 31 genes, referred to as autophagy-related genes (ATGs), were identified as being directly involved in the execution of autophagy (Mizushima, 2007; Xie and Klionsky, 2007). At least 16 members of this gene

family have been identified in humans. The role of a large number of these genes has been deciphered. Our understanding of the molecular regulation of the autophagy process originates from the characterization of these genes and proteins in yeast, many of which have counterparts in mammals. The core autophagic machinery comprises 18 Atg proteins, which represent three functional and structural units: (1) the Atg9 cycling system (Atg9, Atg1 kinase complex [Atg1 and Atg13], Atg2, Atg18, and Atg27); (2) the phosphatidylinositol 3-kinase (PI3K) complex (Atg6/VPS30), Atg14, VPS15, and VPS34; and (3) the ubiquitin-like protein system (Atg3–5, Atg7, Atg8, Atg10, Atg12, and Atg16) (Minibayeva *et al.*, 2012). In addition to these core Atg proteins, 16 other proteins are essential for certain pathways or in different species.

An alternate abbreviated system of Atg proteins follows. Autophagic proteins generally function in four major groups: the Atg1 kinase complex, the VPS34 class III phosphatidylinositol 3-kinase complex, two ubiquitin-like conjugation systems involving Atg8 and Atg12, and a membrane-trafficking complex involving Atg9 (Florey and Overholtzer, 2012). In mammalian cells, the key upstream kinase that regulates the induction of most forms of autophagy is the Atg1 homologue ULK1, which forms a complex with Atg13, FIP200, and Atg101. Among the Atg proteins, Atg9 is the only multispanning membrane protein essential for autophagosome formation.

It needs to be noted that autophagy proteins are also involved in non-autophagic functions such as cell survival, apoptosis, modulation of cellular traffic, protein secretion, cell signaling, transcription, translation, and membrane reorganization (Subramani and Malhotra, 2013). This subject is discussed in detail later in this chapter.

Abnormal Proteins

Intracellular proteins are subjected to continuous turnover through coordinated synthesis, degradation, and recycling of their component amino acids. Proteins can undergo degradation by the proteasome or by lysosomes. Proteins are degraded by macroautophagy, microautophagy or chaperone-mediated autophagy (CMA). CMA is especially efficient in the degradation of damaged or abnormal proteins, fulfilling its role in quality control. However, proteolytic systems in certain cases fail to adequately dispose of deleterious proteins, which results in protein aggregation and neuronal demise, causing neurodegenerative diseases.

The presence of unfolded or misfolded proteins in cells in not uncommon. It is estimated that approximately 30% of newly-synthesized proteins are unfolded or incorrectly folded. It appears that protein folding is an exceedingly complex process because the transition from a linear sequence of amino acids to a correctly fully-folded, three-dimensionally active protein requires at least favorable physiological environment and assistance from other biological molecules. It is known, for example, that low molecular weight chemical chaperones stabilize a protein as it folds into the proper structural form (Ferreon *et al.*, 2012).

In order to understand the damage (e.g., Alzheimer's disease) caused by the accumulation of unfolded or misfolded proteins, it is important to identify and measure the quantity of such proteins. It is relevant to determine the amount of misfolded proteins that cause cell damage or cell death. One method to visualize the interplay between fully folded and unfolded forms of proteins is by using a designed fluorescent tagged small molecule

(folding probe) (Liu *et al*., 2014). This probe specifically binds to the folded, functional protein, but not to misfolded forms of the protein. Thus, the quantification method can determine the comparative amount of folded proteins versus misfolded proteins in a cell.

In most cases, autophagy is able to degrade misfolded proteins. Information to correct protein misfolding is available. In certain cases, specific molecules (pharmacoperones) can correct protein misfolding in cells. An example of such therapeutic effect was reported by Janovick *et al*. (2013). They reported the rescue and expression of a misfolded G-protein coupled receptor (hormone), that contained a single amino acid change; a negatively-charged glutamic acid was substituted by a positively-charged lysine. This modification resulted in the misfolding and misrouting of the gonadal protein (GnRHR). By using 1N3 (a small molecule), they accomplished proper folding of the misfolded protein and restored normal gonodal function in the mutant mice. The normal function resulted from correct routing of the protein to the plasma membrane instead of it routing to the endoplasmic reticulum. It also became clear that misfolded protein was forming oligomers with wild type GnRHR protein, effectively rendering the latter useless and becoming a target for the cell's quality control machinery. It is concluded that small molecules (e.g., 1N3) can be tried for the treatment of genetic diseases associated with misfolded proteins.

A different type of autophagy protein, intrinsically disordered or unstructured proteins, is discussed below. Some autophagy proteins have intrinsically disordered regions (IDRs) and are called IDRPs. They are predicted to be in approximately 30% of the prokaryotic proteins and approximately 47% of eukaryotic proteins (Dunker *et al*., 2008). IDRPs have negligible folded tertiary structure or stable secondary structure elements such as α-helix and β-sheets. The importance of the IDRs in cellular processes has so far been overlooked, as biological roles and mechanisms of most of these regions are poorly understood. These regions play an important role in autophagy, and this role has not been adequately investigated.

In contrast to Atgs, IDRPs are poorly conserved. IDRs seem to have diverse functions in different homologues. Recent studies indicate that IDRs facilitate protein–protein interactions (Mei *et al*., 2013). The importance of this role becomes apparent when one considers that many or even most Atgs function via formation of multi-protein complexes. These complexes initiate autophagy initiation, autophagosome nucleation, and autophagosome expansion, maturation, and fusion with lysosomes.

Potential protein partners that might interact with the disordered regions have been identified (Mei *et al*., 2013). For example, a BCL2 homology 3 domain (BH3D) (within the key autophagy Beclin 1 protein) is an IDR. BH3D undergoes a conformational change from coil to α-helix upon binding to BCL2. The C-terminal half of this BH3D constitutes the binding motif, which serves to anchor the interaction of the BH3D to BCL2. Finally, the high preponderance of IDRs in autophagy proteins implies that these regions play a significant role in the autophagic functions. It needs to be noted that mutations implicated in major diseases, including cancer and neurodegenerative and cardiovascular disorders, map to IDRs (Uversky *et al*., 2008).

Protein Degradation Systems

There are two major protein degradation pathways in eukaryotic cells: the ubiquitin–proteasome system and the autophagy–lysosome system. Both of these systems are

characterized by selective degradation. The ubiquitin–proteasome system (UPS) is responsible for degradation of short-lived proteins, and is involved in the regulation of various cellular signaling pathways. Autophagy is a regulatory mechanism for degrading large proteins with longer half-lives, aggregates, and defective cellular organelles. Ubiquitin binding proteins such as p62 and NBR1 regulate autophagy dynamics. These adaptor proteins decide the fate of protein degradation through either UPS or the autophagy–lysosome pathway. Many degenerative conditions, such as Huntington's, Parkinson's, Alzheimer's, amyotrophic lateral sclerosis, and diabetes, are due to defective clearance of mutated protein aggregates or defective organelles through autophagy.

Beclin 1

Beclin 1 (from Bcl-2 interacting protein) is a 60-kDa coiled-coil protein that contains a Bcl-2 homology-3 domain, a central coiled-coil domain, and an evolutionary conserved domain. Beclin 1 was originally discovered not as an autophagy protein but as an interaction partner for the anti-apoptotic protein Bcl-2. The function of Beclin 1 in autophagy was first suspected due to its 24.4% amino acid sequence identity with the yeast autophagy protein Atg6. Beclin 1 was found to restore autophagic activity in Atg6-disrupted yeast, becoming one of the first identified mammalian genes to positively regulate autophagy. Subsequent studies demonstrated that Beclin 1 is a haploinsufficient tumor-suppressor gene that is either monoallelically deleted or shows reduced expression in several different cancers (Yue *et al*., 2003).

Beclin 1 is also involved in several other biological functions, and in human conditions including heart disease, pathogen infections, impact on development, and neurodegeneration. These functions will not be discussed in this chapter because only the role of this gene (protein) in autophagy is relevant here. The central role of Beclin 1 complexes is in controlling human VPS34-mediated vesicle trafficking pathways including autophagy. Beclin 1 and its binding partners control cellular VPS34 lipid kinase activity that is essential for autophagy and other membrane trafficking processes, targeting different steps of the autophagic process such as autophagosome biogenesis and maturation (Funderburk *et al*., 2010). Beclin 1-depleted cells cannot induce autophagosome formation. In conclusion, the crucial regulator of autophagy is Beclin 1 (the mammalian homologue of yeast Atg6), which forms a multiprotein complex with other molecules such as UVRAG, AMBRA-1, Atg14L, Bif-1, Rubicon, SLAM, IP3, PINK, and survivin; this complex activates the class III phosphatidylinositol-3-kinase (Petiot *et al*., 2000).

Non-Autophagic Functions of Autophagy-Related Proteins

The importance of non-autophagic biological functions of autophagy-related proteins is beginning to be realized. These proteins (e.g., ubiquitin-like proteins Atg8 and Atg12) play an important role in various aspects of cellular physiology, including protein sorting, DNA repair, gene regulation, protein retrotranslation, apoptosis, and immune response (Ding *et al*., 2011). They also play a role in cell survival, modulation of cellular traffic, protein secretion, cell signaling, transcription, translation, and membrane reorganization (Subramani and Malhotra, 2013). Apparently, these proteins and their conjugates possess a different, broader role that exceeds autophagy.

The interactions of ubiquitin-like proteins with other autophagy-related proteins and other proteins are summarized below. For example, six Atg8 orthologues in humans interact

with at least 67 other proteins. Non-autophagy-related proteins that interact with Atg8 and LC3 include GTPases, and affect cytoskeletal dynamics, cell cycle progression, cell polarity, gene expression, cell migration, and cell transformation (Ding *et al.*, 2011). Non-lipidated LC3 and non-lipidated Atg8 regulate viral replication and yeast vacuole fusion, respectively (Tamura *et al.*, 2010). Atg5 and Atg12–Atg5 conjugates suppress innate antiviral immune signaling. Based on these and other functions, ubiquitin-like proteins in their conjugated and unconjugated forms modulate many cellular pathways, in addition to their traditional role in autophagy (Subramani and Malhotra, 2013).

In addition to ubiquitin-like Atg proteins, other Atg-related proteins are involved in non-autophagic functions; these are summarized below. UNC-51, the homologue of human ULK1, regulates axon guidance in many neurons. Atg16L1 positively modulates hormone secretion in PC12 cells, independently of autophagic activity (Ishibashi *et al.*, 2012). Atg16L1, Atg5, Atg7, and LC3 are genetically linked to susceptibility to Crohn's disease, a chronic inflammatory condition of the intestinal tract (Cadwell *et al.*, 2009). Atg5, Atg7, Atg4B, and LC3 are involved in the polarized secretion of lysosomal enzymes into an extracellular resorptive space, resulting in the normal formation of bone pits or cavities (bone resorption) (Deselm *et al.*, 2011).

The wide variety of functions of Atg-related proteins in typical non-autophagic cellular activities (some of which are enumerated here) indicates that the autophagic machinery is enormously complex and more versatile than presently acknowledged. Indeed, much more effort is needed to better understand the role of this machinery in health and disease, which eventually may allow us to delay the aging process and provide us with effective therapeutics.

Microtubule-Associated Protein Light Chain 3

Microtubule-associated protein chain 3 (LC3) is a mammalian homologue of yeast Atg8. It was the first mammalian protein discovered to be specifically associated with autophagosomal membranes. Although LC3 has a number of homologues in mammals, LC3B is most commonly used for autophagy (macroautophagy) assays because it plays an indispensable role in autophagy formation, making it a suitable marker for the process.

The cytoplasm contains not only LC3-I but also an active form (LC3-II). Immediately after synthesis of the precursor protein (pro-LC3), hAtg4B cleaves a C-terminal 22-amino acid fragment from this precursor form to the cytosolic form LC3-I. Afterwards, LC3-I is transiently conjugated to membrane-bound phosphatidylethanolamine (PE) to generate LC3-II, which localizes in both the cytosolic and intralumenal faces of autophagosomes. Owing to its essential role in the expansion step of autophagosome formation, LC3-II is regarded as the most reliable marker protein for autophagy. Following fusion with lysosomes, intralumenally-located LC3-II is degraded by lysosomal hydrolases, and cytosolically-oriented LC3-II is delipidated by hAtg4B, released from the membrane, and finally recycled back to LC3-I (Karim *et al.*, 2007). Divergent roles of LC3 (or Beclin 1) in tumorigenesis have been reported. For example, LC3 expression is either decreased in brain cancer (Aoki *et al.*, 2008) and ovary cancer (Shen *et al.*, 2008) or increased in esophageal and gastrointestinal neoplasms (Yoshioka *et al.*, 2008). LC3 is also associated with a poor outcome in pancreatic cancer (Fujita *et al.*, 2008), whereas its expression is associated with a better survival in glioblastoma patients with a poor performance score (Aoki *et al.*, 2008). It has also been reported that LC3-II protein expression is inversely correlated with melanoma thickness, ulceration, and mitotic

rate (Miracco *et al*., 2010). These and other studies imply that the clinical impact of LC3 is associated with the tumor type, tissue context, and other factors.

MONITORING AUTOPHAGY

A number of methods are available to monitor autophagy; such monitoring can be accomplished by using electron microscopy, biochemical protocols, and detection of relevant protein modifications through SDS-PAGE and western blotting. Autophagy can be monitored by detecting autophagosomal proteins such as LC3. LC3 is a specific marker protein of autophagic structure in mammalian cultured cells. The appearance of this protein-positive puncta is indicative of the induction of autophagy. One such method consists of monitoring autophagy by detecting LC3 conversion from LC3-I to LC3-II by immunoblot analysis because the amount of LC3-II is clearly correlated with the number of autophagosomes. Endogenous LC3 is detected as two bands following SDS-PAGE and immunoblotting: one represents cytosolic LC3-I and the other, LC3-II that is conjugated with phosphatidylethanolamine, is present on isolation membranes and autophagosomes but much less so on autolysosomes (Mizushima and Yoshimori, 2007). According to Kadowaki and Karim (2009), the LC3-I to LC3-II ratio in the cytosol (cytosolic LC3 ratio), but not in the homogenate, is an easy quantitative method for monitoring the regulation of autophagy. Alternatively, comparison of LC3-II levels between different conditions is a useful method for monitoring autophagy.

Another approach is use of the fluorescent protein GFP-LC3, which is a simple and specific marker. To analyze autophagy in whole animals, GFP-LC3 transgenic mice have been generated (Mizushima and Kuma, 2008). However, the GFP-LC3 method does not provide a convenient measure for assessing autophagic flux. Therefore, another alternative method, tandem fluorescent-tagged LC# (tfLC#), can be used to monitor autophagic flux (Kimura *et al*., 2009).

In spite of the advantages of the LC3 method, it has some limitations. LC3 protein, for example, tends to aggregate in an autophagy-independent manner. LC3-positive dots seen in the light microscope after using the transfected GFP-LC3 method may represent protein aggregates, especially when GFP-LC3 is overexpressed or when aggregates are found within cells (Kuma *et al*., 2007). LC3, in addition, is easily incorporated into intracellular protein aggregates – for example, in autophagy-deficient hepatocytes, neurons, or senescent fibroblasts. Also, LC3 is degraded by autophagy.

In light of the above limitations, it is important to measure the amount of LC3-II delivered to lysosomes by comparing its levels in the presence of or absence of lysosomal protease inhibitors such as E64d and pepstatin A (Mizushima and Yoshimori, 2007). Mizushima and Yoshimori have pointed out pitfalls and necessary precautions regarding LC3 immunoblot analysis. A very extensive update of the assays for monitoring autophagy has been presented by Klionsky *et al*. (2012), who strongly recommend the use of multiple assays to monitor autophagy, and present 17 methods of doing so.

REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species (ROS) are highly reactive forms of molecular oxygen, including the superoxide anion radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical

(Park *et al.*, 2012). ROS are generally produced during normal metabolism of oxygen inside the mitochondrial matrix, which acts as their primary source. Basal levels of ROS serve as physiological regulators of normal cell multiplication and differentiation. If the balance of ROS increases more than the scavenging capacity of the intracellular antioxidant system, the cell undergoes a state of oxidative stress with significant impairment of cellular structures. Excessive levels of ROS, for example, can cause severe damage to DNA and proteins.

The oxidative stress especially targets mitochondria, resulting in the loss of mitochondrial membrane potential and initiating mitochondria-mediated apoptosis. Oxidative stress can also lead to the auto-oxidation of sterols, thereby affecting the cholesterol biosynthetic pathway – mainly the postlanosterol derivatives. The intracellular accumulation of oxysterols directs the cell to its autophagic fate, and may also induce it to differentiate. ROS, in fact, can play contrasting roles: they can initiate autophagic cell death and also function as a survival mechanism through induction of cytoprotective autophagy in several types of cancer cells.

MAMMALIAN TARGET OF RAPAMYCIN (mTOR)

The mammalian target of rapamycin (mTOR), also known as the mechanistic target of rapamycin or FK506-binding protein 12-rapamycin-associated protein 1 (FRAP1), is an ~289-kDa protein originally discovered and cloned from *Saccharomyces cerevisiae* that shares sequence homologues with the phosphoinositide 3-kinase (PI3-kinase) family, which is the key element in response to growth factors. mTOR represents a serine threonine protein kinase that is present in all eukaryotic organisms (Wullschleger *et al.*, 2006). mTOR represents the catalytic subunit of two distinct complexes, mTORC1 and mTORC2 (Zoncu *et al.*, 2011). mTORC1 controls cell growth by maintaining a balance between anabolic processes (e.g., macromolecular synthesis and nutrient storage) and catabolic processes (e.g., autophagy and the utilization of energy stores) (Nicoletti *et al.*, 2011). The receptor–mTOR complex positively regulates cell growth, and its inhibition causes a significant decrease in cell size. The raptor part of the mTOR pathway modulates a large number of major processes, which are discussed here.

Rapamycin binds to the FKBP12 protein, forming a drug–receptor complex which then interacts with and perturbs TOR. TOR is the central component of a complex signaling network that regulates cell growth and proliferation. The components of these complexes exist in all eukaryotes.

As indicated above, mTOR is a major cellular signaling hub that integrates inputs from upstream signaling pathways, including tyrosine kinase receptors, that play a key role in intracellular nutrient sensoring. It serves as the convergent point for many of the upstream stimuli to regulate cell growth and nutrient metabolism, cell proliferation, cell motility, cell survival, ribosome biosynthesis, protein synthesis, mRNA translation, and autophagy (Meijer and Godogno, 2004). Two mammalian proteins, S6 kinase and 4E-BP1, link raptor–mTOR to the control of mRNA translation (Sarbassov *et al.*, 2005).

mTOR also governs energy homeostasis and cellular responses to stress, such as nutrient deprivation and hypoxia. Many studies have demonstrated that the Akt/mTOR-dependent pathway is involved in the process of chemical (platinum)-induced autophagy, in which mTOR is a pivotal molecule in controlling autophagy by activating mTOR (Hu *et al.*, 2012).

Another recent investigation also shows that methamphetamine causes damage to PC12 cells, but this damage can be decreased by using a supplement of taurine via inhibition of autophagy, oxidative stress, and apoptosis (Li *et al.*, 2012).

Abundance of nutrients, including growth factors, glucose, and amino acids, activates mTOR and suppresses autophagy, while nutrient deprivation suppresses mTOR, resulting in autophagy activation. In other words, triggering of autophagy relies on the inhibition of mammalian mTOR, an event that promotes the activation of several autophagy proteins (Atgs) involved in the initial phase of membrane isolation. Among many signaling pathways controlling mTOR activation, phosphoinositide 3-kinase (PI3K) is the key element in response to growth factors. mTORC1 and Atg1–ULK complexes constitute the central axis of the pathways that coordinately regulate growth and autophagy in response to cellular physiological and nutritional conditions. The negative regulation of mTORC1 by Atg1–ULK stresses further the intimate cross-talk between autophagy and cell growth pathways (Jung *et al.*, 2010).

ROLE OF AUTOPHAGY IN TUMORIGENESIS AND CANCER

Malignant neoplasms constitute the second most common cause of death in the United States, and malignant brain tumors contribute 2.4% of cancer-related deaths. An estimated 20,340 new cases of primary central nervous system tumors were diagnosed in 2012 in the United States alone, and resulted in approximately 13,110 deaths. Despite considerable advances in multimodal treatment of tumors in the past five decades, there has been only a minimal improvement in the median survival time of brain-malignancy patients. Causative factors for the poor survival rate include the highly invasive nature of brain malignant tumors, making them intractable to complete surgical resection, and resistance to standard chemotherapy and radiotherapy. This difficulty in remedying cancer underscores the need to pursue prosurvival signaling mechanisms that contribute to the resistance to cancer development; such alternative therapies include the use of autophagy.

Autophagy defects are linked to many diseases, including cancer, and its role in tumorigenesis, being tissue- and genetic context-dependent, is exceedingly complex. Metabolically stressed tumor cells rely on autophagy for survival and reprogramming of their metabolism to accommodate rapid cell growth and proliferation (Lozy and Karantza, 2012). To accomplish this goal, specific catabolic reactions (e.g., aerobic glycolysis and glutaminolysis) are upregulated to provide needed energy and rebuild new complex macromolecules such as proteins, nucleic acids, and lipids.

Autophagy has complex and paradoxical roles in antitumorigenesis, tumor progression, and cancer therapeutics. Initially, two principal lines of evidence connected autophagy and cancer: it was found that (1) the *BECN1* gene is monoallelically deleted in several types of cancers, and (2) autophagy can function to promote tumor cell survival, but can also contribute to cell death. In other words, autophagy can be both tumorigenic and tumor suppressive. Its exact role in each case is dependent on the context and stimuli. Autophagy can be upregulated or suppressed by cancer therapeutics, and upregulation of autophagy in cancer therapies can be either prosurvival or prodeath for tumor cells.

It is known that autophagy maintains cellular integrity and genome stability. Loss of autophagy genes perturbs this homeostasis, thereby potentially priming the cell for tumor

development. The following autophagy genes are frequently mutated in human cancers (Liu and Ryan, 2012): *BECN1*, *UVRAG*, *SH3GLB1* (Bif-1), *Atg2B*, *Atg5*, *Atg9B*, *Atg12*, and *RAB7A*. Mutations in *Atg2B*, *Atg5*, *Atg9B*, and *Atg12* have been reported in gastric and colorectal cancers (Kang *et al.*, 2009). The expression of Bif-1 is downregulated in gastric and prostate cancers (Takahashi *et al.*, 2010). Mutations of *UVRAG* have been found in colon cancer (Knaevelsrud *et al.*, 2010).

Autophagy is associated with both cancer progression and tumor suppression. The molecular mechanisms underlying these two phenomena have been elucidated. It is known that cancer cells generally tend to have reduced autophagy compared with their normal counterparts and premalignant lesions. Therefore, for autophagy to induce cancer progression, it will have to be activated. This is accomplished, for example, by the KRAS oncogene, which is known to induce autophagy. It has been shown that autophagy is activated constitutively in oncogenic KRAS-driven tumors, and that this cellular event is required for the development of pancreatic tumors (Yang *et al.*, 2011).

The discovery that the autophagic-related gene *BECN1* suppresses tumor growth stimulated significant interest from cancer biologists in this previously unexplored therapeutic process. This interest has resulted in both intensive and extensive research efforts to understand the role of autophagy in cancer initiation, progression, and suppression. Pharmacological or genetic inactivation of autophagy impairs KRAS-mediated tumorigenesis. It has been shown that transmembrane protein VMP1 (vacuole membrane protein 1), a key mediator of autophagy, is a transcriptional target of KRAS signaling in cancer cells (Lo Ré *et al.*, 2012). It regulates early steps of the autophagic pathway. In fact, KRAS requires VMP1 not only to induce but also to maintain autophagy levels in cancer. PI3K–AKT1 is the signaling pathway mediating the expression and promoter activity of VMP1 upstream of the GLI3–p300 complex.

The *BECN 1* gene is deleted in ~40% of prostate cancers, ~50% of breast cancers, and ~75% of ovarian cancers (Liang *et al.*, 1999). In addition, reduced expression of Beclin 1 has been found in other types of cancers, including human colon cancer, brain tumors, hepatocellular carcinoma, and cervical cancer. It can be concluded that a defective autophagic process is clearly linked to cancer development.

Autophagy is associated with resistance to chemotherapeutics such as 5-fluorouracil and cisplatin. It is recognized that tumors and the immune systems are intertwined in a competition where tilting the critical balance between tumor-specific immunity and tolerance can finally determine the fate of the host (Townsend *et al.*, 2012). It is also recognized that defensive and suppressive immunological responses to cancer are exquisitely sensitive to metabolic features of rapidly growing tumors.

On the other hand, autophagy may increase the effectiveness of anticancer radiotherapy. It is known that some malignancies become relatively resistant to repeated radiotherapy, and may eventually recover self-proliferative capacity. This problem can be diminished by inducing autophagy through Beclin 1 overexpression in conjunction with radiotherapy. It is known that autophagy enhances the radiosensitization of cancer cells rather than protecting them from radiation injury and cell death. It is also known that autophagy inhibits the growth of angiogenesis in cancer cells. It should also be noted that autophagic cell death occurs in many cancer types in response to various anticancer drugs. In other words, autophagy can serve as a pathway for cellular death. Based on the two opposing roles of autophagy, it is

poised at the intersection of life and death. It is apparent that we need to understand and modulate the autophagy pathway to maximize the full potential of cancer therapies.

As mentioned earlier, autophagy is frequently upregulated in cancer cells following standard treatments (chemotherapy, radiotherapy), showing as prosurvival or prodeath for cancer cells (reviewed by Liu and Ryan, 2012). Treatment with rapamycin, rapamycin analogues, and imatinib shows a prodeath effect, while treatment with radiation, tamoxifen, camptothecan, and proteasome inhibitors results in the survival of cancer cells. The effect of autophagy seems to be different in distinct tumor types, at various stages of tumor development, and even within different regions of the same tumor. It is concluded that, generally, either overactivation or underactivation of autophagy contributes to tumorigenesis, and that autophagy limits tumor initiation, but promotes establishment and progression.

ROLE OF AUTOPHAGY IN IMMUNITY

The eradication of invading pathogens is essential in multicellular organisms, including humans. During the past two decades there has been rapid progress in the understanding of the innate immune recognition of microbial components and its critical role in host defense against infection. The innate immune system is responsible for the initial task of recognizing and destroying potentially dangerous pathogens. Innate immune cells display broad antimicrobial functions that are activated rapidly upon encountering microorganisms (Franchi *et al*., 2009).

Autophagy can function as a cell's defense against intracellular pathogens. It is involved in almost every key step, from the recognition of a pathogen to its destruction and the development of a specific adaptive immune response to it. Autophagy, in addition, controls cell homeostasis and modulates the activation of many immune cells, including macrophages, dendritic cells, and lymphocytes, where it performs specific functions such as pathogen killing or antigen processing and presentation (Valdor and Macian, 2012).

The autophagy pathway is linked to one or more aspects of immunity. Studies have shown that autophagy is regulated by pathways that are critical for the function and differentiation of cells of the immune system, including Toll-like receptors (TLRs). TLRs were the first class of immune receptors identified as regulators in cells of the innate immune system, and play a crucial role in many aspects of the immune response. They are broadly expressed in immune cells, particularly in antigen-presenting cells, and recognize pathogen-associated molecular patterns such as lipopolysaccharides, viral double-stranded RNA, and unmethylated CPG islands (Harashima *et al*., 2012). Initiation of TLR signaling induces release of inflammatory cytokines, maturation of dendritic cells, and activation of adaptive immunity. Cancer cells also express functional TLRs. TLR4 signaling, for example, promotes escape of human lung cancer cells from the immune system by inducing immune suppressive cytokines and promoting resistance to apoptosis (He *et al*., 2007). In contrast, TRL3 signaling induces antitumor effects. Akt activation can render cancer cells resistant to antitumor cellular immunity (Hähnel *et al*., 2008). The implication is that Akt inactivation increases the susceptibility of cancer cells to immune surveillance.

TLRs also have been shown to induce autophagy in several cell types, including neutrophils (Xu *et al*., 2007). Activation of the TLR downstream signaling proteins MyD88 and Trif appears to be involved in the induction of autophagy. These proteins are recruited together

with Beclin 1 to TLR4, which promotes the dissociation of the Beclin 1–Bc12 complex and induces autophagosome formation (Shi and Kehri, 2008). MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. TLRs have also been shown to promote a process involving the autophagy machinery termed LC3-associated phagocytosis (Valdor and Macian, 2012). The uptake of cargo containing TLR ligands by macrophages leads to the recruitment of LC3 on the phagosome surface, promoting degradation of the pathogens by enhancing phagosome–lysosome fusion in the absence of autophagosome formation (Sanjuan *et al.*, 2009).

In fact, the study of TLRs showed that pathogen recognition by the innate immune system is specific, relying on germline-encoded pattern-recognition receptors that have evolved to detect components of foreign pathogens (Akira *et al.*, 2006). TLRs recognize conserved structures in pathogens, which leads to the understanding of how the body senses pathogen invasion, triggers innate immune responses, and primes antigen-specific adaptive immunity (Kawai and Akira, 2010). The adaptive immune system relies on a diverse and specific repertoire of clonally selected lymphocytes. Additional studies are needed to better understand the mechanisms that regulate autophagy in immune cells and the role this process plays in the establishment of immune responses against foreign pathogens.

AUTOPHAGY AND SENESCENCE

Cellular senescence is a biological state in which cells have lost the ability to undergo mitosis, but remain metabolically active for a long time. Three types of senescence have been reported:

1. Replicative senescence, caused by telomere shortening after a genetically predetermined number of cell divisions in non-transformed cells (Shay and Roninson, 2004).
2. Oncogene-induced senescence, which involves the capacity of cells to undergo senescence in the presence of oncogenes (e.g., Ras) (Lee *et al.*, 1999).
3. Premature senescence, occurring through exposure of cells to exogenous cytotoxic agents causing DNA damage (Gewirtz, 2014).

It is known that the cytotoxic response of autophagy to stress and stress-induced senescence evades cell death. However, autophagy can be either a cytoprotective or cytotoxic response to chemotherapy or radiotherapy. Some information is available regarding a relationship between autophagy and senescence. That there is a cross-talk between autophagy and apoptosis has also been established, and this is discussed elsewhere in this chapter.

An increase of autophagic vacuoles and senescence has been observed in the bile duct cells of patients with primary biliary cirrhosis (Sasaki *et al.*, 2010). The generation of autophagic vesicles in dying senescent keratinocytes has also been reported (Gosselin *et al.*, 2009), and autophagy markers in senescent endothelial cells have been found. More importantly, Young *et al.* (2009) reported the upregulation of autophagy-related genes during oncogene-induced senescence, and that inhibition of autophagy delayed the senescence phenotype. Recently, Goehe *et al.* (2012) reported that treatment of breast cancer cells and colon cancer cells with doxorubicin or camptothecin resulted in both autophagy and senescence.

It is concluded that both autophagy and senescence are collaterally induced by chemotherapy in cancer cells. In contrast, interference with ROS generation, ATM activation, and

induction of p53 or p21 suppresses both autophagy and senescence (Goehe *et al.*, 2012). Both autophagy and senescence signal to the immune system the presence of tumor cells that require elimination. In addition, both autophagy and senescence enhance the effect of chemotherapy on cancer cells. Although autophagy accelerates the senescence process by possibly providing an additional source of energy, senescence can occur independently of autophagy.

ROLE OF AUTOPHAGY IN VIRAL DEFENSE AND REPLICATION

Viruses and other pathogens induce dramatic changes in the intracellular environment. Infected cells activate certain defense pathways to combat these pathogens. Conversely, pathogens interfere with defense processes and utilize cellular supplies for pathogen propagation. Autophagy, for example, plays an antiviral role against the mammalian vesicular stomatitis virus, and the phosphatidylinositol 3-kinase–Akt signaling pathway is involved in this defense process (Shelly *et al.*, 2009). Many virus types, including herpes simplex virus 1 and Sindbis virus, have been observed inside autophagic compartments for degradation (Orvedahl *et al.*, 2007).

Autophagy is an essential component of *Drosophila* immunity against the vesicular stomatitis virus (Shelly *et al.*, 2009). Recently, an interesting role of the RNAse L system and autophagy in the suppression or replication of the encephalomyocarditis virus or vesicular stomatitis virus was reported (Chakrabarti *et al.*, 2012). At a low multiplicity of infection, induction of autophagy by RNAse L suppresses virus replication; however, in subsequent rounds of infection, autophagy promotes viral replication. RNAse is a virus-activated host RNAse pathway that disposes of or processes viral and cellular single-stranded RNAs. However, it has not been established whether autophagy itself is sufficient to control viral replication in all cases; the participation of other cell death phenomena in this defense process cannot be disregarded. On the other hand, autophagy is, for example, actively involved in influenza A virus replication (Zhou *et al.*, 2009). Mouse hepatitis virus and polio virus sabotage the components of the mammalian autophagy system, which normally is important in innate immune defense against intracellular pathogens. In other words, autophagic machinery (which normally would function to eliminate a virus) may promote viral assembly (Jackson *et al.*, 2005). However, Zhao *et al.* (2007) indicate that mouse hepatitis virus replication does not require the autophagy gene *Atg5*.

The survival of HIV depends on its ability to exploit the host cell machinery for replication and dissemination, to circumvent the cell's defense mechanisms or to use them for its replication. Autophagy plays a dual role in HIV-1 infection and disease progression. Direct effects of HIV on autophagy include the subversion of autophagy in HIV-infected cells and the induction of hyper-autophagy in bystander CD4+ T cells. HIV proteins modulate autophagy to maximize virus production (Killian, 2012). On the other hand, HIV-1 protein also disrupts autophagy in uninfected cells and thus contributes to CD4+ T cell death and viral pathogenesis.

It has also been reported that HIV-1 downregulates autophagy regulatory factors, reducing both basal autophagy and the number of autophagosomes per cell (Blanchet *et al.*, 2010). The HIV negative elongation factor (Nef) protein protects HIV from degradation by inhibiting autophagosome maturation (Kyei *et al.*, 2009). It has been shown that the foot and mouth disease virus induces autophagosomes during cell entry to facilitate infection, but does not provide membranes for replication (Berrym *et al.*, 2012).

Another example of a virus that uses a component of autophagy to replicate itself is the hepatitis C virus (HCV) (Sir *et al.*, 2012). HCV perturbs the autophagic pathway to induce the accumulation of autophagosomes in cells (via the PI3KC3-independent pathway) and uses autophagosomal membranes for its RNA replication. Other positive-strand RNA viruses (poliovirus, dengue virus, rhinoviruses, and nidoviruses) also use the membrane of autophagic vacuoles for their RNA replication (Sir and Ou, 2010). Suppression of LC3 and Atg7 reduces the HCV RNA replication level; these two proteins are critical for autophagosome formation. There is still controversy regarding the contrasting roles of autophagy in pathogen invasion; the mechanisms governing activation of autophagy in response to virus infection require further elucidation.

ROLE OF AUTOPHAGY IN INTRACELLULAR BACTERIAL INFECTION

Post-translation modifications of cell proteins (e.g., ubiquitination) regulate the intracellular traffic of pathogens. Ubiquitination involves the addition of ubiquitin to the lysine residues of target proteins, resulting in endocytosis and sorting events (Railborg and Stenmark, 2009). Several strategies have been developed by pathogenic bacteria to interfere with the host's ubiquitination and thus to achieve successful infection. Some types of bacteria act directly on the ubiquitination pathway by mimicking host cell proteins, while others (e.g., *Escherichia coli*, *Shigella flexneri*) act indirectly by expressing or interfering with the host ubiquitinating pathway. The other defense by the cell against bacterial infection is through autophagy; this is described below.

Autophagy serves as a double-edged sword; on the one hand it eliminates some pathogens and bacterial toxins, while on the other hand some pathogens can evade or exploit autophagy for survival and replication in a host. Recently, it has become clear that the interaction between autophagy and intracellular pathogens is highly complex. The components of the autophagy machinery also play roles in infection in a process different from the canonical autophagy pathway (formation of a double-membrane autophagosome and the involvement of more than 35 autophagy-related proteins, including the LC3 mammalian autophagy marker). There is an alternative autophagy pathway that is relevant to infection. For example, a subset of autophagy components can lead to LC3 conjugation onto phagosomes (Cemma and Brumell, 2012). In other words, the process of LC3-associated phagocytosis (LAP) results in the degradation of the cargo by promoting phagosome fusion with lysosomes. It is likely that both the LAP process and the canonical system operate simultaneously or selectively as host defenses against infection. Examples of bacteria the growth of which is suppressed by autophagy include *Escherichia coli* (Cooney *et al.*, 2010), *Salmonella typhimurium* (Perrin *et al.*, 2004), *Streptococcus pyogenes* (Virgin and Levine, 2009), and *Mycobacterium tuberculosis* (Randow, 2011); examples of bacteria that exploit autophagy for replication include *Staphylococcus aureus*, *Legionella pneumophila*, and *Yersinia pseudotuberculosis*; examples of bacteria that can evade targeting by autophagy/LAP include *Listeria monocytogenes* (Randow, 2011), *Shigella flexneri* (Virgin and Levine, 2009), and *Burkholderia pseudomallei*.

ROLE OF AUTOPHAGY IN HEART DISEASE

Heart failure is one of the leading causes of morbidity and mortality in industrialized countries. Myocardial stress due to injury, valvular heart disease, or prolonged hypertension induces pathological hypertrophy, which contributes to the development of heart failure and sudden cardiac death (Ucar *et al.*, 2012).

It has been reported that autophagy is an adaptive mechanism to protect the heart from hemodynamic stress. In fact, autophagy plays a crucial role in the maintenance of cardiac geometry and contractile function (Nemchenko *et al.*, 2011). Cardiac-specific loss of autophagy causes cardiomyopathy. Impaired autophagy has been found in a number of heart diseases, including ischemia/reperfusion injury. Excessive and uncontrolled autophagy leads to loss of functional proteins, depletion of essential organic molecules, oxidative stress, loss of ATP, the collapse of cellular catabolic machinery, and, ultimately, the death of cells in the heart. Autophagic elimination of damaged organelles, especially mitochondria, is crucial for proper heart function, whereas exaggerated autophagic activity may foster heart failure. Therefore, a delicate balance of autophagy maintains cardiac homeostasis, whereas an imbalance leads to the progression of heart failure.

A consensus on whether autophagy is cardioprotective or leads to hypertrophy and heart failure is lacking. In any case, autophagy is an important process in the heart. Various studies indicate that autophagy has a dual role in the heart, where it can protect against or contribute to cell death depending on the stimulus. It occurs at low basal levels under normal conditions, and is important for the turnover of organelles. Autophagy is upregulated in the heart in response to stress such as ischemia/reperfusion. Studies of ischemia/reperfusion injury indicate that ROS and mitochondria are critical targets of injury, as opening of the mitochondrial permeability transition pore culminates in cell death. However, Sciarretta *et al.* (2011) indicate that autophagy is beneficial during ischemia but harmful during reperfusion.

It has been shown that mitophagy mediated by Parkin is essential for cardioprotection (Huang *et al.*, 2011). The sequestration of damaged mitochondria depends on Parkin, which averts the propagation of ROS-induced ROS release and cell death. The implication is that mitochondrial depolarization and removal through mitophagy is cardioprotective. The sequestration of damaged cell materials into autophagosomes is essential for cardioprotection. An increased number of autophagosomes is a prominent feature in many cardiovascular diseases, such as cardiac hypertrophy and heart failure (Zhu *et al.*, 2007). Recently, Gottlieb and Mentzer (2013) have ably reconciled contradictory findings and concluded that the preponderance of evidence leans towards a beneficial role of autophagy in the heart under most conditions.

Recently, it was reported that autophagy plays a role in the onset and progression of alcoholic cardiopathy (Guo and Ren, 2012). Adenosine monophosphate-activated protein kinase (AMPK) plays a role in autophagic regulation and subsequent changes in cardiac function following an alcoholic challenge. It is known that AMPK promotes autophagy via inhibition of mTORC1 by phosphorylating the mTORC1-associated protein Raptor and tuberous sclerosis complex 2.

MicroRNAs (miRNAs) also play a role in cardiomyopathy and heart failure. These endogenous small molecules regulate their target gene expression by post-transcriptional regulation of messenger RNA. Recently, it was demonstrated that hypertrophic conditions induced the

expression of the miR-212/132 family in cardiomyocytes, and both of these molecules regulated cardiac hypertrophy and cardiomyocyte autophagy (Ucar *et al.*, 2012). Cardiac hypertrophy and heart failure in mice can be rescued by using a pharmacological inhibitor of miR-132.

Inflammation is also implicated in the pathogenesis of heart failure. Some information is available regarding the mechanism responsible for initiating and integrating inflammatory responses within the heart. Mitochondrial DNA plays an important role in inducing and maintaining inflammation in the heart. Mitochondrial DNA that escapes from autophagy cells autonomously leads to Toll-like receptor (TLR) 9-mediated inflammatory responses in cardiomyocytes, and is capable of inducing myocarditis and dilated cardiomyopathy (Oka *et al.*, 2012). Pressure overload induces the impairment of mitochondrial cristae morphology and functions in the heart. It is known that mitochondria damaged by external hemodynamic stress are degraded by the autophagy/lysosome system in cardiomyocytes (Nakai *et al.*, 2007). It is also known that increased levels of circulating proinflammatory cytokines are associated with disease progression and adverse outcomes in patients with chronic heart failure.

ROLE OF AUTOPHAGY IN NEURODEGENERATIVE DISEASES

Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) are the major neurodegenerative conditions causing dementia and movement disorders in the aging population. All three diseases are characterized by the presence of abnormal protein aggregates and neuronal death, although the etiology of AD is distinct from that of PD and HD.

It is known that epigenetic dysregulation and transcriptional dysregulation are pathological mechanisms underlying neurological diseases. It is also known that histone deacetylase (HDAC) inhibitor 4b preferentially targets HDAC1 and HDAC3, ameliorating, for example, HD (Jia *et al.*, 2012). HDACs are enzymes that remove acetyl groups from lysine amino acid on a histone. Several studies have identified HDAC inhibitors (4b) as candidate drugs for the treatment of neurodegenerative diseases, including HD.

Familial AD mutations increase the amyloidogenicity of the amyloid beta peptide, placing disruption of amyloid precursor protein (APP) metabolism and amyloid beta production at the center of AD pathogenesis (Pickford *et al.*, 2008). An increase in the production of both APP and amyloid beta, and a decrease in the degradation of APP, contributes to AD.

PD is a progressive neurodegenerative disorder caused by the interaction of genetic and environmental factors. It is characterized by the loss of dopaminergic neurons. The available evidence indicates that mitochondrial dysfunction, environmental toxins, oxidative stress, and abnormal accumulation of cytoplasmic proteinaceous materials can contribute to disease pathogenesis. These proteins tend to aggregate within Lewy bodies. The loss of dopaminergic neurons in the substantia nigra may be partly due to the accumulation of aggregated or misfolded proteins or mitochondrial dysfunction. Prevention of such accumulation or degeneration of dysfunctional mitochondria might prevent the occurrence of apoptosis. Mutations in the DJ-1 oncogene are also implicated in the pathogenesis of this disease. This oncogene is neuroprotective by activating the ERK1/2 pathway and suppressing mTOR in the dopaminergic neurons, leading to enhanced autophagy.

One of the major constituents of Lewy bodies is a protein called alpha-synuclein. This protein is likely to be a toxic mediator of pathology in PD because wild-type

alpha-synuclein gene duplications, which increase its expression levels, cause rare cases of autosomal dominant PD (Winslow and Rubinsztein, 2011). Overexpression of alpha-synuclein increases mutant huntingtin aggregation. Mutant huntingtin is an autophagy substrate, and its level increases when autophagy is compromised. Even physiological levels of this protein negatively regulate autophagy.

HD is characterized by the accumulation of mutant huntingtin (the protein product of the *IT15* gene) in intraneuronal inclusions, primarily in the brain but also peripherally. The increase is caused by the appearance of cytoplasmic (neutrophil) and nuclear aggregates of mutant huntingtin, and selective cell death in the striatum and cortex (DiFiglia *et al.*, 1997). HD is recognized as a toxic gain-of-function disease, where the expansion of the polyQ stretch within huntingtin confers new deleterious functions on the protein. Loss of normal huntingtin function is thought to be responsible for HD.

Amyotrophic lateral sclerosis (ALS) is the fourth most common neurodegenerative disease. It is characterized by progressive loss of upper and motor neurons. The following genes and proteins have been reported to be involved in familial ALS: superoxide dismutase 1, als2, TAR DNA binding protein of 43 kDa, and optineurin (Da Cruz and Cleveland, 2011). Accumulation of ubiquitinated inclusions containing these gene products is a common feature in most familial ALS models, and is also a pathologic hallmark of sporadic ALS. Failure to eliminate detrimental proteins is linked to pathogenesis of both familial and sporadic types of ALS. Dysfunction of the 26S proteasome in motor neurons is sufficient to induce cytopathological phenotypes of ALS (Tashiro *et al.*, 2012). This evidence indicates that dysfunction of the ubiquitin–proteasome system primarily contributes to the pathogenesis of sporadic ALS. In other words, proteasomes, but not autophagy, fundamentally govern the development of ALS, in which TDP-43 and FUS proteinopathy plays a crucial role (Tashiro *et al.*, 2012). The role of autophagy in AD, PD, and HD is further elaborated below.

Loss of autophagy-related genes results in neurodegeneration and abnormal protein accumulation. Autophagy is important in avoiding, or at least delaying, the development of age-related diseases such as neurodegeneration and cancer. In fact, autophagy is an essential pathway in postmitotic cells, such as neurons, that are particularly susceptible to the accumulation of defective proteins and organelles. Neuron-specific disruption of autophagy results in neurodegenerative diseases, including AD, PD, HD, ALS, and prion diseases. Tissue-specific genetic manipulation of autophagy of the brain causes neuronal accumulation of misfolded proteins and an accelerated development of neurodegeneration.

One of the prominent features of AD is the accumulation of autophagic vacuoles in neurons, suggesting dysfunction in this degradation pathway. Autophagy is normally efficient in the brain, as reflected by the low number of brain autophagic vacuoles at any given moment (Nixon and Yang, 2011). In contrast, brains of AD patients exhibit prominent accumulation of such vacuoles in association with dystrophic neuritis and deformed synaptic membranes (Yu *et al.*, 2005).

The majority of PD is idiopathic, with no clear etiology. The available evidence indicates that mitochondrial dysfunction, environmental toxins, oxidative stress, and abnormal protein accumulation can contribute to disease pathogenesis. The loss of dopaminergic neurons in the substantial nigra may be partly due to the accumulation of aggregated or misfolded proteins, or mitochondrial dysfunction. Prevention of such accumulations or degradation of dysfunctional mitochondria might prevent the occurrence of apoptosis. Mutations in the DJ-1 oncogene are

also implicated in the pathogenesis of this disease. DJ-1 is neuroprotected by activating the ERL1/2 pathway and suppressing mTOR in the dopaminergic neurons, leading to enhanced autophagy. Upregulation of autophagy has the potential to be a therapeutic strategy for disorders. This genetic method for autophagy upregulation is mTOR-independent. The development of genetic-based therapeutic strategies aimed at stimulating the autophagic clearance of aggregated proteins can be used both in the treatment of neurodegenerative diseases and in lifespan extension (Zhang *et al*., 2010). Several studies have identified histone deacetylose (HDAC) inhibitors (4b) as candidate drugs for the treatment of neurological diseases, including HD.

CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS

The cross-talk between autophagy and apoptosis is exceedingly complex, and various aspects of this phenomenon are still being understood. A brief introduction to the apoptosis pathway is in order. The significant functions of apoptosis (type 1 programmed cell death) are embodied in its maintenance of organism homeostasis and metabolic balance, and organ development. Morphological changes and death in apoptotic cells are caused by caspases, which cleave 400 proteins. The earliest recognized morphological changes in apoptosis involve condensation of cytoplasm and chromatin, DNA fragmentation, and cell shrinkage. The plasma membrane convolutes or blebs in a florid manner, producing fragments of a cell (apoptotic bodies). The fragments are membrane bound, and contain nuclear parts. The apoptotic bodies are rapidly taken up by nearby cells and degraded within their lysosomes.

There are two established signaling pathways that result in apoptosis. In the extrinsic pathway, apoptosis is mediated by death receptors on the cell surface, which belong to the TNF receptor superfamily and are characterized by extracellular cysteine-rich domains and extracellular death domains. In other words, the extrinsic pathway is induced by cell death receptor pathways such as TRAIL or FAS ligand. The cell surface receptors form a multiprotein complex called the death-inducing signaling complex (DISC).

The intrinsic pathway, on the other hand, is mediated by mitochondria in response to apoptotic stimuli, such as DNA damage, irradiation and some other anticancer agents (Zhan *et al*., 2012), serum deprivation, cytochrome c, SMAC/DIABLO (a direct inhibitor of apoptosis-binding protein), AIF (apoptosis-inducing factor that promotes chromatin condensation), and EndoG (endonuclease G that facilitates chromatin condensation). Cytochrome c binds to and activates Apaf-1 (apoptotic protease activating factor-1) protein in the cytoplasm. This induces the formation of an apoptosome that subsequently recruits the initiator procaspase-9, yielding activated caspase-9, and finally mediates the activation of caspase-3 and caspase-7 (Tan *et al*., 2009). It is apparent that diverse stimuli cause release of mitochondrial proteins to activate the intrinsic apoptosis pathway leading to MOMP and the release of cytochrome c and other apoptogenic proteins; MOMP is regulated by the Bcl family of proteins. In summary, in both pathways activated caspases cleave and activate other downstream cellular substrates as explained above.

Under stress conditions, prosurvival and prodeath processes are simultaneously activated and the final outcome depends on the complex cross-talk between autophagy and apoptosis. Generally, autophagy functions as an early induced cytoprotective response,

favoring stress adaptation by removing damaged subcellular constituents. It is also known that apoptotic stimuli induce a rapid decrease in the level of the autophagic factor activating molecule in Beclin 1-regulated autophagy (AMBRA-1) (Pagliarini *et al.*, 2012). Such AMBRA-1 decrease can be prevented by the simultaneous inhibition of caspases and calpains. Caspases cleave AMBRA-1 at the D482 site, while calpains are involved in complete AMBRA-1 degradation. AMBRA-1 levels are critical for the rate of apoptosis induction.

Autophagy can trigger caspase-independent cell death by itself, or by inducing caspase-dependent apoptosis. Autophagy can protect cells by preventing them from undergoing apoptosis. Autophagy also protects cells from various other apoptotic stimuli. Although the exact mechanism underlying this protection is not known, the role of damaged mitochondrial sequestration has been suggested; this prevents released cytochrome c from being able to form a functional apoptosome in the cytoplasm (Thorburn, 2008). There is a close connection between the autophagic machinery and the apoptosis machinery. Is it possible that there is simultaneous activation of these two types of death processes? In fact, autophagy is interconnected with apoptosis, as the two pathways share key molecular regulators (Eisenberg-Lener *et al.*, 2009). For example, it has been reported that autophagy regulates neutrophil apoptosis in an inflammatory context-dependent manner, and mediates the early pro-apoptotic effect of TNF-α in neutrophils. Neutrophils are a major subset of circulating leukocytes, and play a central role in defense against bacterial and fungal infections.

The concept of the presence of cross-talk between autophagy and apoptosis is reinforced by the indication that common cellular stresses activate various signaling pathways which regulate both of these cell death programs. ROS induce apoptosis and regulate Atg4, which is essential for autophagy induction. In addition, Atg5 promotes both apoptosis and autophagy induction. In addition to Atg5, several other signal transduction pathways (Bcl2 regulator) can elicit both of those cell death mechanisms. The transcription factor p53 is another such molecule.

Several additional recent studies have revealed additional information regarding the molecular mechanisms underlying the cross-talk between autophagy and apoptosis. An interesting study of the effect of ganoderic acid (a natural triterpenoid) on melanoma cells was recently carried out by Hossain *et al.* (2012). This study indicated that ganoderic acid induced orchestrated autophagic and apoptotic cell death as well as enhanced immunological responses via increased HLA class II presentation in melanoma cells. In other words, this treatment initiated a cross-talk between autophagy and apoptosis as evidenced by increased levels of Beclin 1 and LC3 proteins.

Another study investigated the effect of taurine on methamphetamine (METH)-induced apoptosis and autophagy in PC12 cells, and the underlying mechanism (Li *et al.*, 2012). METH, a commonly abused psychostimulant, induces neuronal damage by causing ROS formation, apoptosis, and autophagy. Taurine, in contrast, decreases METH-induced damage by inhibiting autophagy, apoptosis, and oxidative stress through an mTOR-dependent pathway. It is known that mTOR is the major negative regulator of autophagy.

The cross-talk between autophagy and apoptosis is indicated by the involvement of Beclin 1 in both of these programmed cell death types. Autophagy and apoptosis are two dynamic and opposing (in most cases) processes that must be balanced to regulate cell death and survival. Available evidence clearly indicates that cross-talk between autophagy and apoptosis does exist, and that in its presence the former precedes the latter. Also,

autophagy may delay the occurrence of apoptosis. Many studies indicate that cancer cells treated with an anticancer drug induce both autophagy and apoptosis. In addition, normal cells exposed to cancer-causing agents tend to invoke defense by inducing both autophagy and apoptosis. Moreover, cancer cells exposed to anticancer agents induce autophagy, but in the absence of autophagy these cells develop apoptosis. This concept is confirmed by a recent study by Li *et al.* (2012), which indicated that oridonin (an anticancer agent) upregulates *p21* (an antitumor gene) expression and induces autophagy and apoptosis in human prostate cancer cells, and that autophagy precedes apoptosis, thus protecting such treated cells from apoptosis by delaying the onset of the latter. To substantiate the above conclusions, several other recently published reports are described below.

Co-regulation of both autophagy and apoptosis using bis-benzimidazole derivatives has been reported (Wang *et al.*, 2012). These compounds are potent antitumor agents. The implication is that autophagy and apoptosis act in synergy to exert tumor cell death. In another study, it was shown that low-density lipoprotein receptor-related protein-1 (LRP1) mediates autophagy and apoptosis caused by *Helicobacter pylori* in the gastric epithelial cell line AZ-521 (Yahiro *et al.*, 2012). This study also proposes that the cell surface receptor, LRP1, mediates vacuolating cytotoxin-induced autophagy and apoptosis; this toxin induces mitochondrial damage leading to apoptosis. In these cells, the toxin triggers formation of autophagosomes, followed by autolysosome formation. Recently it was reported that death-associated protein kinase (DAPK) induces autophagy in colon cancer cells in response to treatment with histone deacetylase inhibitor (HDACi), while in autophagy-deficient cells DAPK plays an essential role in committing cells to HDACi-induced apoptosis (Gandesiri *et al.*, 2012).

Further evidence supporting the cross-talk between autophagy and apoptosis was recently reported by Visagie and Joubert (2011). They demonstrated the induction of these two programmed cell death mechanisms in the adenocarcinoma cell line MCF-7, which was exposed to 2-methoxyestradiol-bis-sulfamate (2-MeDE2bis MATE), a 2-methoxyestradiol derivative (an anticancer agent). The presence of apoptosis was indicated in this morphological study by growth inhibition, presence of a mitotic block, membrane blebbing, nuclear fragmentation, and chromatin condensation, which are hallmarks of this type of cell death. Simultaneously, this drug induced autophagy, shown by increased lysosomal staining.

Organic compounds have also been used to determine the cross-talk between autophagy and apoptosis. A few examples follow. Pterostilbene (a naturally occurring plant product) activates autophagy and apoptosis in lung cancer cells by inhibiting epidermal growth factor receptor and its downstream pathways (Chen *et al.*, 2012). Gui *et al.* (2012) used glyphosate (a herbicide linked to Parkinson's disease) to induce autophagy and apoptosis in PC12 cells, and found that the *Beclin 1* gene was involved in cross-talk between the mechanisms governing the two programmed cell death types. Two plant products, dandelion root extract and quinacrine, mediate autophagy and apoptosis in human pancreatic cancer cells and colon cancer cells, respectively (Ovadje *et al.*, 2012; Mohaptra *et al.*, 2012). Hirsutanol A, a compound from the fungus *Chondrostereum*, inhibits cell proliferation, elevates ROS level, and induces autophagy and apoptosis in breast cancer MCF-7 cells (Yang *et al.*, 2012).

A switch from apoptosis to autophagy is not uncommon during chemoresistance by cancer cells. It is known that defective apoptosis is an important mechanism underlying chemoresistance by cancer cells. Such resistance is associated with profound changes in cell death responses, and a likely switch from apoptosis to autophagy. This switch involves

balancing the deletion of multiple apoptotic factors by upregulation of the autophagic pathway and collateral sensitivity to the therapeutic agent. Ajabnoor *et al*. (2012) have reported that reduction of apoptosis occurring in the MCF-7 breast cancer cells upon acquisition of paclitaxel resistance is balanced by upregulation of autophagy as the principal mechanism of cytotoxity and cell death; this sensitivity is associated with mTOR inhibition. Upregulation of the autophagic pathway gives rise to rapamycin resistance. Also, loss of expression of caspase-7 and caspase-9 is observed in these cells.

It is known that the cell survival mechanism is driven by Beclin 1-dependent autophagy, while cell death is controlled by caspase-mediated apoptosis. Both of these processes share regulators such as Bcl-2, and influence each other through feedback loops. The question is whether autophagy and apoptosis coexist at the same time at the same stress level. To elucidate the role of regulatory components involved in both autophagy and apoptosis, and better understand the cross-talk between these two programmed cell death mechanisms, Kapuy *et al*. (2013) have explored the systems level properties of a network comprising cross-talk between autophagy and apoptosis, using a mathematical model. They indicate that a combination of Bcl-2-dependent regulation and feedback loops between Beclin 1 and caspases strongly enforces a sequential activation of cellular responses depending upon the intensity and duration of stress levels (transient nutrient starvation and growth factor withdrawal). This study also shows that amplifying loops for caspase activation involving Beclin 1-dependent inhibition of caspases and cleavage of Beclin 1 by caspases not only make the system bistable but also help to switch off autophagy at high stress levels. In other words, autophagy is activated at lower stress levels, whereas caspase activation is restricted to higher levels of stress. Apparently, autophagy precedes apoptosis at lower stress levels, while at a very high stress level apoptosis is activated instantaneously and autophagy is inactivated. According to this observation, autophagy and apoptosis do not coexist at the same time at the same stress level.

In summary, it is clear that a close relationship exists between autophagy and apoptosis, and that autophagy and apoptosis are not mutually exclusive pathways. They can act in synergy, or can counteract or even balance each other. Both share many of the same molecular regulators (Bcl-2). However, stress (e.g., nutrient deficiency, growth factor withdrawal) levels tend to affect autophagy and apoptosis differently from each other, resulting in mutual balancing. Thus, in a clinical setting it is difficult to predict the outcome of inhibition or activation of one form of programmed cell death (autophagy) without considering that of the other (apoptosis) (Eisenberg-Lerner *et al*., 2009). Because autophagy is involved not only in cell death but also (and mostly) in cell survival, and apoptosis leads only to cell death, an understanding of the critical balance between these two types of cellular processes is required to design anticancer therapeutics. The dual role of autophagy depends on the context and the stimuli. It has even been proposed that not only autophagy and apoptosis but also programmed necrosis may jointly decide the fate of cells of malignant neoplasms (Ouyang *et al*., 2012). Further investigations are required to understand the interplay between these two important cellular processes.

AUTOPHAGY AND UBIQUITINATION

Ubiquitin is a small (76-amino acid) protein that is highly conserved and widely expressed in all eukaryotic cells. Ubiquitination involves one or more covalent additions

to the lysine residues of target proteins. Ubiquitination is a reversible process due to the presence of deubiquitinating enzymes (DUBs) that can cleave ubiquitin from modified proteins. Post-translational modification of cell proteins, including ubiquitin, are involved in the regulation of both membrane trafficking and protein degradation. Ubiquitination is also implicated in the autophagy pathway (Kirkin *et al.*, 2009).

Successful invasion of the host cell by pathogenic microorganisms depends on their ability to subvert intracellular signaling to avoid triggering the cell's immune response. The host cell, under normal conditions, possesses pathways (xenophagy) that protect it from infection. Post-translation modifications (ubiquitination) play a role in the activation of xenophagy. A link between ubiquitination and the regulation of autophagy has been established (Dupont *et al.*, 2010). It is also known that p62 proteins target protein aggregates for degradation via autophagy. Pathogens, however, have developed mechanisms that subvert a cell's defense systems (xenophagy), replicating themselves. *Mycobacterium tuberculosis*, for example, prevents inflammasome activation (Master *et al.*, 2008) Other mechanisms involve interference with the host cell ubiquitination, membrane injury, and impairment of SUMOylation.

AGGRESOME: UBIQUITIN PROTEASOME AND AUTOPHAGY SYSTEMS

The ubiquitin proteasome system (UPS) removes non-functional, damaged, and misfolded proteins from the cell. When the capacity of the proteasome is impaired and/or when the amounts of misfolded proteins exceed the capacity of proteasome, they accumulate in the aggresome, the mechanism of which is explained below. Aggresomes are localized in the proximity of the microtubule-organizing center. Microtubule-associated histone deacetylase 6 (HDAC6) mediates this process. Through its ubiquitin-binding BUZ finger domain, HDAC6 binds to and facilitates the transport of polyubiquitinated misfolded proteins along microtubules to the aggresome (Kawaguchi *et al.*, 2003). Aggresome removal is mediated by ubiquitin-binding proteins such as p62/SQSTM1 and NBR1. These adaptor proteins through their ubiquitin-binding protein (UBA) are responsible for the fate of protein degradation either through the UPS or via autophagy (Komatsu and Ichimura, 2010). E3-ubiquitin ligases play a key role in the execution of autophagy (Chin *et al.*, 2010). Recently, it was reported that in response to proteasome inhibition, the E3-ubiquitin ligase TRIM50 localizes and promotes the recruitment and aggregation of polyubiquitinated proteins to the aggresome (Fusco *et al.*, 2012). Fusco and colleagues showed TRIM50 co-localizes, interacts with, and increases the level of p62, which is a multifunctional adaptor protein involved in various cellular processes including the autophagic clearance of polyubiquitinated protein aggregates. The implication of this information is that in the absence of proteasome activity, TRIM50 fails to drive its substrates to proteasome-mediated degradation and promotes their storage in the aggresome for subsequent removal by p62-mediated autophagy. It is known that the accumulation of polyubiquitinated protein aggregates is associated with neurodegenerative disorders and other protein aggregation diseases. It is also known that p62 is a component of inclusion bodies in neurodegenerative diseases and liver diseases.

AUTOPHAGY AND NECROPTOSIS

Necroptosis (type 3 programmed cell death) is one of the three basic cell death pathways. The functions of necroptosis include the regulation of normal embryonic development, T cell proliferation, and chronic intestinal inflammation. The molecular mechanisms underlying TNF-α induced necroptosis and autophagy have been deciphered, and are elaborated below.

Necrostatin-1 (Nec-1), targeting serine–threonine kinase receptor-interacting protein-1 (RIP1), is a specific inhibitor of necroptosis which is dependent on RIP1/3 complex activation (Degtcrev *et al.*, 2008). Tumor necrosis factor alpha (TNF-α) induces necroptosis and autophagy.

It was recently found that TNF-α administration causes mitochondrial dysfunction and ROS production (Ye *et al.*, 2012). Mitochondrial dysfunction led to necroptosis and autophagy in murine fibrosarcoma L929 cells. Nec-1 represses, whereas pan-caspase inhibitor z-VAD-fmk (z-VAD) increases, RIP1 expression. This increase, in turn, enhances TNF-α induced mitochondrial dysfunction and ROS production. It has also been shown that TNF-α administration and zVAD induce cytochrome c release from mitochondria, whereas Nec-1 blocks this release (Ye *et al.*, 2012).

In addition to apoptosis, necroptosis and autophagy are implicated in controlling both innate and adaptive immune functions. It has been demonstrated that the death of cells following ligation of death receptors (a subfamily of cell surface molecules related to TNF receptor 1) is not exclusively the domain of caspase-dependent apoptosis (Lu and Walsh, 2012). In these cells, cell death occurs via necroptosis.

MITOCHONDRIAL FUSION AND FISSION

Mitochondria form highly dynamic organelles that are continuously fusing and dividing to control their size, number, and morphology. The balance between these two processes regulates their shape. Loss of mitochondrial fusion generates many small mitochondria, while their inability to divide results in elongated mitochondria in most cells (Kageyama *et al.*, 2012).

The central components that mediate mitochondrial dynamics are three conserved dynamin-related GTPases (Kageyama *et al.*, 2011). In mammals, mitochondrial fusion is mediated by mitofusion 1 and 2, and Opal, which are located in the outer and inner membranes, respectively. Mitochondrial division is mediated by Drpl, which is mainly located in the cytosol. Drpl is recruited to the mitochondrial surface by other outer membrane proteins (e.g., Mff, MiD49) (Otera *et al.*, 2010; Palmer *et al.*, 2011). The importance of information on functions of Mfn2 and Opal becomes evident considering that mutations in these genes cause neurodegenerative disorders. In other words, alternations in mitochondrial fusion and fission are associated with neurodevelopmental abnormalities.

Mitochondria are highly dynamic cellular organelles involved in a wide variety of physiological functions, including ATP production, apoptosis, calcium and iron homeostasis, aging, lipid metabolism, and the production of reactive oxygen species. Although mitochondria are generally thought to be morphologically static, they alter their morphology continuously in response to various cellular signals, and this phenomenon is termed

mitochondrial dynamics (Zungu *et al.*, 2011). These alterations involve mitochondrial division (fission) and the merging of individual mitochondria (fusion). Contact sites between the inner and outer mitochondrial membranes consist of components of the mitochondrial permeability transition pore, which serves as the site for fission and fusion (Reichert and Neupert, 2004).

Under certain starvation conditions (e.g., amino acid depletion) mitochondria may escape autophagosomal degradation through extensive fusion. Such mitochondrial fusion under starvation conditions provides enough ATP necessary for cell survival. Downregulation of the mitochondrial fission protein Drpl is considered to be responsible for the fusion (Rambold *et al.*, 2011a). The process of fusion tends to result in the interconnected mitochondrial network through their elongation. As expected, pharmacological and genetic inhibition of mTOR leads to increased mitochondrial fusion. It is known that mTOR controls mitochondrial fusion. However, other signaling pathways (e.g., AMPK and PKA) may also be involved in starvation-induced mitochondrial fusion (Rambold *et al.*, 2011b).

SELECTIVE AUTOPHAGIES

Autophagy is a more selective process than originally anticipated. This type of autophagy distinguishes cargo to be degraded from its functional counterpart. Autophagy adaptors such as p62 and NBR1 provide mechanistic insight into this process. These adaptors are selectively degraded by autophagy, and are able to act as cargo receptors for degradation of ubiquitinated substrates and misfolded proteins. The autophagy adaptor p62 interacts directly with the adaptor protein ALFY (autophagy-linked FYE protein, encoded by the gene located on chromosome 4q21) and both bring the cargo in contact with the core autophagy machinery, allowing the formation of the autophagosomal membrane around the cargo, allowing its sequestration (Iskason *et al.*, 2013). A direct interaction between these adaptors and the autophagosomal marker protein LC3 is required for specific recognition of substrates and efficient selective autophagy (Johansen and Lemark, 2011). The best described adaptor protein is yeast Atg11 involved in the Ctv pathway. The cargo consists of ubiquitinated protein aggregates. ALFY is mainly located in the nucleus under normal conditions, but is transferred to the cytoplasm as protein aggregates upon cellular stress. These receptors seem not be involved in the bulk degradative autophagy.

A brief description of the proteins p62 and NBR1 is now given. The human p62 protein is 440 amino acids long and contains and N-terminal PB1 domain followed by a ZZ-type zinc finger domain, nuclear localization signals, nuclear export signal, LC3-interacting region, KIR motifs, and a C-terminal Ub-associated domain (Johansen and Lemark, 2011). p62 harbors active nuclear import and export signals and, as stated above, shuttles between the nucleus and cytoplasm.

NBR1 (neighbor of BRCA1 gene 1) is a protein that is ubiquitously expressed and highly conserved in eukaryotes. This protein is associated with cellular signaling pathways. NBR1 is a binding partner of autophagy-related protein 8 (ATG8) family proteins including LC3. The ATG8 functions in autophagosome formation, similar to yeast ATG8. NBR1 functions as a cargo adaptor for autophagic degradation of ubiquitinated substrates in a similar way as carried out by p62. Recent studies indicated that NBR1 is located in Lewy bodies and glial

cytoplasmic inclusions in multiple system atrophy, suggesting that it has a binding preference for α-synucleinopathy-related molecules (Odagiri *et al.*, 2012).

Specific or selective autophagy requires specific receptors to engage the substrate with the autophagy machinery, such as Atg32 for mitophagy and Atg19 for the cytoplasm to vacuole targeting pathway. Autophagy exhibits significant versatility in its selectivity to degrade cell components, which is discussed below.

Allophagy

In sexual reproduction, gamete fusion leads to the combination of two nuclear genomes, but the fate of paternal mitochondrial DNA requires explanation. Cumulative evidence indicates that in most animals, including humans, paternal mitochondria usually are eliminated during embryogenesis, a process termed allophagy, which is accomplished through autophagy.

A number of mechanisms have been proposed to explain allophagy. Some years ago Gyllenstein *et al.* (1991) hypothesized that according to the "simple dilution model," the paternal mitochondrial DNA (present at a much lower copy number) is simply diluted away by the excess of oocyte mitochondrial DNA, and consequently the former is hardly detectable in the offspring. On the other hand, according to the "active degradative process", the paternal mitochondrial DNA or mitochondria themselves are selectively eliminated (either before or after fertilization) by autophagy, preventing their transmission to the next generation (Al Rawi *et al.*, 2012).

As indicated above, uniparental inheritance of mitochondrial DNA is observed in many sexually reproducing species, and may be accomplished by different strategies in different species. Sato and Sato (2012, 2013) have proposed the following strategies.

1. Diminished content of mitochondrial DNA during spermatogenesis
2. Elimination of mitochondrial DNA from mature sperms
3. Prevention of sperm mitochondria from entering the oocyte
4. Active degradation of the paternal mitochondrial DNA in the zygote
5. Selective degradation of the whole paternal mitochondria (mitophagy) in the zygote.

The most feasible mechanism to accomplish this goal in mammals is as follows. Sperm-derived mitochondria and their DNA enter the oocyte cytoplasm during fertilization and temporarily coexist in the zygote alongside maternal mitochondria. However, very shortly after fertilization, paternal mitochondria are eliminated from the embryo. Thus, mitochondrial DNA is inherited solely from the oocyte from which mammals develop. This also means that some human mitochondrial diseases are caused by maternal mitochondrial DNA mutations.

The embryo of the *Caenorhabditis elegans* nematode has been extensively used as an experimental model for exploring the role of autophagy in the degradation of paternal organelles (Al Rawi *et al.*, 2012). It has been shown that paternal mitochondrial degradation depends on the formation of autophagosomes a few minutes after fertilization. This macroautophagic process is preceded by an active ubiquitination of some spermatozoon-inherited organelles, including mitochondria. The signal for such degradation is polyubiquitination of paternal mitochondria. Sato and Sato (2012) have also reported selective allophagy in such embryos.

It should be noted that the elimination of paternal mitochondrial DNA is not universal. Paternal inheritance of mitochondrial DNA, for example, has been reported in sheep and lower primates (St. John and Schatten, 2004; Zhao *et al.*, 2004). A recent study using mice carrying human mitochondrial DNA indicated that this DNA was transmitted by males to the progeny in four successive generations, confirming the paternal transmission of mitochondrial DNA (Kidgotko *et al.*, 2013). Apparently, human mitochondrial DNA safely passed via the male reproductive tract of several mice in several generations. This and a few other studies invoke a question regarding the existence of a specific mechanism responsible for paternal mitochondrial DNA transmission. Another pertinent, more important, unanswered question is: why are paternal mitochondria and/or their DNA eliminated from embryos? One hypothesis is that paternal mitochondria are heavily damaged by ROS prior to fertilization, and need to be removed to prevent potentially deleterious effects in the next generation (Sato and Sato, 2013).

Axonopathy (Neuronal Autophagy)

Selective degradation of axons under pathological conditions is termed axonopathy, which is directly linked to CNS and spinal cord neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis. They exhibit axonal degeneration early in the disease course; examples are degeneration of nigrostriatal projection tracts in Parkinson's disease and corticospinal tracts in amyotrophic lateral sclerosis.

Neurons have developed specific mechanisms for regulating autophagy. However, neuronal autophagic activities can be altered by pathological conditions including neurodegenerative diseases as shown by the accumulation of autophagosomes (Rubinszstein *et al.*, 2005). Large numbers of autophagosomes are frequently found in axonal dystrophic terminals of degenerating neurons (Yue, 2007). Autophagy is more pronounced in axons than in the cell body and dendrites under excitotoxic insult. It has been proposed that p62/SQSTM1 (a putative autophagic substrate) can serve as a marker for evaluating the impairment of autophagic degradation (Yue, 2007). Autophagosomes formed in the distal ends of axons may undergo retrograde axonal transport back to the cell body where lysosomes are usually located for completion of degradation.

Sequential features of axonal degeneration are elaborated below. The distal part of the lesioned axon undergoes initial axonal stability, followed by rapid degeneration and blebbing of the remaining axons, microtubule disassembly, and phagocytic clearance of the lesion site (Knöferle *et al.*, 2010). In contrast to this mechanism, axon degeneration occurs within the first few minutes after lesion, such as in the case of acute axonal degeneration in the spinal cord. One of the putative initiating steps in axonal degeneration is the influx of extracellular calcium, which destabilizes the axon and transmits apoptotic signals to the neuronal soma (Ziv and Spira, 1995). The role of calcium and autophagosomes in axonopathy is discussed below.

Autophagosomes play a critical role in the axonopathy process, and calcium plays a crucial role in their formation. This process has been investigated in the axonal degeneration in the optic nerve *in vivo* (Knöferle *et al.*, 2010). It was shown that mechanical injury to the optic nerve induced extracellular calcium entry to the axolemma via calcium channels,

which resulted in rapid increase of Ca^{2+}. This results in secondary generation of autophagosomes and axonal degradation.

Crinophagy

Disposal of excess secretory granules containing insulin by fusion of these granules with lysosomes is termed crinophagy. The β-cells in the pancreatic islets are involved in the storage of insulin secretory granules and instant secretion of insulin. These cells must maintain an optimal insulin concentration, which is maintained by insulin biosynthesis and its intracellular degradation. Such degradation is carried out via crinophagy, that is, β-cell lysosomes are subjected to glucose-dependent alterations. At low or physiological glucose concentration, secretory granules containing insulin are common in β-cell lysosomes.

As mentioned above, crinophagy in these cells is glucose dependent, and variations in glucose concentration affect the balance between insulin biosynthesis and secretion, which is under direct molecular control. It has been demonstrated that intracellular degradation of insulin and crinophagy are regulated by COX-2 activity that is maintained by endogenous nitric oxide (NO) (Sandberg and Borg, 2006). It has also been demonstrated that incubation of isolated pancreatic islets with interleukin 1β (IL-1β) enhances the intracellular degradation of insulin (Sandberg and Borg, 2006). It is known that IL-1β causes expression of inducible NOs in pancreatic islets.

It is concluded that considerable amounts of insulin are degraded within the pancreatic β-cells at low or physiological glucose concentrations, whereas there is virtually no degradation at a high glucose concentration (Halban and Wollheim, 1980). This mechanism seems to control the intracellular degradation of insulin and crinophagy in pancreatic β-cells.

Glycophagy

The delivery of glycogen to lysosomes for degradation is termed glycophagy. Three types of enzymes convert glucose into uridine diphosphoglucose, the primary intermediate in glycogen synthesis. The glucose residue of the intermediate molecule is transferred by glycogen to the free hydroxyl group on carbon 4 of a glucose residue at the end of a growing glycogen chain. Glycogen functions as a reserve for glucose, and provides an intracellular energy reserve in many types of cell. Glycogen is especially abundant in liver and muscle cells. As much as 10% by weight of the liver can be glycogen. The presence of glycogen particles in the vicinity of the smooth endoplasmic reticulum membranes in the liver as well as in the sarcoplasmic reticulum membranes in muscle is commonly seen using electron microscopy (Hayat, personal observation). Glycogen is also present in lysosomes of mammalian cells where it is directly hydrolyzed by lysosomal acid alpha-glucosidase (acid maltase). Deficient glucosidase causes severe glycogen storage diseases (Pompe disease, cardiopathologies).

Normally, synthesis and degradation of glycogen are highly regulated according to need. Accumulation of glycogen tends to cause a severe glycogen storage disease, Pompe disease, in multiple tissue types, especially in skeletal and cardiac muscles. The build-up of glycogen forms a large mass that interrupts the contractile proteins of the skeletal muscle fibers, affecting muscle contraction (Fukuda *et al.*, 2006) and causing muscular weakness and eventual tissue destruction. Other glycogen diseases include Anderson disease (Chen and Burchell, 1995), Tarui disease (Nakajima *et al.*, 1995), and Lafora disease (Andrade *et al.*, 2007).

Some information is available explaining glycogen trafficking to the lysosomes and its degradation. Autophagy seems to be involved in this process. The starch-binding domain-containing protein 1 (Stbd 1) (genethonin 1) participates in this mechanism by anchoring glycogen to intracellular membranes via its N-terminus (Janecek, 2002; Jiang *et al.*, 2011). Degradation of glycogen occurs by removing glucose residues catalyzed by glycogen phosphorylase. Stbd 1 targets two autophagy-related proteins, GABARAP and GABARAPL 1. Stbd 1 acts as a cargo receptor for glycogen. The Atg8 family interacting motif (AIM) in Stbd 1 is responsible for its interaction with GABARAPL 1 (Jiang *et al.*, 2011). Stbd 1 is thought to function as a cargo binding protein that delivers glycogen to lysosomes in an autophagic pathway (glycophagy). In fact, Stbd 1 is considered to be a glycophagy marker.

Lipophagy

The vast majority of studies of autophagy in the past rightfully have emphasized its role in cellular energy balance, cellular nutritional status, cellular quality control, remodeling, and cell defense. In most of these studies emphasis was placed on the role of autophagy in supplying energy through degradation of proteins to obtain amino acids required to maintain protein synthesis under extreme nutritional conditions. However, the contribution of autophagy to maintain cellular energetic balance is not solely dependent on its capacity to provide free amino acids (Singh and Cuervo, 2012). Free amino acids are a relatively inefficient source of energy when oxidized to urea and carbon dioxide. In contrast, free fatty acids and sugars are more efficient in supplying energy, especially the former through lipophagy.

Lipophagy is a selective form of autophagy and refers to the degradation of lipid droplets by stimulating autophagy. Lipid droplets are intracellular storage deposits for neutral lipids that are widely present in cells ranging from bacteria to humans. These droplets are considered to be organelles enclosed by a polar lipid monolayer membrane. They contain the hydrophobic core of triglycerides, diacyglycerol, cholesterol ester, and other esters. Mobilization of lipids inside the lipid droplets occurs through lipolysis. Cells activate lipolysis when they need energy and also when lipid storage becomes too large. The synthesis of fatty acids and phospholipids occurs in the smooth endoplasmic reticulum (SER).

Autophagy has been implicated in the degradation of several types of intracellular components, but only relatively recently have cytoplasmic lipid droplets been added to the list. This process of lipophagy has raised the likelihood that autophagy is involved in the regulation of lipoprotein assembly and contributes to both intracellular and whole-body lipid homeostasis (Christian *et al.*, 2013). Thus, autophagy is thought to be partially responsible for the upregulation or downregulation of very low density lipoprotein (VLDL) assembly. This means that autophagy is involved in the regulation of lipid accumulation during adipocyte differentiation.

Lipophagy breaks down triglycerides and cholesterol stored in lipid droplets, regulating intracellular lipid content. This degradation supplies free fatty acids required to sustain cellular mitochondrial levels of ATP. In other words, lipophagy maintains cellular energy homeostasis. Intracellular lipids, in addition, function as structural components of membrane building blocks for hormones, and mediators of cell signaling. The amount of lipid targeted for autophagic degradation depends on the nutritional status.

Another important function of autophagy is in liver diseases which are characterized by the accumulation of triglycerides and irregular lipid metabolism within the liver. It has been

reported that suppression of autophagy pathway leads to the accumulation of lipid droplets in hepatocytes and other cell types (Singh *et al.*, 2009).

Aberrant autophagy is also involved in conditions of deregulated lipid homeostasis in metabolic disorders such as metabolic syndrome of aging (Christian *et al.*, 2013). Lipophagy is also functionally involved in hypothalamic neurons and macrophage foam cells (Kaushik *et al.*, 2011; Ouimet and Marcel, 2012). A variety of proteins (Rab and PAT) are also associated with the lipid droplet membrane. PAT proteins regulate cytosolic lipase-mediated lipolysis, a major pathway for regulating lipid homeostasis (Fujimoto *et al.*, 2008). Impaired lipophagy, indeed, is a fundamental mechanism of disorders of lipid metabolism such as obesity, diabetes, and atherosclerosis. The initial accumulation of excess lipid is referred to as steatosis (Czaja, 2010).

The role of lipophagy in the alcohol-induced liver is discussed later. In addition to the role played by lipophagy in the above-mentioned diseases, the role of lipid accumulation in cardiovascular diseases was recently studied by Kim *et al.* (2013). Epigallocatechin gallate (EGCG) is a major polyphenol in green tea, which has beneficial health effects in the prevention of cardiovascular disease. These authors suggest that EGCG regulates ectopic lipid accumulation through a facilitated lipophagy flux. Treatment with EGCG increases the formation of LC3-II and autophagosomes in bovine aortic endothelial cells. Activation of CaMKKβ is required for EGCG-induced LC3-II formation. This effect is due to cytosolic C++ load. It is concluded that EGCG induces lipophagy through a reduction in the accumulation of lipid droplets in endothelial cells. It is known that impairment of the lysosomal degradation process reduces autophagic flux leading to serious disorders in cardiovascular and metabolic tissues (Singh and Cuervo, 2011).

The following questions still remain to be answered and are open for future studies (Singh and Cuervo, 2012):

1. Is there any similarity between the signaling pathways that regulate lipophagy and those for other types of autophagy?
2. What is the molecular mechanism underlying the selective targeting of the lipid droplets by lipophagy?
3. Is there a subset of lipid droplets that is targeted by lipophagy?
4. Is there a difference between the lipid products produced by lipophagy and those arising from lipolysis?
5. How does the switch take place from a stimulatory to an inhibitory effect of free fatty acids on lipophagy?
6. Does upregulation of lipophagy protect cells from lipotoxicity?
7. Does defective hypothalamic lipophagy contribute to the reduced food intake at an advanced age?
8. What is the potential of developing a therapeutic intervention against metabolic disorders by organ-specific targeting of this process?

Role of Lipophagy in Alcohol-Induced Liver Disease

An interesting role of lipophagy and mitophagy in chronic ethanol-induced hepatic steatosis has been reported (Eid *et al.*, 2013). It is known that chronic alcohol intake may induce alcoholic disease, ranging from early-stage steatosis (fatty liver) to steatohepatitis, fibrosis,

cirrhosis, and finally hepatic cancer (Yan *et al.*, 2007). Rats fed with 5% ethanol in liquid diet for 10 weeks showed large lipid droplets and damaged mitochondria in steatolic hepatocytes (Eid *et al.*, 2013). Moreover, hepatocyte steatosis was associated with enhanced autophagic vacuole formation compared to control hepatocytes. In addition, LC3 (a marker for autophagosomes) demonstrated an extensive punctate pattern in hepatocytes of these experimental rats.

Furthermore, PINK1 (a sensor damaged mitochondria, mitophagy) as well as LAMP-2 (a marker of autolysosomes) were expressed in these rats. This information provides clear evidence of ethanol toxicity because of the accumulation of lipid droplets in the cytoplasm of hepatocytes involving lipogenesis and lipolysis. Elevated levels of lipophagy and mitophagy reduce hepatocyte cell death under acute ethanol toxicity (Ding *et al.*, 2011).

In conclusion, the enhanced autophagic sequestration of accumulated lipid droplets presence of endogenous LC3-II, LAMP-2, PINK1, pan cathepsin, and cytochrome c under chronic ethanol toxicity. Nevertheless, the available information is insufficient to explain the relationship between lipophagy and canonical autophagy as well as between lipophagy and cytosolic lipolysis. The deciphering of the molecular mechanism underlying such differences may provide new therapeutic tools.

Mitophagy

It is thought that after its endosymbiosis from an α-proteobacterial ancestor, the mitochondrial genome was streamlined into a small, bioenergetically specialized genetic system, allowing an individual mitochondrion to respond through gene expression to alterations in membrane potential and maintain oxidative phosphorylation. Replication and transcription of mitochondrial DNA is initiated from a small noncoding region, and is regulated by nuclear-encoded proteins that are post-translationally imported into mitochondria. Mitochondria possess a unique genetic system that is able to translate the mitochondria-encoded genes into 13 protein subunits of the electron chain. Mercer *et al.* (2011) have presented analysis of the mitochondrial transcription across multiple cell lines and tissues, revealing the regulation, expression, and processing of mitochondrial RNA. This information should help in the understanding of the exceedingly complex functions of mitochondria. The major functions of mitochondria are summarized below.

Mitochondria fulfill central roles in oxidative phosphorylation, and in energy metabolism, in the synthesis of amino acids, lipids, heme, and iron sulfur clusters, in ion homeostasis and in thermogenesis. The most important role of mitochondria is to provide energy to aerobic eukaryotic cells by oxidative phosphorylation. Thus, these organelles are essential for growth, division, and energy metabolism in these cells. Each cell usually contains hundreds of mitochondria, and without these organelles even cancer cells are unable to grow, multiply, and survive *in vivo*. Mitochondrial dysfunction is strongly linked to numerous neurodegenerative and muscular disorders, myopathies, obesity, diabetes, cancer, and aging. Minimizing mitochondrial dysfunction is thus of major importance for counteracting the development of numerous human disorders and the aging process.

Mitochondria also play a crucial role in apoptosis and autophagy. It is apparent that mitochondria are central to the two fundamental processes of cell survival and cell death. Mitophagy plays a major role in the specific recognition and removal of damaged

mitochondria, and thus in mitochondrial quality control. The quality control of mitochondria does occur naturally at different levels. On the molecular level dysfunctional mitochondria are recognized and degraded within cells by autophagy. Mitochondria can be degraded both by non-selective autophagy and by mitophagy. Engulfment of mitochondria by autophagosomes is observed under starvation conditions as well as when mitochondrial function is impaired.

Mitochondrial turnover is necessary for cellular homeostasis and differentiation. Mitochondria are replaced every 2–4 weeks in rat brain, heart, liver, and kidney. The removal of dysfunctional mitochondria is achieved through mitophagy. Mitophagy is responsible for the removal of mitochondria during terminal differentiation of red blood cells and T cells. Mitochondria are recognized for selective mitophagy either by PINK1 and Parkin or mitophagic receptors Nix and Bnip3 and their accompanying modulators (Novak, 2012). The former mitophagy recognizes mitochondrial cargo through polyubiquitination of mitochondrial proteins. Nix functions as a regulated mitophagy receptor. These two modes of capturing mitochondria function at different efficiencies, from partial to complete elimination of mitochondria. In addition to autophagy machinery, proteins associated with mitochondrial fusion and fission regulate mitochondrial morphology, which is discussed elsewhere in this chapter.

A number of factors required for mitophagy have been identified and their role in this process has been analyzed. NIX (a BH3 domain containing protein) acts as a mitochondrial receptor required for mitochondrial clearance in some types of cells (e.g., reticulocytes). Many studies have shown that PINK1 and Parkin are involved in mitophagy. Mitochondrial depolarization induced by protonophore CCCP, downregulation of PINK1, and ROS, induces mitophagy as well as non-selective autophagy. More importantly, mitochondrial fission is necessary for the induction of mitophagy.

Nucleophagy

Parts of the cell nucleus can be selectively degraded without killing the cell, by a process termed nucleophagy. The cell nucleus is an organelle bounded by a double membrane, which undergoes drastic reorganization during major cellular events such as cell division and apoptosis. The process of nucleophagy is best described in the budding yeast *Saccharomyces cerevisiae*. Under certain conditions, the removal of damaged or non-essential parts of the nucleus or even an entire nucleus (differentiation or maturation of certain cells) is necessary to promote cell longevity and normal function; such degradation and recycling are accomplished via nucleophagy (Mijaljica and Devenish, 2013). Autophagic degradation of the nucleus in mammalian cells as a "housecleaning" under normal and disease conditions has been studied (Mijaljica *et al.*, 2010).

Molecular mechanisms underlying the formation of nucleus–vacuole junctions that mediate nucleophagy in the yeast have been deciphered. This mediation is accomplished through specific interactions between Vac8p on the vacuole membrane and Nvj1p in the nuclear envelope. Electron microscopy has shown that portions of the nucleolus are sequestered during nucleophagy (Mijaljica *et al.*, 2012).

Morphologically, during nucleophagy, a nuclear bleb containing the nuclear cargo is pinched off from the nucleus and directly engulfed and sequestered into an invagination of

the vacuolar membrane rather than packaged into autophagosome-like vesicles. It has been shown that upon nitrogen starvation the initiation of piecemeal micronucleophagy of the nucleus (PMN) occurs, as stated above, at the nucleus–vacuole junction between the outer nuclear membrane protein, Nvj1p, and the vacuolar membrane protein, Vac8p. Recently, it was demonstrated that induction of PMN can be detected as early as after 3 h of nitrogen starvation (Mijaljica *et al.*, 2012). Mijaljica and co-workers employed a genetically encoded nuclear fluorescent reporter (n-Rosella).

The PMN occurs through a series of morphologically distinct steps: (1) a nucleus–vacuole junction is formed at the nuclear envelope (both inner and outer membranes are involved); (2) simultaneous invagination of the vacuolar lumen occurs; (3) the nuclear derived double membranous structure containing nuclear material undergoes fission and is degraded by vacuolar hydrolases. This efficient process requires core *ATG* genes. All four components of the Atg8p–phosphatidylethanolamine conjugation system (Atg3, Atg4, Atg7, and Atg8) have been reported to be essential for efficient late nucleophagy.

The role of lipid trafficking membrane proteins in the mechanism of late nucleophagy is important. Kvam and Goldfarb (2004) have proposed that yeast Osh proteins play a general role in lipid trafficking at membrane contact sites between different organelles including the nucleus and vacuole. Roberts *et al.* (2003) have shown that upon nitrogen starvation and concomitant increased expression of Nvj1p, two proteins – Osh1 and Tsc13p – were required for PMN. In spite of the known molecular mechanisms discussed above, the specific conditions under which various cell nucleus components such as nucleoli, chromosomes, chromatin, histones, nuclear pore complexes, and nucleoplasm are degraded are not known.

Pexophagy

The selective degradation of peroxisomes by autophagy is referred to as pexophagy. The number of peroxisomes in a cell is tightly regulated in response to changes in metabolic status. They can be rapidly and selectively degraded when methanol-grown cells are placed in conditions of repression of methanol metabolism (e.g., glucose) by a process termed micropexophagy (van Zutphen *et al.*, 2008). Degradation of peroxisomes is also observed when the cells are placed in an ethanol medium; this is termed macropexophagy. In other words, micropexophagy is induced by glucose, and macropexophagy is induced by ethanol. The micro- and macropexophagy pathways are morphologically similar to the micro- and macroautophagy pathways, respectively. On the other hand, phthalate esters can cause a marked proliferation of peroxisomes. It has been demonstrated in yeast that protein trafficking, lipid trafficking, or both as directed by Sar1p are essential for micro- and macropexophagy (Schroder *et al.*, 2008). Stasyk *et al.* (2008) have presented methods for monitoring peroxisome status in yeast. Autophagic degradation of peroxisomes can be monitored with electron microscopy as well as by using biochemical assays for peroxisome markers. Several types of membrane dynamics during pexophagy can be visualized simultaneously under live cell imaging.

Pexophagy has been extensively studied in the methylotrophic yeast *Pichia pastoris*, which is capable of growth on methanol as a sole source of carbon and energy. There are two types of pexophagy: (1) micropexophagy through microautophagy; and (2) macropexophagy through macroautophagy. The induction of these two pathways depends on the carbon source in the methylotrophic yeast (Ano *et al.*, 2005). During micropexophagy,

peroxisomes are incorporated directly into the vacuoles by invagination; during macropexophagy, in contrast, peroxisomes are sequestered primarily by inclusion within newly-formed membranes. Subsequently, the peroxisome-containing pexophagosome fuses with the vacuole to deliver its cargo. Micropexophagy is more sensitive to ATP depletion than is macropexophagy, implying that former process requires a higher level of ATP.

It has been shown in yeast that PpAtg9 is essential for formation of the sequestering membranes that engulf the peroxisomes for degradation within the vacuole (Chang *et al*., 2005). Upon the onset of micropexophagy, PpAtg11 recruits PpAtg9 to the perivascular structure, which acts as the site of formation of the sequestering membrane presumably by causing segmentation of the vacuole. These membranes subsequently engulf the peroxisomes and eventually fuse with the help of PpAtg1 and PpVac8 to incorporate the peroxisomes into the vacuole for degradation (Chang *et al*., 2005).

Reticulophagy

Reticulophagy is responsible for the selective sequestration of portions of the endoplasmic reticulum (ER) with associated ribosomes. ER is a highly complex organelle, composed of a single continuous phospholipid membrane and flattened peripheral sheets with associated ribosomes. Almost all eukaryotic cells contain a discernible amount of ER because it is needed for the synthesis of plasma membrane proteins and proteins of the extracellular matrix. While detoxification of drugs, fatty acid and steroid biosynthesis, and Ca^{2+} storage occur in the smooth ER, most of the folding and post-translational processing of membrane-bound and secreted proteins takes place in the ER. Ribosomes that are present free in the cytosol mainly translate cytoplasmic proteins, whereas ribosomes associated with the ER membrane synthesize proteins that are secreted or reside in one of the organelles of the endomembrane system. As these newly synthesized proteins are cotranslationally translated into the ER, a substantial proportion of these proteins remain located in this compartment (Cebollero *et al*., 2012).

The ER stress signal, along with other signals (e.g., oxidative signal), is involved in autophagy. The former is involved in membrane formation and fusion, including autophagosome formation, autophagosome–lysosome fusion, and degradation of intra-autophagosomal contents by lysosomal hydrolases. ER stress is also involved in amplifying ROS production (Rubio *et al*., 2012). The study by Rubio *et al.* (2012) indicated that apical ER photodamage in murine fibrosarcoma cells generated ROS via mitochondria, which contributed to the processes of reticulophagy.

The unfolded protein response (UPR) is a form of intracellular signaling triggered by the ER stress. ER stress occurs under various physiological and pathological conditions where the capacity of the ER to fold proteins becomes saturated, for example as a response to incompetent or aggregation prone proteins, Ca^{2+} flux across the ER membrane, glucose starvation, or defective protein secretion or degradation (Hoyer-Hansen and Jaattela, 2007). Glucose starvation results in reduced protein glycosylation, and hypoxia causes reduced formation of disulfide bonds. ER stress resulting from the accumulation of unfolded or misfolded proteins threatens cell survival and the ER to nucleus signaling pathway; this pathway is called the UPR. The UPR reduces global protein synthesis and induces the synthesis of chaperone proteins and other proteins, which increase the ER capacity to fold its client proteins (Hoyer-Habsen and Jaattela, 2007). To prevent the accumulation of misfolded polypeptides in the ER,

chaperone proteins are thought to assist in the folding of the nascent polypeptides or recognize the misfolded proteins and mediate their refolding (Braakman and Bulleid, 2011). However, under certain conditions, unfolded proteins accumulate in the ER. At least two interconnected mechanisms are available to cope with such undesirable protein aggregation: (1) the UPR and (2) ER-associated degradation (ERAD) (Bernales *et al.*, 2006a; Romisch, 2005).

The UPR signaling is transduced into cytoplasmic and nuclear actions aimed at increasing the protein folding capacity of the ER and eliminating the proteins that remain misfolded and accumulated in the ER. The UPR also initiates inhibition of general translation and upregulation of genes encoding ER chaperones and components of ERAD machinery (Cebollero *et al.*, 2012). ERAD, in turn, recognizes misfolded proteins and translocates them into the cytoplasm where they are degraded by the ubiquitin–proteasome system. When the function of the ER is not restored, it may lead to cell death by apoptosis or autophagy depending on the cell type and the stimulus (Momoi, 2006).

Ribophagy

Selective degradation of ribosomes is termed ribophagy. Ribosomes are essential components of all cells and constitute the translation engine of the cell. Protein synthesis is catalyzed by ribosomes, which are composed of large complexes of RNA and protein molecules. Each ribosome is composed of one large subunit (60S) and one small subunit (40S) in eukaryotes, while prokaryotic ribosomes are made up of 50S and 30S subunits. Although these two types of ribosomes differ in size and number in eukaryotes and prokaryotes, both have the same function. Before protein synthesis can begin, the corresponding mRNA molecule must be produced by DNA transcription. This is followed by the binding of the small subunit to the mRNA molecule at a start codon that is recognized by an initiator tRNA molecule. Then the large subunit binds to complete the ribosome, and initiates the elongation phase of protein synthesis.

Ribosome turnover occurs both under normal conditions and under starvation. Under normal nutrient-rich conditions, large amounts of ribosomal subunits are assembled, which raises the possibility for the need of the removal of excess ribosomes in response to changing environmental conditions (Bakowska-Zywicka *et al.*, 2006). The ribophagy pathway could also target defective ribosomes under normal growth conditions (Cebollero *et al.*, 2012). This is a quality control function. It is also known that the autophagy of ribosomal proteins is involved in antibacterial function. Some information on the pathway of normal ribosome turnover, especially the role of rRNA decay, is available. *Arabidopsis* RNS2 (a conserved ribonuclease of the RNAse T2 family) is necessary for normal decay of rRNA (Macintosh and Bassham, 2011). The absence of RNS2 results in longer-lived rRNA and its accumulation in the yeast vacuoles and ER, showing constitutive autophagy. This evidence supports the concept that RNS2 participates in a ribophagy-like mechanism that targets ribosomes for recycling under normal growth conditions (Macintosh and Bassham, 2011).

Regarding the role of ribophagy during starvation, cells are subjected to energy shortage and need to save available energy. The beginning of the construction of ribosomes in the cell nucleus and the subsequent translation they carry out require considerable energy. Therefore, cells need to save energy, which is accomplished by removing ribosomes and terminating translation and protein synthesis. Ribophagy begins by separating the two

subunits of a ribosome. It has also been suggested that Ubp3/Bre5 (discussed later) regulates different types of selective autophagies during starvation (Beau *et al.*, 2008).

It is important to identify the genes required for ribophagy. Kraft *et al.* (2008) indicated the involvement of two proteins, ubiquitin-specific protease 3 (Ubp3) enzyme and Ubp3-associated cofactor (Bres), in the selective degradation of ribosomes, but not for bulk autophagy. They also indicated that ribophagy affects the entire 60S subunit, but not the 40S subunit, suggesting differential degradation of large and small subunits. These authors, furthermore, demonstrated the involvement of Atg1 and Atg7 in the transport of ribosomes to the vacuole in the yeast *S. cerevisiae*. It also has been reported that the Ubp3/Bre5 complex interacts with Atg19 protein and modulates its ubiquitination (Baxter *et al.*, 2005).

It is concluded that ribosome degradation relies on both ribophagy and non-selective autophagy. The evidence presented there and from other studies confirms a cross-talk between selective autophagy and ubiquitin-dependent processes. The majority of cellular proteins and most other cell components are eventually degraded and recycled in a cell either by autophagy or the ubiquitin–proteasome pathway or by a combination of these two systems. In fact, there is a connection between autophagy and ubiquitin modification and destruction by the proteasome pathways of protein degradation.

Xenophagy

The successful invasion of the host cell by pathogenic microorganisms depends on their ability to subvert intracellular signaling to avoid triggering the cell's immune response. The host cell, under normal conditions, possesses pathways (xenophagy) that protect it from infection. Post-translation modifications (ubiquitination) play a role in the activation of xenophagy. A link between ubiquitination and the regulation of autophagy has been established (Dupont *et al.*, 2010). It is also known that p62 proteins target protein aggregates for degradation via autophagy. Pathogens, however, have developed mechanisms that subvert the cell's defense systems (xenophagy), replicating themselves. *Mycobacterium tuberculosis*, for example, prevents inflammasome activation (Master *et al.*, 2008). Other mechanisms involve interference with the host cell ubiquitination, membrane injury, and impairment of SUMOylation.

Zymophagy

Pancreatic acinar cells are highly differentiated cells which synthesize and secrete digestive enzymes into the pancreatic juice. These digestive enzymes are initially produced as inactive enzymes (zymogens) and stored in zymogen granules until exocytosis. These granules can be harmful if activated prematurely because the release of these enzymes can hydrolyze tissue parenchyma, resulting in pancreatitis (Grasso *et al.*, 2011). VMP1 interacts with Beclin 1/Atg6 through its hydrophilic C-terminal region, which is necessary for early steps of autophagosome formation. Thus, the involvement of VMP1 is implicated in the induction of autophagy during this disease. VMP1 also interacts with the ubiquitin-specific proteases (USPs), indicating close cooperation between the autophagy pathway and the ubiquitin machinery required for selective autophagosome formation (Grasso *et al.*, 2011). Ubiquitination and ubiquitin-receptors such as p62

(SQSTAM1) play a part in vesicular traffic in pancreatitis. In fact, a VMP1-USP4-p62 molecular pathway is involved in mitophagy.

As explained above, if zymogen granules prematurely release the digestive enzymes in the acinar cells, the result could be pancreatitis. Under normal physiological conditions selective autophagy (zymophagy) degrades the activated zymogen granules, avoiding the release of digestive enzymes into the cytoplasm and thus preventing further trypsinogen activation and cell death. In other words, zymophagy has a critical function in secretory homeostasis and cell response to injury by selective degradation of altered secretory granules in acute pancreatitis.

In conclusion, zymophagy protects the pancreas from self-digestion. It is a selective form of autophagy, a cellular process to specifically detect and degrade secretory granules containing activated enzymes before they can digest the organ (Vaccaro, 2012). Zymophagy is activated in pancreatic acinar cells during pancreatitis-induced vesicular transport alteration to sequester and degrade potentially deleterious, activated zymogen granules.

References

Agarraberes, F.A., Dice, J.F., 2001. A molecular chaperon complex at the lysosomal membrane is required for protein translocation. J. Cell Biol. 114, 2491–2499.

Ajabnoor, G.M.A., Crook, T., Coley, H.M., 2012. Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. Cell Death Dis. 3, 1–9.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Al Rawi, S., Louvet-Vallee, S., Djeddi, A., et al., 2012. Allophagy: a macroautophagic process degrading spermatozoid-inherited organelles. Autophagy 8, 421–423.

Andrade, D.M., Turnball, J., Minassian, B.A., 2007. Lafora disease, seizures and sugars. Acta Myol. 26, 83–86.

Ano, Y., Hattori, T., Kato, N., et al., 2005. Intracellular ATP correlates with mode of pexophagy in *Pichia pastoris*. Biosci. Biotechnol. Biochem. 69, 1527–1533.

Aoki, H., Kondo, Y., Aldape, K., et al., 2008. Monitoring autophagy in glioblastoma with antibody against isoform B of human microtubule-associated protein 1 light chain 3. Autophagy 4, 467–475.

Ave, E.L., Walker, S.A., Manifava, M., et al., 2008. Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J. Cell Biol. 182, 685–701.

Bakowska-Zywicka, K., Tyczewska, A., Twardowski, T., 2006. Mechanism of peptide bond formation on the ribosome – controversions. In Polish. Postepy Biochem. 52, 166–172.

Baxter, B.K., Abeliovich, H., Zhang, X., et al., 2005. Atg19p ubiquitination and the cytoplasm to vacuole trafficking pathway in yeast. J. Biol. Chem. 280, 39067–39076.

Beau, I., Esclatine, A., Codogno, P., 2008. Lost to translation: when autophagy targets mature ribosomes. Trends Cell Biol. 18, 311–314.

Bernales, S., Papa, F.R., Walter, P., 2006a. Intracellular signaling by the unfolded protein response. Annu. Rev. Cell Develop. Biol. 22, 487–508.

Bernales, S., McDonald, K.L., Walter, P., 2006b. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. PLoS Biol. 4 (12), 2311–2324.

Berrym, S., Brooks, E., Burman, A., et al., 2012. Foot-and-mouth disease virus induces autophagosomes during cell entry via a Class III phosphatidylinositol 3-kinase-independent pathway. J. Virol. 86, 12940–12953.

Blanchet, F.P., Morris, A., Nikolic, D.S., et al., 2010. Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic cells impairs early innate and adaptive immune responses. Immunity 32, 654–669.

Braakman, I., Bulleid, N.J., 2011. Protein folding and modification in the mammalia endoplasmic veticulum. Annu. Rev. Biochem. 80, 71–99.

Brodsky, F.M., 1988. Living with clathrin: its role in intracellular membrane traffic. Science 242, 1396–1402.

Cadwell, K., Patel., K.K., Komatsu, M., et al., 2009. A common role for Atg16L1, Atg5, and Atg7 in small intestinal Paneth cells and Crohn disease. Autophagy 5, 250–252.

Cebollero, E., Reggiori, F., Kraft, C., 2012. Reticulophagy and ribophagy: regulated degradation of protein production factories. Int. J. Cell Biol. 2012, 182834.
Cemma, M., Brumell, J.H., 2012. Interactions of pathogenic bacteria with autophagy systems. Cur. Biol. 22, 540–545.
Chakrabarti, A., Gosh, P.K., Banerjee, S., et al., 2012. RNase L triggers autophagy in response to viral infections. J. Virol. 86, 11311–11321.
Chang, T., Schroder, L.A., Thomson, J.M., et al., 2005. PpAtg9 encodes a novel membrane protein that traffics to vacuolar membranes, which sequester peroxisomes during pexophagy in *Pichia pastoris*. Mol. Biol. Cell 16, 4941–4953.
Chen, G., Hu, X., Zhang, W., et al., 2012. Mammalian target of rapamycin regulates isoliquiritigenin-induced autophagic and apoptotic cell death in adenoid cystic carcinoma cells. Apoptosis 17, 90–101.
Chen, Y., Yu, L., 2012. Autophagic lysosome reformation. Exp. Cell Res. 319, 142–146.
Chen, Y.-T., Burchell, A., 1995. Glycogen storage diseases. In: Scriver, C.R., Beaudet, A.L., Sly, W.S. (Eds.), The Metabolic and Molecular Basis of Inherited Disease. McGraw-Hill, New York, pp. 935–965.
Chin, I.S., Olzmann, J.A., Li, L., 2010. Parkin-mediated ubiquitin signaling in aggresome formation and autophagy. Biochem. Soc. Trans. 38, 144–149.
Christian, P., Sacco, J., Adeli, K., 2013. Autophagy: emerging roles in lipid homeostasis and metabolic control. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1831, 819–824.
Cooney, R., Baker, J., Brain, O., et al., 2010. NOD2 stimulation induces autophagy in dendritic cells influences bacterial handling and antigen presentation. Nat. Med. 16 (1), 90–97.
Cuervo, A.M., 2009. Chaperone-mediated autophagy: selectivity pays off. Trends Endocrinol. Metab. 21, 142–150.
Cuervo, A.M., Dice, J.F., 1996. A receptor for the selective uptake and degradation of proteins by lysosomes. Science 273, 501–503.
Czaja, M.J., 2010. Autophagy in health and disease. 2. Regulation of lipid metabolism and storage by autophagy: pathophysiological implications. Am. J. Physiol. Cell Physiol. 298, C973–C978.
Da Cruz, S., Cleveland, D., 2011. Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. Curr. Opin. Neurobiol. 21, 904–919.
Degterev, A., Hitomi, J., Germscheid, M., et al., 2008. Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.
DeSelm, C.J., Miller, B.C., Zou, W., et al., 2011. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. Dev. Cell 21, 966–974.
Deter, R.L., de Duve C., 1967. Influence of glucagon, and inducer autophagy, on some physical properties of rat liver lysosomes. J. Cell Biol. 33, 437–499.
DiFiglia, M., Sapp, E., Chase, K.O., et al., 1997. Aggregation of Huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. Science 277, 1990–1993.
Ding, F., Yin, Z., Wang, H.R., 2011. Ubiquitination in Rho signaling. Curr. Top. Med. Chem. 11, 2879–2887.
Dice, J., 1990. Peptide sequences that target cytosolic proteins for lysosomal proteolysis. Trends Biochem. Sci. 15, 305–309.
Doyle, K.M., Kennedy, D., Gorman, A.M., et al., 2011. Unfolded proteins and endoplasmic reticulum stress in neurodegenerative disorders. J. Cell. Mol. Med. 15, 2025–2039.
Dupont, N., Temime-Smaali, N., Lafont, F., 2010. How ubiquitination and autophagy participate in the regulation of the response to bacterial infection. Cell 102, 621–634.
Eid, N., Ito, Y., Maemura, K., et al., 2013. Elevated autophagic sequestration of mitochondria and lipid droplets in steatotic hepatocytes of chronic ethanol-treated rats: an immunohistochemical and electron microscopic study. J. Mol. Histol. 44, 311–326.
Eisemberg-Lerner, A., Bialik, S., Simon, H.-U., et al., 2009. Life and death partners: apoptosis, autophagy, and the cross-talk between them. Cell Death Differ. 16, 966–975.
Ferreon, A.C.M., Moosa, M.M., Gambin, Y., et al., 2012. Counter acting chemical chaperone effects on the single-molecule α-synuclein structural landscape. Proc. Natl Acad. Sci. USA 109, 17826–17831.
Florey, O., Overholtzer, M., 2012. Autophagy proteins in macroendocytic engulfment. Trends Cell Biol. 22, 376–380.
Franchi, L., Eigenbrod, T., Munoz-Planillo, R., et al., 2009. The inflammasome: a caspase-1 activation platform regulating immune responses and disease pathogenesis. Nat. Immunol. 10, 241–255.
Fujimoto, T., Ohsaki, Y., Cheng, J., et al., 2008. Lipid droplets: a classic organelle with new outfits. Histochem. Cell Biol. 130, 263–279.
Fujita, N., Itoh, T., Omori, H., et al., 2008. The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. Mol. Biol. Cell 19, 2092–2100.

Funderburk, S.F., Wang, Q.J., Yue, Z., 2010. The Beclin 1–VPS34 complex at the crossroads of autophagy and beyond. Trends Cell Biol. 20, 355–362.
Fukuda, T., Roberts, A., Ahearn, M., et al., 2006. Autophagy and lysosomes in Pompe disease. Autophagy 2, 318–320.
Fusco, C., Micale, L., Egorov, M., et al., 2012. The E3-ubiquitin ligase TRIM50 interacts with HDAC6 and promotes the sequesteration and clearance of ubiquitinated proteins into the aggresome. PLoS ONE 7 (issue), e40440.
Gandesiri, M., Chakilam, S, Ivanovska, J., et al., 2012. Dapk plays an important role in panobinostat-induced autophagy and commits cells to apoptosis under autophagy deficient conditions. Apoptosis 17, 1300–1315.
Gao, W., Kang, J.H., Liao, Y., et al., 2010. Biochemical isolation and characterization of tubulovesicular LC3-positive autophagosomal compartment. J. Biol. Chem. 285, 1371–1383.
Gewirtz, D.A., 2014. Autophagy and senescence in cancer therapy. J. Cell. Physiol. 229, 6–9.
Goehe, R.W., Di, X., Sharma, K., et al., 2012. The autophagy-senescence connection in chemotherapy: most tumor cells (self) eat before they die. J. Pharmacol. Exp. Ther. 343, 763–778.
Gosselin, K., Deruy, E., Martien, S., et al., 2009. Senescent keratinocytes die by autophagic programmed cell death. Am. J. Pathol. 174, 423–435.
Gottlieb, R.A., Mentzer Jr., R.M., 2013. Autophagy: an affair of the heart. Heart Fail. Rev. 18, 575–584.
Grasso, D., Ropolo, A., Lo Re, A., et al., 2011. Zymophagy, a novel selective autophagy pathway mediated by VMP1-USP9x-p 62, prevents pancreatic cell death. J. Biol. Chem. 286, 8308–8324.
Gui, Y.-X., Fan, X.-N., Wang, H.-M., et al., 2012. Glyphosate induced cell death through apoptotic mechanisms. Neurotoxicol. Teratol. 34, 344–349.
Guo, R., Ren, J., 2012. Deficiency in AMK attenuates ethanol-induced cardiac contractile dysfunction through inhibition of autophagosome formation. Cardiovasc. Res. 94, 480–491.
Gyllenstein, U., Wharton, D., Joseffson, A., et al., 1991. Paternal inheritance of mitochondrial DNA in mice. Nature 352, 255–257.
Hähnel, P.S., Thaler, S., Antunes, E., et al., 2008. Targeting AKT signaling sensitizes cancer to cellular immunotherapy. Cancer Res. 68 (10), 3899–3906.
Halban, P.A., Wollheim, C.B., 1980. Intracellular degradation of insulin stores by rat pancreatic islets *in vitro:* an alternative pathway for homeostases of pancreatic insulin content. J. Biol. Chem. 255, 6003–6006.
Harashima, N., Inao, T., Imamura, R., et al., 2012. Roles of PI3K/Akt pathway and autophagy in TLR3 signaling-induced apoptosis and growth arrest of human prostate cancer. Cancer Immunol. Immunother. 61, 667–676.
He, W., Liu, Q., Wang, L., et al., 2007. TLR4 signaling promotes immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance. Mol. Immunol. 44, 2850–2859.
Hocking, L.J., Whitehouse, A., Helfrich, M.H., 2012. Autophagy: a new player in skeletal maintenance? J. Bone Miner. Res. 27, 1439–1447.
Hoffman, W.H., Shacka, J.J., Andjelkovic, A.V., 2012. Autophagy in the brains of young patients with poorly controlled TIDM and fatal diabetic ketoacidosis. Exp. Mol. Path. 93, 273–280.
Hossain, A., Radwan, F.F.Y., Doonan, B.P., et al., 2012. A possible crosstalk between autophagy and apoptosis in generating an immune response in melanoma. Apoptosis 17, 1066–1078.
Hoyer-Hansen, M., Jaattela, M., 2007. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell Death Differ. 14, 193–205.
Hu, S.-Y., Tai, C.C., Li, Y.H., et al., 2012. Progranulin compensates for blocked IGF-1 signaling to promote myotube hypertrophy in C2C12 myoblast via the PI3K/Akt/mTOR pathway. FEBS Lett. 586 (19), 3485–3492.
Huang, C., Andres, A.M., Ratliff, E.P., et al., 2011. Preconditioning involves selective mitophagy mediated by Parkin and p26/SQSTM1. PLoS ONE 6, 20975.
Isaksen, P., Holland, P., Simonsen, A., 2013. The role of ALFY in selective autophagy. Cell Death Differen. 20, 12–20.
Ishibashi, K., Uemura, T., Waguri, S., et al., 2012. Atg16L1, an essential factor for canonical autophagy, participates in hormone secretion from PC12 cells independently of autophagic activity. Mol. Cell. Biol. 23, 3193–3202.
Jackson, W.T., Giddings Jr., T.H., Taylor, M.P., et al., 2005. Subversion of cellular autophagosomal machinery by RNA viruses. PLoS Biol. 3, 861–871.
Janecek, S., 2002. A motif of a microbial starch-binding domain found in human genethonin. Bioinformatics 18, 1534–1537.
Janovick, J., Stewart, D.M., Jacob, D., et al., 2013. Restoration of testis function in hypogonadotropic hypogonadal mice harboring a misfolded GnrHR mutant by pharmacoperones drug therapy. Proc. Natl Acad. Sci. USA 110, 21030–21035.

Jia, H., Kast, R.J., Steffan, J.S., et al., 2012. Selective histone deacetylase (HDAC) inhibition imparts beneficial effects in Huntington's disease mice: implications for the ubiquitin-proteasomal and autophagy systems. Hum. Mol. Genet. 21, 5280–5293.

Jiang, S., Wells, C.D., Roach, P.J., 2011. Starch-binding domain-containing protein 1 (Stbd1) and glycogen metabolism: identification of the Atg8 family interacting motif (AIM) in Stbd1 required for interaction with GABARAPL1. Biol. Chem. Res. Commun. 413, 420–425.

Johansen, T., Lemark, T., 2011. Selective autophagy mediated by autophagic adaptor proteins. Autophagy 7 (3), 279–296.

Jung, C.H., Ro, S.H., Cao, J., et al., 2010. mTOR regulation of autophagy. FEBS Lett. 585, 1287–1295.

Kadowaki, M., Karim, M.R., 2009. Cytosolic LC3 ratio as a quantitative index of macroautophagy. Methods Enzymol. 452, 199–213.

Kageyama, Y., Zhang, Z., Sesaki, H., 2011. Mitochondrial division: molecular machinery and physiological functions. Curr. Opin. Cell Biol. 23, 427–434.

Kageyama, Y., Zhang, Z., Roda, R., et al., 2012. Mitochondrial division ensures the survival of postmitotic neurons by suppressing oxidative damage. J. Cell Biol. 197, 535–551.

Kang, M.R., Kim, M.S., Oh, J.E., et al., 2009. ATG2B, ATG5, ATG9B, and ATG12 in gastric and colorectal cancers with microsatellite instability. J. Pathol. 217, 702–706.

Kapuy, O., Vinod, P.K., Mandl, J., et al., 2013. A cellular stress-directed bistable switch controls the crosstalk between autophagy and apoptosis. Mol. BioSyst. 9, 296–306.

Karim, R., Kanazawa, T., Daigaku., Y., et al., 2007. Cytosolic LC3 ratio as a sensitive index of macroautophagy in isolated rat hepatocytes and H4-11-E cells. Autophagy 3, 553–560.

Kaushik, S., Rodriguez-Navarro, J.A., Arias, E., et al., 2011. Autophagy in hypothalamic AgRP neurons regulates food intake and energy balance. Cell Metab. 14, 173–183.

Kawaguchi, Y., Kovacs, J.J., McLaurin, A., et al., 2003. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 115, 727–788.

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. 11, 373–384.

Kidgotko, O.V., Kustova, M.Y., Sokolova, V.A., et al., 2013. Transmission of human mitochondrial DNA along the paternal lineage in transmitochondrial mice. Mitochondrion 13, 330–335.

Killian, M.S., 2012. Dual role of autophagy in HIV-1 replication and pathogenesis. Aids Res. Therap. 9, 1–13.

Kim, H.-S., Montana, V., Jang, H.J., et al., 2013. Epigallocatechin-gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. J. Biol. Chem. 288, 22693–22705.

Kimura, S., Fujita, N., Noda, T., Yoshimori, T., 2009. Monitoring autophagy in mammalian cultured cells through the dynamics of LC3. Methods Enzymol. 452, 1–12.

Kirkin, V., McEwan, D., Novak, I., et al., 2009. A role for ubiquitin in selective autophagy. Mol. Cell 34, 259–269.

Klionsky, D.J., 2012. A human autophagy interaction network. Autophagy 8, 439–441.

Klionsky, D.J., Cuervo, A.M., Seglen, P.O., 2007. Methods for monitoring autophagy from yeast to human. Autophagy 3, 181–206.

Klionsky, D.J., Abdalla., F.C., Abeliovich, H., et al., 2012. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 8, 445–544.

Knaevelsrud, H., Ahlquist, T., Merok, M.A., et al., 2010. UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy. Autophagy 6, 863–870.

Knöferle, J., Koch, J.C., Ostendorf, T., et al., 2010. Mechanisms of acute axonal degeneration in the optic nerve *in vivo*. PNAS 107, 6064–6069.

Komatsu, M., Ichimura, Y., 2010. Selective autophagy regulates various cellular functions. Genes Cells 15, 923–2010.

Kraft, C., Deplazes, A., Sohrman, M., et al., 2008. Mature ribosomes are selectively degraded upon starvation by an autophagy pathway requiring the Ubp3p/Bre5p ubiquitin protease. Nat. Cell Biol. 10, 602–610.

Kroemer, G., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell 40, 280–293.

Kuma, A., Matsui, M., Mizushima, N., 2007. LC3, and autophagosome marker, can be incorporated into protein aggregates independent of autophagy. Autophagy 3, 323–328.

Kvam, E., Goldfarb, D.S., 2004. Nvj1p is the outer-nuclear-membrane receptor for oxysterol-binding protein homolog Osh1p in *Saccharomyces cerevisiae*. J. Cell Sci. 117, 4959–4968.

Kyei, G.B., Dinkins, C., Davis, A.S., et al., 2009. Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages. J. Cell Biol. 186, 255–268.
Lee, A.C., Fenster, B.E., Ito, H., et al., 1999. Ras proteins induce senescence by altering the intracellular levels of reactive species. J. Biol. Chem. 274, 7936–7940.
Li, Y., Hu, Z., Chen, 2012. Taurine attenuates methamphetamine-induced autophagy and apoptosis in PC12 cells through mTOR signaling pathway. Toxicol. Lett. 215, 1–7.
Liang, X.H., Jackson, S., Seaman, M., et al., 1999. Induction of autophagy and inhibition of tumorigenesis by Beclin 1. Nature 402, 672–676.
Liu, E.Y., Ryan, K.M., 2012. Autophagy and cancer: issues we need to digest. J. Cell Sci. 125, 2349–2358.
Liu, Y., Tan, Y.L., Zhang, X., et al., 2014. Small molecular probes to quantify the functional fraction of a specific protein in a cell with minimal folding equilibrium shifts. Proc. Natl Acad. Sci. USA 111, 4449–4454.
Lo Ré, A.E., Fernández-Barrena, M.G., Almada, L.L., et al., 2012. Novel AKT1–GL13–VWP1 pathway mediates KRAS oncogene-induced autophagy in cancer cells. J. Biol. Chem. 287, 25325–25334.
Lozy, F., Karantza, V., 2012. Autophagy and cancer cell metabolism. Rev. Cell Dev. Biol. 23, 395–401.
Lu, J.V., Walsh, C.M., 2012. Programmed necrosis and autophagy in immune function. Immunol. Rev. 249, 205–217.
Luo, S., Rubinsztein, D.C., 2010. Apoptosis blocks Beclin 1-dependent autophagosome synthesis: an effect rescued by Bcl-xL. Cell Death Differ. 17 (268), 277.
Macintosh, G.C., Bassham, D.C., 2011. The connection between ribophagy and ribosomal RNA decay. Autophagy 7 (6), 662–663.
Master, S., Rampini, S., Davis, A., et al., 2008. *Mycobacterium tuberculosis* prevents inflammasome activation. Cell Host Microbe 3, 224–232.
McLaughlin, M., Vandenbroeck, K., 2011. The endoplasmic reticulum protein folding factor and its chaperones: new targets for drug discovery. Brit. J. Pharmcol. 162, 328–345.
Mei, Y., Minfei, S., Gaurav, S., et al., 2013. Intrinsically disordered regions in autophagy proteins. Proteins 82, 265–278.
Meijer, A.J., Godogno, P., 2004. Regulation and role of autophagy in mammalian cells. Int. J. Biochem. Cell Biol. 36, 2445–2462.
Mercer, T.R., Neph, S., Dinger, M.E., et al., 2011. The human mitochondrial transcriptome. Cell 146, 645–658.
Mijaljica, D., Devenish, R.J., 2013. Nucleophagy at a glance. J. Cell Sci. 126, 4325–4330.
Mijaljica, D., Prescott, M., Devenish, R.J., 2010. The intricacy of nuclear membrane dynamics during nucleophagy. Nucleus 1, 213–223.
Mijaljica, D., Prescott, M., Devenish, R.J., 2012. A late form of nucleophagy in *Saccharomyces cerevisiae*. PLoS ONE 7 (6), e40013.
Minibayeva, F., Dmitrieva, S., Ponomareva, A., et al., 2012. Oxidative stress-induced autophagy in plants: the role of mitochondria. Plant Physiol. Biochem. 59, 11–19.
Miracco, C., Meng, G.C., Franchi, A., et al., 2010. Beclin 1 and LC3 autophagic gene expression in cutaneous melanocytic lesins. Hum. Pathol. 41, 503–512.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2973.
Mizushima, N., Kuma, A., 2008. Autophagosomes in GFP-LC3 transgenic mice. Methods Mol. Biol. 445, 119–124.
Mizushima, N., Yoshimori, T., 2007. How to interpret LC3 immunoblotting? Autophagy 3, 542–545.
Mohapatra, P., Preet, R., Das, D., et al., 2012. Quinacrine-mediated autophagy and apoptosis in colon cancer cells is through a p53- and p21-dependent mechanism. Oncol. Res. 20, 81–91.
Momoi, T., 2006. Conformational diseases and ER stress-mediated cell death: apoptotic cell death and autophagic cell death. Curr. Mol. Med. 6, 111–118.
Nakai, A., Yamaguchi, O., Takeda, T., et al., 2007. The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat. Med. 13, 619–624.
Nakajima, H., Hamasaki, T., Tarui, S., 1995. Phosphofructokinase: recent advances in molecular biology. Muscle Nerve 3, S28–S34.
Nemchenko, A., Chiong, M., Turer, A., et al., 2011. Autophagy as a therapeutic target in cardiovascular disease. J. Mol. Cell. Cardiol. 300, H2123–H2134.
Nicoletti, F., Fagone, P., Meroni, P., et al., 2011. mTOR as a multifunctional therapeutic target in HIV infection. Drug Discov. Today 16, 715–721.
Nixon, R.A., Yang, D.S., 2011. Autophagy failure in Alzheimer's disease – locating the primary defect. Neurobiol. Dis. 43, 38–45.

Novak, I., 2012. Mitophagy: a complex mechanism of mitochondrial removal. Antioxid. Redox Signal. 17, 794–802.
Odagiri, S., Tanji, K., Mori, F., et al., 2012. Autophagic adaptor protein NBR1 is localized in Lewy bodies and glial cytoplasmic inclusions and is involved in aggregate formation in α-synucleinopathy. Acta Neuropathol. 124, 173–186.
Ohsumi, Y., Mizushima, N., 2004. Two ubiquitin-like conjugation systems essential for autophagy. Semin. Cell Develop. Biol. 15, 231–236.
Oka, T., Hikoso, S., Yamaguchi, O., et al., 2012. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. Nature 485, 251–256.
Olsen, M., Kornbluth, S., 2001. Mitochondria in apoptosis and human disease. Curr. Mol. Med. 1, 91–122.
Orvedahl, A., Alexander, D., Talloczy, Z., et al., 2007. HSV-1 1CP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 1, 23–35.
Otera, H., Wang, C., Cleland, M.M., et al., 2010. Mff is an essential factor for mitochondrial recruitment of Drpl during mitochondrial fission in mammalian cells. J. Cell Biol. 191, 1141–1158.
Ouimet, M., Marcel, Y.L., 2012. Regulation of lipid droplet cholesterol efflux from macrophage foam cells. Arterioscler. Thromb. Vasc. Biol. 32, 575–581.
Ouyang, L., Shi, Z., Zhao, S., et al., 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy, and programmed necrosis. Cell Prolif. 45, 487–498.
Ovadje, P., Chochkeh, M., Akbari-Asl, P., et al., 2012. Selective induction of apoptosis and autophagy through treatment with dandelion root extract in human pancreatic cancer cells. Pancreas 41, 1039–1047.
Pagliarini, V., Wirawan, E., Romagnoli, A., et al., 2012. Proteolysis of Ambra1 during apoptosis has a role in the inhibition of the autophagic pro-survival response. Cell Death Differ. 19, 1495–1504.
Palmer, C.S., Osellame, L.D., Latine, D., et al., 2011. MiD49 and Mid51, new components of the mitochondrial fission machinery. EMBO Rep. 12, 565–573.
Park, S-H., Kim, J-H., Chi, G.Y., et al., 2012. Induction of apoptosis and autophagy by sodium selenite in A549 human lung carcinoma cells through generation of reactive oxygen species. Toxicol. Lett. 212, 252–261.
Perrin, A.J., Jiang, X., Birmingham, C.L., et al., 2004. Recognition of bacteria in the cytosol of mammalian cells by the ubiquitin system. Curr. Biol. 14, 806–811.
Petiot, A., Ogier-Denis, E., Blommaart, E.F., et al., 2000. Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. J. Biol. Chem. 275, 992–998.
Pickford, F., Masliah, E., Britschgi, M., 2008. The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid β accumulation in mice. J. Clin. Invest. 118, 2190–2199.
Railborg, C., Stenmark, H, 2009. The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature 458, 445–452.
Rambold, A.S., Kostelecky, B., Elia, N., et al., 2011a. Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. Proc. Natl Acad. Sci. USA 108, 10190–10195.
Rambold, A.S., Kostelecky, B., Lippincott-Schwartz, J., 2011b. Together we are stronger. Fusion protects mitochondria from autophagosomal degradation. Autophagy 7 (12), 1568–1569.
Randow, F., 2011. How cells deploy ubiquitin and autophagy to defend their cytosol from bacterial invasion. Autophagy 7 (3), 304–309.
Reichert, A.S., Neupert, W., 2004. Mitochondriomics or what makes us breathe. Trends Genet. 20, 555–562.
Roberts, P., Moschitch-Moshkovitz, S., Kvam, E., et al., 2003. Piecemeal microautophagy of nucleus in *Saccharomyces cerevisiae*. Mol. Biol. Cell 14, 129–141.
Romanov, J., Walczak, M., Lbiricu, I., et al., 2012. Mechanism and functions of membrane binding by the Atg5–Atg12/Atg16 complex during autophagosome formation. EMBO J. 31, 4304–4317.
Romisch, K., 2005. Endoplasmic reticulum-associated degradation. Annu. Rev. Cell Develop. Biol. 21, 435–456.
Rong, Y., McPhee, C.K., Deng, S., et al., 2011. Spinster is required for autophagic lysosome reformation and mTOR reactivation following starvation. Proc. Natl Acad. Sci. USA 108, 7826–7831.
Rubinszstein, D.C., DiFiglia, M., Heintz, N., et al., 2005. Autophagy and its possible roles in nervous system diseases, damage, and repair. Autophagy 1, 11–22.
Rubio, N., Coupienne, I., Di Valentin, E., et al., 2012. Spatiotemporal autophagic degradation of oxidatively damaged organelles after photodynamic stress is amplified by mitochondrial reactive oxygen species. Autophagy 8, 1312–1324.
Sandberg, M., Borg, L.A.H., 2006. Intracellular degradation of insulin and crinophagy are maintained by nitric oxide and cyclo-oxygenase 2 activity in isolated pancreatic islets. Biol. Cell 98 (5), 307–315.

Sanjuan, M.A., Milasta, S., Green, D.R., 2009. Toll-like receptor signaling in the lysosomal pathways. Immunol. Rev. 227, 203–220.
Sarbassov, D.D., Ali, S.M., Sabatini, D.M., 2005. Growing roles for the mTor pathway. Curr. Opin. Biol. 17, 596–603.
Sasaki, M., Miyakoshi, M., Sato, Y., et al., 2010. Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab. Invest. 90, 835–843.
Sato, M., Sato, K., 2012. Maternal inheritance of mitochondrial DNA. Autophagy 8, 424–425.
Sato, M., Sato, K., 2013. Maternal inheritance of mitochondrial DNA by diverse mechanisms to eliminate paternal mitochondrial DNA. Biochim. Biophys. Acta 1833, 1979–1984.
Schroder, L.A., Ortiz, M.V., Dunn Jr., W.A., 2008. The membrane dynamics of pexophagy are influenced by Sarlp in *Pichia pastoris*. Mol. Biol. Cell 19, 4888–4899.
Sciarretta, S., Hariharan, N., Monden, Y., et al., 2011. Is autophagy in response to ischemia and reperfusion protective or detrimental for the heart? Pediatr. Cardiol. 32, 275–281.
Shay, J.W., Roninson, I.B., 2004. Hallmarks of senescence in carcinogenesis and cancer therapy. Oncogene 23, 2919–2933.
Shelly, S., Lukinova, N., Bambina, S., et al., 2009. Autophagy is an essential component of *Drosophila* immunity against vesicular stomatitis virus. Immunity 30, 588–598.
Shen, Y., Li, D.D., Wang, L.L., et al., 2008. Decreased expression of autophagy-related proteins in malignant epithelial ovarian cancer. Autophagy 16, 1067–1068.
Shi, C.S., Kehri, J.H., 2008. MyD88 and Trif target Beclin 1 to trigger autophagy in macrophages. J. Biol. Chem. 283, 33175–33182.
Singh, R., Cuervo, A.M., 2011. Autophagy in the cellular energetic balance. Cell Metab. 13, 495–504.
Singh, R., Cuervo, A.M., 2012. Lipophagy: connecting autophagy and lipid metabolism. Int. J. Cell Biol. 2012, 282041.
Singh, R., Kaushik, S., Wang, Y., et al., 2009. Autophagy regulates lipid metabolism. Nature 458, 1131–1135.
Sir, D., Ou, J.H., 2010. Autophagy in viral replication and pathogenesis. Mol. Cells 29, 1–7.
Sir, D., Kuo, C-F., Tian, Y., et al., 2012. Replication of hepatitis C virus RNA on autophagosomal membranes. J. Biol. Chem. 287, 18036–18043.
Stasyk, O.V., Nazarko, T.Y., Sibirny, A.A., 2008. Methods of plate pexophagy monitoring and positive selections for ATG gene cloning in yeasts. Methods Enzymol. 451, 229–239.
St. John, J.C., Schatten, G., 2004. Paternal mitochondrial DNA transmission during nonhuman primate nuclear transfer. Genetics 167, 897–905.
Subramani, S., Malhotra, V., 2013. Non-autophagic roles of autophagic-related proteins. EMBO Rep. 14, 143–151.
Takahashi, Y., Coppola, D., Matsushita, N., et al., 2010. Bif-1 interacts with Beclin 1 through UVRAG and regulates autophagy and tumorigenesis. Nat. Cell Biol. 9, 1142–1151.
Tamura, N., Oku, M., Sakai, Y., 2010. Atg8 regulates vacuolar membrane dynamics in a lipidation-independent manner in *Pichia pastoris*. J. Cell Sci. 123, 4107–4116.
Tan, M.L., Ooi, J.P., Ismail, N., et al., 2009. Programmed cell death pathways and current antitumor targets. Pharmaceut. Res. 26, 1547–1560.
Tashiro, Y., Urushitani, M., Inoue, H., et al., 2012. Motor neuron-specific disruption of proteasome, but not autophagy replicates amyotrophic lateral sclerosis. J. Biol. Chem. 287, 42984–42994.
Thorburn, A., 2008. Apoptosis and autophagy: regulatory connections between two supposedly different processes. Apoptosis 13 (1), 1–9.
Townsend, K.N., Hughson, L.R., Schlie, K., et al., 2012. Autophagy inhibition in cancer therapy: metabolic considerations for antitumor immunity. Immunol. Rev. 249, 176–194.
Ucar, A., Gupta, S.K., Fiedler, J., et al., 2012. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. Nat. Commun. 3, 1078.
Uttenweiler, A., Schwarz, H., Neumann, H., et al., 2007. The vacuolar transporter chaperone (VTC) complex is required for microautophagy. Mol. Biol. Cell 18, 166–175.
Uversky, V., Oldfield, C., Dunker, A., 2008. Intrinsically disordered proteins in human disease: introducing the D2 concept. Annu. Rev. Biophys. 37, 215–246.
Vaccaro, M.I., 2012. Zymophagy: selective autophagy of secretory granules. Int. J. Cell Biol. 2012, 396705.
Valdor, R., Macian, F., 2012. Autophagy and the regulation of the immune response. Pharmacol. Res. 66, 475–483.
van Zutphen, T., van der Klei, I.J., Kiel, J.A., 2008. Pexophagy in *Hansenula polymorpha*. Methods Enzymol. 451, 197–215.

Velikkakath, A.K.G., Nishimora, T., Oita, E., et al., 2012. Mammalian Atg2 proteins are essential for autophagosome formation and important for regulation of size and distribution of lipid droplets. Mol. Biol. Cell 23, 896–909.
Virgin, H.W., Levine, B., 2009. Autophagy genes in immunity. Nat. Immunol. 10, 461–470.
Visagie, M.H., Joubert, A.M., 2011. 2-Methoxyestradiol-bis-sulfamate induces apoptosis and autophagy in a tumorigenic breast epithelial cell line. Mol. Cell. Biochem. 357, 343–352.
Wang, X.-J., Chu, N.-Y., Wang, Q.-H., 2012. Newly synthesized bis-benzimidazole derivatives exerting anti-tumor activity through induction of apoptosis and autophagy. Bioorg. Med. Chem. Lett. 22, 6297–6300.
Winslow, A.R., Rubinsztein, D.C., 2011. The Parkinson disease protein α-synuclein inhibits autophagy. Autophagy 7, 429–431.
Wullschleger, S., Loewith, R., Hall, M.N., 2006. TOR signaling in growth and metabolism. Cell 124, 471–484.
Xia, H.G., Zhang, L., Chen, G., et al., 2010. Control of basal autophagy by calpain1 mediated cleavage of ATG5. Autophagy 6 (1), 61–66.
Xie, Z., Klionsky, D.J., 2007. Autophagosome formation: core machinery and adaptations. Nat. Cell Biol. 9, 1102–1109.
Xu, Y., Jagannath, C., Liu, X.D., et al., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144.
Yahiro, K., Satoh, M., Nakano, M., et al., 2012. Low-density lipoprotein receptor-related protein-1 (LRP1) mediates autophagy and apoptosis caused by *Helicobacter pylori* VacA. J. Biol. Chem. 287, 31104–31115.
Yan, M., Zhu, P., Liu, H.M., et al., 2007. Ethanol induced mitochondria injury and permeability transition pore opening: role of mitochondria in alcoholic liver disease. World J. Gastroenterol. 13, 2352–2356.
Yang, F., Chen, W.D., Deng, R., et al., 2012. Hirsutanol A induces apoptosis and autophagy via reactive oxygen species accumulation in breast cancer MCF-7 cells. J. Pharmacol. Sci. 119, 214–220.
Yang, S., Wang, X., Contino, G., et al., 2011. Pancreatic cancers require autophagy for tumor growth. Genes Dev. 25, 717–729.
Ye, Y-C., Wang, H-J., Yu, L., et al., 2012. RIP1-mediated mitochondrial dysfunction and ROS production contributed to tumor necrosis factor alpha-induced L929 cell necroptosis and autophagy. Inter. Immunopharm. 14, 674–682.
Yoshioka, A., Miyata, H., Doki, Y., et al., 2008. LC3: and autophagosome marker, is highly expressed in gastrointestinal cancer. Int. J. Oncol. 33, 461–468.
Young, A.R.J., Narita, M., Ferreira, M., et al., 2009. Autophagy mediates the mitotic senescence transition. Genes Develop. 23, 798–803.
Yu, W.H., Cuervo, A.M., Kumar, A., et al., 2005. Macroautophagy – a novel beta-amyloid peptide-generating pathway activated in Alzheimer's disease. J. Cell Biol. 171, 87–98.
Yue, Z., 2007. Regulation of neuronal autophagy in axon. Autophagy 3 (2), 139–141.
Yue, Z., Jin, S., Yang, C., et al., 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl Acad. Sci. USA 100, 15077–15082.
Zhan, Y., Gong, K., Chen, C., et al., 2012. P38 MAP kinase functions as a switch in MS-275-induced reactive oxygen species-dependent autophagy and apoptosis in human colon cancer cells. Free Rad. Biol. Med. 53, 532–543.
Zhang, S., Clabough, E.B.D., Sarkar, S., et al., 2010. Deletion of the Huntingtin polyglutamine stretch enhances neuronal autophagy and longevity in mice. PLoS Genet. 6, 1–15.
Zhao, X., Li, N., Guo, W., et al., 2004. Further evidence for paternal inheritance of mitochondrial DNA in the sheep (*Ovis aries*). Heredity 93, 399–403.
Zhao, Z., Trackray, L.B., Miller, B.C., et al., 2007. Coronavirus replication does not require the autophagy gene Atg5. Autophagy 3 (6), 581–585.
Zhou, Z., Jiang, X., Liu, D., et al., 2009. Autophagy is involved in influenza a virus replication. Autophagy 5, 321–328.
Zhu, H., Tannous, P., Johnstone, J.L., et al., 2007. Cardiac autophagy is a maladaptive response to hemodynamic stress. J. Clin. Invest. 117, 1782–1793.
Ziv, N.E., Spira, M.E., 1995. Axotomy induced a transient and localized elevation of the free intracellular calcium concentration to the millimolar range. J. Neurophysiol. 74, 2625–2637.
Zoncu, R., Efevan, A., Sabatini, D.M., 2011. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat. Rev. Mol. Cell Biol. 12, 21–35.
Zungu, M., Schisler, J., Willis, M.S., 2011. Regulation of mitochondrial fusion and fission by ubiquitin and small ubiquitin-like modifier and their potential relevance in the heart. Circ. J. 75, 2513–2521.

CHAPTER

2

Regulation of Autophagy by Amino Acids

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OUTLINE

Abstract

Insulin and amino acids are two major regulators of macroautophagy. They both act through the MTOR complex 1 (MTORC1) signaling pathway to inhibit macroautophagy upstream of the ULK1 complex that initiates the autophagosome formation. In contrast to insulin that stimulates MTORC1 via its receptor and class I phosphatidylinositol 3-kinase, amino acids can activate MTORC1 by several mechanisms involving cell surface G-protein-coupled receptors, t-RNA synthetases, small Rag GTPases and the mitochondrial enzyme glutamate dehydrogenase. The location of MTORC1 at the lysosomal membrane as well as the positioning of lysosomes in cell is an important aspect of the regulation of kinase activity. In addition, amino acids regulate autophagy by controlling the dissociation of the Beclin-1–Bcl-2 by c-Jun N-terminal kinase 1 (JNK1).

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In the absence of amino acids, JNK1 triggers the dissociation of the Beclin-1–Bcl-2 complex by phosphorylating Bcl-2. Then Beclin-1 associates with other members of the PI3K complex (PI3K CIII/Vps15, ATG14L and AMBRA1) to nucleate the autophagosomal membrane in a coordinated manner with the ULK1 complex.

INTRODUCTION

Macroautophagy (hereafter referred to as autophagy) occurs at a basal rate in most cells, where it acts as a cytoplasmic quality control mechanism to eliminate protein aggregates and damaged organelles (Mizushima and Komatsu, 2011). The physiological importance of basal autophagy in maintaining tissue homeostasis has been demonstrated in conditional brain and liver *Atg* knockout mouse models (Mizushima and Komatsu, 2011). These studies have also demonstrated the role of autophagy in preventing the deposition of aggregate-prone proteins in the cytoplasm, and the contribution of autophagy to the elimination of ubiquitinated proteins that are efficient substrates for the proteasome (Rubinsztein *et al.*, 2011). The anti-aging role of autophagy probably depends, at least in part, on its quality control function on the cytoplasmic structure that limits the deposition of aggregate-prone proteins and the formation of damaging reactive oxygen species (ROS) by mitochondria (Rubinsztein *et al.*, 2011).

On the other hand, when the supply of nutrients is limited, the stimulation of autophagy contributes to the lysosomal recycling of nutrients to maintain protein synthesis and glucose synthesis from amino acids (in the liver), substrates for oxidation and ATP production in the mitochondria (Meijer and Codogno, 2009) and inhibition of the default apoptotic pathway (Kroemer *et al.*, 2010). *In vivo*, at birth the sudden interruption of the supply of nutrients via the placenta triggers autophagy in newborn mouse tissues to maintain energy homeostasis and survival (Mizushima and Komatsu, 2011). Starvation-induced autophagy is also important in adult mammalian muscle and liver to degrade proteins in order to produce amino acids for the hepatic synthesis of glucose which is needed as substrate for energy production in brain and erythrocytes (Meijer and Codogno, 2009).

Amino acids are potent physiological feedback inhibitors of autophagy so that autophagic flux decreases with increasing amino acid concentration (Meijer and Codogno, 2009). They carry out this function by inhibiting the formation of autophagosomes. In this chapter, we will discuss the amino acid- and starvation-dependent regulation of autophagy that mostly impinges on two complexes that contain MTOR and Beclin-1 as core proteins.

OVERVIEW OF THE INSULIN-AMINO ACID-MTOR SIGNALING PATHWAY

The major signaling pathway controlling autophagy is the insulin-amino acid-MTOR signaling pathway (Kim and Guan, 2011; Laplante and Sabatini, 2012) (Figure 2.1). This pathway converges at the Ser/Thr kinase signaling complex MTORC1 (mammalian target of rapamycin complex 1), containing the central kinase MTOR associated with raptor (regulatory associated protein of MTOR, a protein that acts as a scaffold for MTOR-mediated phosphorylation of MTOR substrates), the protein mLST8 of unknown function, the inhibitory proteins PRAS40 and Deptor, and a few other proteins regulating the assembly and

stability of MTORC1. As part of MTORC1, MTOR activity is inhibited by rapamycin which is not the case when MTOR is a part of another complex, named MTORC2 (Kim and Guan, 2011; Laplante and Sabatini, 2012).

Upstream of MTOR, the activation of the insulin-signaling pathway starts at the plasma membrane with the binding of insulin to its receptor, which allows the recruitment of IRS1/IRS2 (IRS, insulin receptor substrate) and the activation of class I phosphatidylinositol 3-kinase (PI3K CI), producing PtdIns(3,4)P_2 (phosphatidylinositol 3,4-biphosphate) and PtdIns(3,4,5)P_3 (phosphatidylinositol 3,4,5-triphosphate). Both PtdIns(3,4)P_2 and PtdIns(3,4,5)P inhibit autophagy, in contrast to PtdIns(3)P (phosphatidylinositol 3-phosphate) (the product of PI3K CIII) which is essential for autophagosome formation (Meijer and Codogno, 2009). The plasma membrane lipid PtdIns(3,4,5)P_3 activates the phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (PKB), which regulate MTOR and autophagy through the inhibition of the heterodimer TSC1/TSC2 (tuberous sclerosis complex). The activity of MTOR is in turn inhibited by the heterodimer TSC1/TSC2 (tuberous sclerosis complex): it acts as a GTPase-activating protein complex for the small G-protein Rheb (Ras homolog enriched in brain). $Rheb^{GTP}$, not $Rheb^{GDP}$, binds and activates MTOR. PKB phosphorylates TSC2, which inactivates the TSC1/TSC2 complex and stimulates MTOR. In addition, PKB also inhibits autophagy by phosphorylation of Beclin-1 (Wang *et al.*, 2012), and exerts long-term control on autophagy by phosphorylating, and inhibiting, FoxO3, a transcription factor involved in the synthesis of some ATG proteins. For activation of MTOR, the presence of insulin (or other growth factors) alone is not sufficient: the presence of amino acids is indispensable (Meijer and Codogno, 2009).

Downstream of MTOR, the second part of the insulin-signaling pathway involves components such as S6K (70 kDa S6 kinase), 4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1), eIF2 (eukaryotic initiation factor 2)-kinase, and eEF-2 kinase, proteins that are involved in regulating protein synthesis (Figure 2.1). Apart from being engaged in protein synthesis, S6K is also able to phosphorylate, and inhibit, IRS1 (Kim and Guan, 2011; Laplante and Sabatini, 2012): this feedback inhibition may be considered as a mechanism to prevent overactivation of the MTOR pathway in order to allow some autophagy to continue even under nutrient-rich conditions (Meijer and Codogno, 2009). At the same time, MTOR inhibits autophagy in the shortterm by phosphorylating, and inactivating, ULK1, the mammalian homologue of yeast Atg1, which in the inactive form is part of a protein complex also containing Atg13, FIP200 (the mammalian homologue of Atg17) and Atg101 (Mizushima and Komatsu, 2011). MTOR also phosphorylates Atg13 which stabilizes this protein complex. Under autophagy-inducing conditions – e.g., starvation – inhibition of MTOR results in dephosphorylation of ULK1 and Atg13, followed by dissociation of the protein complex upon which ULK1 becomes active. Long-term regulation of autophagy by MTOR occurs by phosphorylation of the transcription factor EB (TFEB), a master regulator of the synthesis of ATG proteins (an ATG set different from that controlled by FoxO3) and of lysosomal biogenesis, preventing translocation of TFEB to the nucleus (Settembre *et al.*, 2012). The activity of TFEB is also inhibited by nutrient- and growth factor-dependent extracellular signal-regulated kinase 2 (Erk2) in an MTOR-independent fashion. Apart from its role in the opposite regulation of protein synthesis and autophagy, the insulin-MTOR pathway regulates major metabolic pathways such as the metabolism of glucose, lipids and ATP production (Laplante and Sabatini, 2012; Meijer and Codogno, 2009).

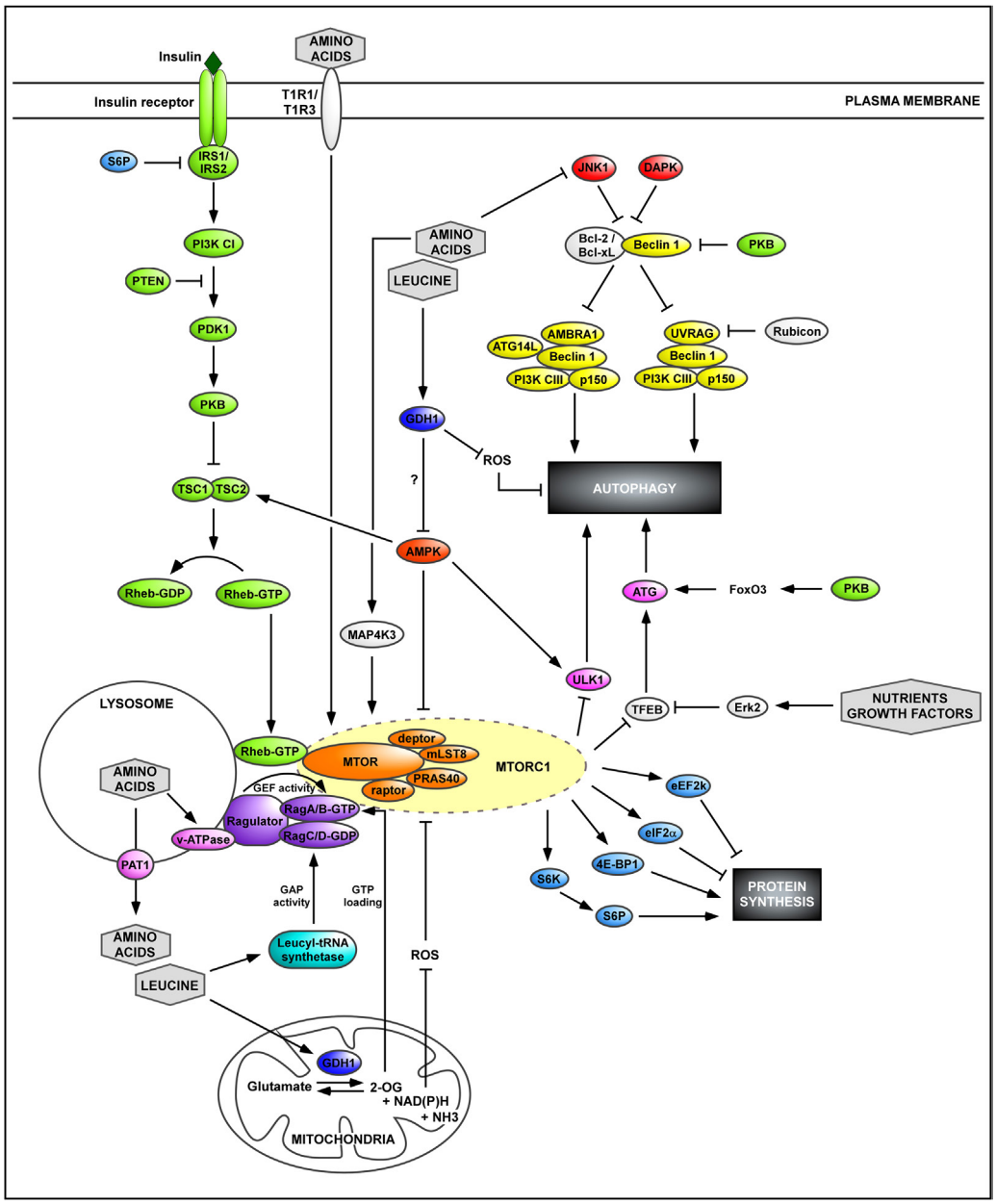

FIGURE 2.1 Amino acids input to MTORC1 and Beclin-1 for the regulation of autophagy. MTOR and Beclin-1 are two major modulators of autophagy regulated by amino acids with opposite effects on autophagy: whereas MTOR inhibits autophagy, Beclin-1 positively regulates this process. To be active, the Ser/Thr kinase MTOR-containing complex 1, MTORC1, has to localize at the lysosomal membrane, where its coactivator Rheb-GTP resides. This phenomenon is regulated by the small GTPases of the Rag family: in response to amino acids, Rag promotes the translocation of MTOR to the lysosomal membrane and its consecutive activation. Rag proteins are

heterodimers of two subunits, RagA/B and RagC/D, where RagA/B is linked to GTP and RagC/D to GDP in the most active form of the dimer. The Rag GTPases are regulated by the v-ATPase, Ragulator and leucyl-tRNA synthetase. In response to amino acids (intralysosomal pool), the v-ATPase, present in the lysosomal membrane, changes its conformation and recruits Ragulator, which displays a guanine nucleotide exchange factor (GEF) activity towards RagA/B, resulting in the formation of RagA/B-GTP. In the presence of leucine (cytosolic pool), leucylation of leucyl-tRNA synthetase reveals its GTPase-activating protein (GAP) activity towards RagC/D, resulting in the formation of RagC/D-GDP. The activity of Rag is also influenced by glutamate dehydrogenase (GDH1), a mitochondrial enzyme directly regulated by leucine, as the production of 2-oxoglutarate by GDH1 stimulates the loading of RagB with GTP. Moreover, GDH1 can also regulate MTOR and autophagy through two other distinct mechanisms: (1) the production of 2-oxoglutarate replenishes the citric acid cycle intermediates, increasing the rate of ATP production and inhibiting AMPK, thereby activating MTOR; (2) the production of NAD(P)H may lead to the reduction of ROS, a potent activator of autophagy which acts through MTOR-dependent and -independent pathways. Probably acting in parallel of the Rag GTPase pathway, MAP4K3 is another protein involved in the regulation of MTOR by amino acids. The extracellular pool of amino acids could be sensed by the plasma membrane receptor of amino acids, T1R1/T1R3, which regulates MTOR and autophagy. The other major complexes regulating autophagy contain the Beclin-1 protein. The core proteins of these two complexes are Beclin-1, PI3K CIII and p150. When associated with ATG14L and AMBRA1, Beclin-1 stimulates the early step autophagosomes formation, downstream of the ULK1 complex. When associated with UVRAG, Beclin-1 positively regulates the formation and the maturation of autophagosomes. Rubicon inhibits the maturation of autophagosomes. In response to amino acids, the kinase JNK1 is inhibited, leading to the formation of a stable complex between Beclin-1 and Bcl-2 which sequesters Beclin-1, in turn inhibiting autophagy.

AMINO ACIDS, MTOR SIGNALING AND THE REGULATION OF AUTOPHAGY

Amino acids play a central role in the regulation of autophagy. The inverse relationship between the concentration of amino acids and the flux through the autophagic pathway was already known in the early days of the studies on hepatic autophagy some 35 years ago (for literature, cf. Meijer and Codogno, 2009). Although the physiological significance of this feedback interaction was clear, at the time there was no clue with regard to the mechanism of this effect. It was wellknown that NH_3, a major product of amino acid catabolism, inhibited autophagic flux because it increased the intralysosomal pH due to its acidotropic properties, and thus inhibited intralysosomal degradation and the fusion between lysosomes and autophagosomes. But this did not account for the ability of amino acids to inhibit the formation of autophagosomes. The first indication that the MTOR pathway was involved in autophagy regulation, and that amino acids stimulated its activity, came from studies with hepatocytes showing that inhibition of autophagy by amino acids (leucine in particular) was accompanied by increased phosphorylation of ribosomal protein S6, a measure of *in situ* S6K activity, in a rapamycin-sensitive manner (Blommaart *et al.*, 1995). Insulin was also able to stimulate S6 phosphorylation, and to inhibit autophagy, but only in the presence of suboptimal, not in the presence of very low or high, amino acid concentrations. These observations have been confirmed, and extended by numerous laboratories, for all eukaryotic cells (Kim and Guan, 2011; Laplante and Sabatini, 2012; Meijer and Codogno, 2009). Interestingly, the starvation hormone glucagon, known to stimulate hepatic autophagic proteolysis in order to provide amino acids for gluconeogenesis, was found to inhibit MTOR signaling (Blommaart *et al.*, 1995). This occurs at least in part through a protein kinase A-dependent mechanism (Meijer and Codogno, 2009).

The mechanism by which amino acids stimulate MTOR activity, and inhibit autophagy, has remained an enigma for a long time (and still is). Amino acids do not appear to stimulate signaling components upstream of MTOR since they do not affect PI3K CI, PKB or TSC1/TSC2, nor do they stimulate MTOR directly (Kim and Guan, 2011; Laplante and Sabatini, 2012). Important for their mechanism of action is that in most cell types, among the various amino acids, leucine, but not the other branched-chain amino acids valine and isoleucine, is most potent in inhibiting autophagy and in stimulating MTOR signaling. Its metabolism is not required and certain nonmetabolizable analogues of leucine are able to mimic its effect (Meijer and Codogno, 2009). In fibroblasts from patients with defects in leucine catabolism, MTOR activity is enhanced (Schriever *et al.*, 2013). Thus, any proposed mechanism of amino acid sensing must account for the high leucine specificity.

Several factors involved in amino acid-MTOR signaling have been described in the past, and they have been reviewed in detail elsewhere (Kim and Guan, 2011; Laplante and Sabatini, 2012; Meijer and Codogno, 2009). These include MAP4K3, Ca^{++} ions, AMPK, PtdIns 3-kinase, the G-protein Rheb, proton-assisted amino acid transporters in the lysosomal membrane, the Rag GTP-ases, p62, phospholipase-D, and inositolphosphate multikinase (IPMK). In none of these studies, however, was the nature of the primary amino acid sensor identified. As will become clear, some of these factors are part of one mechanism responsible for the activation of MTOR activity by amino acids, whereas others participate in mechanisms that are independent of, but act in parallel to, each other.

Rag GTPases, v-ATPase, t-RNA Synthetases and Regulation of Autophagy

A breakthrough in the search for a mechanism of amino acid sensing was the demonstration that the Rag GTPases are essential for the activation of MTOR by amino acids (Kim and Guan, 2011; Laplante and Sabatini, 2012; for reviews) and that active MTORC1 is localized at the lysosomal membrane (Sancak *et al.*, 2010) (Figure 2.1). The Rag proteins form hetero dimers between RagA/B and RagC/D, and in its most active form RagA/B is in the GTP form and RagC/D in the GDP form. It was proposed that the v-ATPase in the lysosomal membrane, in addition to its role in proton pumping, actually functions as the amino acid sensor in MTOR signaling (Zoncu *et al.*, 2011) (Figure 2.1). The v-ATPase responds to an increase in the intralysosomal, rather than the cytosolic, amino acid concentration with a conformational change which causes increased binding of Ragulator, a scaffolding protein complex consisting of five different proteins with guanine nucleotide exchange activity towards RagA and RagB, that anchors the Rag proteins to the lysosomal surface. This promotes the translocation of the MTORC1 complex to the extralysosomal surface, where MTORC1 comes in contact with Rheb and becomes activated. In this mechanism, which requires the proton gradient (Settembre *et al.*, 2012), the activity of the lysosomal proton-assisted amino acid transporter PAT1, responsible for the efflux of amino acids from the lysosomes, controls the concentration of amino acids within the lysosomal lumen and thus the extent of MTORC1 activation (Zoncu *et al.*, 2011) (Figure 2.1). If this mechanism is correct, it must be speculated that among the various amino acids leucine is especially active in inducing the conformation change of the v-ATPase. *In vitro* experiments with isolated lysosomes in which the conditions for binding to the lysosomal membrane, and activation, of MTORC1 were analyzed have suggested that this may indeed be the case (Zoncu *et al.*, 2011).

The fact that MTOR activity is determined by the size of the intralysosomal pool of amino acids implies that the use of compounds such as the v-ATPase inhibitor bafilomycin or the acidotropic agent chloroquine cannot be recommended to estimate autophagic flux by monitoring the accumulation of the autophagosomal marker LC3-II. This is because inhibition of proteolysis within the lysosomes will affect the intralysosomal pool of amino acids, and will lead to underestimation of MTOR activity and overestimation of autophagic flux.

Another very recent study proposes that leucyl-tRNA synthetase is the actual amino acid sensor (Han *et al.*, 2012) (Figure 2.1). The enzyme directly binds to Rag GTPase in a leucine-dependent manner and functions as a GTPase-activating protein for Rag GTPase to activate MTORC1. In this mechanism, leucylation of the tRNA is not required. It is sufficient that leucine binds to the leucine-binding domain of the leucyl-tRNA synthetase and activates the enzyme, as measured by ATP-[^{32}P]PP$_i$ exchange activity (Han *et al.*, 2012). In this context, it is noteworthy that diadenosine tetraphosphate (Ap4A), a byproduct of the tRNA synthetase reaction, was previously proposed as a factor involved in MTOR stimulation by amino acids because Ap4A is a strong inhibitor of AMPK (Meijer, 2008). Thus far, this possibility has not been explored.

It is important to stress that the two mechanisms of amino acid sensing, discussed in the previous paragraphs, detect different pools of amino acids: the v-ATPase senses the intralysosomal pool of amino acids while the leucyl-tRNA synthetase senses cytosolic leucine. It is perfectly possible that these two mechanisms coexist in amino acid sensing.

Glutamate Dehydrogenase and Regulation of Autophagy

On the basis of existing literature, some years ago we hypothesized that glutamate dehydrogenase, in addition to its role in amino acid catabolism, is involved in amino acid sensing and in controlling autophagy (Meijer, 2008; Meijer and Codogno, 2009). The arguments were as follows. This mitochondrial enzyme is specifically activated by leucine. In pancreatic β-cells, the ability of leucine (but not of valine or isoleucine) to stimulate production of insulin and to stimulate rapamycin-sensitive S6K phosphorylation was ascribed to stimulation of glutamate dehydrogenase (Xu *et al.*, 2001). Moreover, a mutation in glutamate dehydrogenase, resulting in overactivation of the enzyme, underlies the hyperinsulinism/hyperammonia (HHS) syndrome (Li *et al.*, 2012). A combination of glutamine (a glutamate donor) and leucine, which maximizes the flux through glutamate dehydrogenase, is most effective in stimulating S6K phosphorylation and in inhibiting autophagic flux in several cell types, including, among others, β-cells and hepatocytes (see Meijer and Codogno, 2009, for literature). Very recent studies with genetic and pharmacological methods have now provided strong experimental evidence that in the course of glutamine metabolism, glutamate dehydrogenase does, indeed, play a crucial role in the activation of MTOR (Figure 2.1). It was also demonstrated that it is the production of 2-oxoglutarate by glutamate dehydrogenase which stimulates the loading of RagB with GTP (Durán *et al.*, 2012). The link between 2-oxoglutarate and MTORC1 was proposed to be prolylhydroxylase which, in an HIF-1α-independent manner, somehow results in increased RagB^{GTP} (Durán *et al.*, 2013).

The importance of glutamate dehydrogenase in the activation of MTOR was also indicated, albeit indirectly, by studies by van der Vos *et al.* (2012) showing that stimulation of glutamine synthetase inhibited MTOR activity, inhibited the translocation of MTOR to the lysosomes, and activated autophagy. Surprisingly, it was concluded that glutamine itself

functions as an inhibitor of MTOR, and thus as an activator of autophagy, a conclusion which is in contrast with existing literature (see above). However, the fact that increased flux through glutamine synthetase results in increased flux through glutamate dehydrogenase, in this case in the direction of amination, i.e., from 2-oxoglutarate to glutamate, because of the use of glutamate for glutamine synthesis, was overlooked.

Apart from a role of glutamate dehydrogenase in the production of 2-oxoglutarate for the activation of MTOR, it is also possible that NADPH, another product of the deamination reaction, activates MTOR, and inhibits autophagy, by scavenging ROS which are predominantly produced by the mitochondrial respiratory chain (Meijer and Codogno, 2009) (Figure 2.1). When the production of ROS exceeds its degradation, excessive ROS levels induce oxidative stress and damage of cellular components including DNA, proteins and lipids. A rise in ROS levels activates autophagy as a protective mechanism, and this occurs in a manner that is sensitive to antioxidants (also in starvation) which, simultaneously, stimulate MTOR activity (Li *et al.*, 2013). Experiments carried out in our laboratories (Lorin *et al.*, 2013) indicate that knockdown of glutamate dehydrogenase does prevent reduction in ROS production by amino acids. By what mechanism oxidative stress may inhibit MTOR is unknown. A possibility is that one or more of the components involved in the amino acid sensing mechanisms, discussed previously, is redox sensitive.

Involvement of glutamate dehydrogenase in amino acid sensing has consequences for the use of chloroquine in autophagic flux measurements because this compound not only raises the intralysosomal pH but, in addition, is also an inhibitor of glutamate dehydrogenase (Lorin *et al.*, 2013). This leads to overestimation of autophagic flux. Along the same line, stimulation of autophagic flux by ammonia (Eng *et al.*, 2010) may be ascribed to the fact that this metabolite drives the glutamate dehydrogenase reaction in the direction of glutamate synthesis.

Other Pathways Involved in the Amino Acid Regulation of Autophagy

Another mechanism through which amino acids may stimulate MTOR and inhibit autophagy is inhibition of AMPK, which acts as a sensor of the cellular energy state (Figure 2.1). AMPK inhibits MTOR by phosphorylating and activating TSC2, which catalyzes the conversion of $Rheb^{GTP}$ to $Rheb^{GDP}$, and by phosphorylating and inactivating Raptor (Kim and Guan, 2011; Laplante and Sabatini, 2012). Apart from these effects on MTOR activity, AMPK also stimulates autophagy by phosphorylating ULK1 (Inoki *et al.*, 2012). Interestingly, ULK1 is thus phosphorylated by both MTOR and AMPK, but at different sites, with opposing effects on ULK1 activity. In some studies (Ghislat *et al.*, 2012; Li *et al.*, 2013), but not all (Durán *et al.*, 2013; Kim *et al.*, 2011a; Wauson *et al.*, 2012), amino acids, leucine in particular, have been shown to inhibit AMPK. Although not directly studied, it is likely that here, too, glutamate dehydrogenase plays an important role, because the net production of 2-oxoglutarate from glutamate serves to replenish citric acid cycle intermediates, which improves the capacity of this process, and thereby increases the rate of ATP production. Because metabolism differs among cell types, e.g., there are differences in the use of oxidizable substrates, it is understandable that the effect of amino acids on AMPK may vary among cell types.

Phospholipase D and inositolphosphate multikinase (IPMK), mentioned earlier as factors involved in the stimulation of MTOR activity by amino acids, may actually participate in the mechanisms of amino acid sensing discussed earlier. Phospholipase D may be part of

the protein complex anchoring MTORC1 to the lysosomal membrane (Wiczer and Thomas, 2012), whereas inositolphosphate multikinase, independent of its catalytic activity, appears to stabilize the binding between MTOR and raptor in the MTORC1 complex through its aminoterminal amino acid sequence which forms a unique mammalian MTOR binding site (Kim *et al.*, 2011b).

MAP4K3 is another protein that acts upstream of MTOR and that becomes activated by amino acids (Figure 2.1). Its role in amino acid sensing is not entirely clear. It is unlikely that this kinase participates in either the v-ATPase or the leucyl-tRNA synthetase mechanism of amino acid sensing. Presumably, MAP4K3 is part of another pathway leading to amino acid-induced MTOR activation (Kim and Guan, 2011).

Some years ago it was proposed that PtdIns 3-kinase (PI3K CIII) is required for amino acid signaling, an effect that is mediated by an amino acid-induced rise in cytosolic Ca^{++}, which results in increased binding of Ca^{++}/calmodulin to, and activation of, PI3K CIII (Gulati *et al.*, 2008). Apart from the fact that a rise in Ca^{++}/calmodulin stimulates autophagy through activation of AMPK by calmodulin-dependent kinase-kinase-β (Ghislat *et al.*, 2012) and that it is likely that cytosolic Ca^{++} decreases rather than increases in the presence of amino acids (Ghislat *et al.*, 2012) (although this issue is controversial) (Wauson *et al.*, 2012), these observations have been puzzling because PI3K CIII also participates in the formation of autophagosomes. A similar problem relates to the proposal that p62 participates in amino acid signaling (Duran *et al.*, 2011) because this protein does not inhibit autophagy but rather is required for this process. In order to solve this problem with PI3K CIII, it was proposed that the enzyme is part of different protein complexes with different functions (Duran *et al.*, 2011; Ktistakis *et al.*, 2012). Perhaps the same is true for p62 because only a small part of the total cellular p62 is bound to the MTORC1 complex through its association with the Rag GTPases (cf. Duran *et al.*, 2011). An alternative explanation (Inoki *et al.*, 2012) may be found in the fact that autophagy itself produces amino acids which act as feedback inhibitors of the process, and can stimulate MTOR activity (Yu *et al.*, 2010). Thus, overexpression of PI3K CIII or p62 activates autophagy, resulting in increased production of amino acids from proteins within the lysosomes. This may increase the size of the intralysosomal pool of amino acids which is sensed by the v-ATPase in the lysosomal membrane and activates MTOR according to the mechanism discussed earlier. Conversely, when PI3K CIII or p62 becomes inhibited, autophagic flux declines, the intralysosomal amino acid pool decreases and MTOR becomes inhibited. The observation that in skeletal muscle of mice deficient of myotubularin, the lipid phosphatase responsible for the degradation of PtdIns(3)P, autophagy is defective and MTOR overactivated (Fetalvero *et al.*, 2013) may be explained similarly. Perhaps initially autophagy is overactivated because of the rise in PtdIns(3)P. This results in increased autophagic proteolysis and a rise in the concentration of lysosomal amino acids, upon which MTOR becomes activated and autophagy inhibited again.

Plasma Membrane Derived Signaling and Regulation of Amino Acid-Dependent Autophagy

In analogy with the situation in yeast, plasma membrane amino acid transporters have been implicated in the sensing of extracellular amino acid availability by mammalian cells (Kim and Guan, 2011). Such a role was attributed to a transport protein that mediates an

exchange between extracellular leucine and intracellular glutamine, which allows leucine to be transported against a concentration gradient (Nicklin *et al.*, 2009). However, it must be pointed out that, as long as the amino acid sensor is intracellular, any process affecting the intracellular concentration of leucine, whether it is its transport across the plasma membrane or the rate of the intracellular metabolism of leucine, will affect the ability of leucine to modulate MTOR activity.

Very recently, it was reported that the G-protein-coupled taste receptor complex T1R1/T1R3, an amino acid receptor in the plasma membrane, originally discovered in gustatory neurons as a detector of the umami flavor and present in many tissues, is an early sensor of extracellular amino acid availability (Wauson *et al.*, 2012) (Figure 2.1). Reduced expression of T1R1/T1R3 impaired the amino acid-induced rise in intracellular Ca^{++}, the activation of MTOR by amino acids, caused mislocalization of MTORC1, and accelerated autophagy under nutrient-rich conditions. Interestingly, the intracellular concentration of amino acids, leucine included, was not affected by knock-down of the taste receptor even though the expression of several plasma membrane amino acid transporters greatly increased under these conditions (Wauson *et al.*, 2012). The question of how inactivation of MTOR and activation of autophagy in T1R1/T1R3 receptor knock-down cells could occur in the absence of changes in intracellular amino acid concentrations remained unanswered. An obvious explanation could be that glutamate dehydrogenase was downregulated after knock-down of the taste receptor. But this is unlikely because this enzyme plays a central role in amino acid catabolism and its downregulation would result in increased intracellular amino acid concentrations under these conditions, which was not observed. Another, plausible, possibility would be that AMPK was activated. However, AMPK was inhibited instead (Wauson *et al.*, 2012). The possibility that leucyl-tRNA synthetase was affected was not explored.

In summary, it is clear that several mechanisms of amino acid sensing, leading to activation of MTOR, can operate. Amino acid sensing by the v-ATPase in the lysosomal membrane was even reconstituted in a cell-free system in which the plasma membrane is not present (Zoncu *et al.*, 2011). Whether the various mechanisms are context and/or cell-type dependent, or act in parallel, is not known.

AMINO ACIDS, BECLIN-1 AND THE REGULATION OF AUTOPHAGY

Beclin-1 (the mammalian homologue of the yeast Atg6) was originally discovered from a mouse brain library using a yeast two-system hybrid with the antiapoptotic protein Bcl-2 as bait (Sinha and Levine, 2008). It is a 60 kDa protein that comprises three specific domains: (1) an N-terminal BH3 domain that binds Bcl-2 and Bcl-2 family proteins by a Bcl-2 binding domain extending from amino acids 88 to 140; (2) a central coil-coiled domain; and (3) an evolutionarily conserved domain essential to mediate autophagy and suppress tumorigenesis. Beclin-1 also contains a nuclear export signal (NES) important to regulate autophagy because only the cytosolic form regulates autophagy. In the cytoplasm, Beclin-1 has been localized at the trans-Golgi network, at the endoplasmic reticulum, at the mitochondria and the perinuclear membrane. *BECN1* is a haplo-insufficient tumor suppressor gene frequently monoallelically deleted in different cancers (breast, prostate, ovarian) (Sinha and Levine, 2008).

Recently, it has been shown that Beclin-1, by controlling the stability of the deubiquitinase USP10, controls the levels of p53, which is another important tumor suppressor (Liu *et al.*, 2011).

Beclin-1 Complexes and Autophagy

Beclin-1 is a protein that can form complexes with a variety of cellular proteins to control different steps in the autophagic pathway (Funderburk *et al.*, 2010) (Figure 2.1). All Beclin-1 complexes are constituted of a core that contains Vps34 or class III phosphatidylinositol 3-kinase (PI3K CIII) and its membrane adaptor Vps15 or p150. When associated with ATG14L and AMBRA1, Beclin-1 regulates an early step in the autophagosome formation downstream of the ULK1 complex. The production of PtdIns3P by PI3K CIII is important for the elongation of the autophagosomal membrane and initiation of autophagy. The interaction of PtdIns3P with the FYVE-domains containing proteins WIPIs (mammalian orthologues of the yeast Atg18) and DFCP1 is crucial during the biogenesis of autophagosomes (Mizushima and Komatsu, 2011). However, the mechanism by which PtdIns3P controls the formation of autophagosomes remains to be clarified. In a separate complex with UVRAG, Beclin-1 can control the formation and maturation of autophagosomes. Rubicon, which contains a conserved RUN domain, negatively regulates the maturation of autophagosomes when present in a complex with UVRAG. Rubicon has been also identified in Beclin-1 complexes with ATG14L; thus, it cannot be excluded that Rubicon also controls the initiation of autophagy. Interestingly, viral proteins are able to target different Beclin-1 complexes to interfere with different steps of the autophagic pathway.

Many cellular proteins have been shown to interact with Beclin-1 and to modulate autophagy. The reader can consult recent reviews on the topic (Funderburk *et al.*, 2010; Sinha and Levine, 2008). Here we will only consider proteins belonging to the Bcl-2 family because the Beclin-1–Bcl-2 interaction is regulated by amino acids.

Initial studies by the Beth Levine group reported that Bcl-2 blocks the induction of autophagy by starvation by interaction with the BH3 domain of Beclin-1 (Pattingre *et al.*, 2005) (Figure 2.1). Following on with the study, it has been shown that BH3 proteins and BH3 mimetics disrupt the interaction between Beclin-1 and Bcl-2/Bcl-xL (Maiuri *et al.*, 2007). The interaction can also be disrupted by the phosphorylation of the BH3 domain of Beclin-1 by death-associated protein kinase (DAPK) (Zalckvar *et al.*, 2009). Viral forms of Bcl-2 have a stronger inhibitory effect on autophagy than the cellular form of the protein because of its higher affinity for Beclin-1. Interaction of Beclin-1 with Bcl-2 family members weakens its interaction with PI3K CIII, but whether the binding of Bcl-2 to Beclin-1 interferes with the interaction of Beclin-1 with other members of the complex is unclear.

Regulation of the Activity of the Beclin-1 Complex during Starvation

The dissociation of the Beclin-1–Bcl-2 complex at the ER is necessary to trigger autophagy in response to nutrient starvation. The dissociation is dependent on the phosphorylation of a triad of amino acids present in the nonstructural loop of the cellular form of Bcl-2 by the c-jun N-terminal kinase 1 (JNK1) (Wei *et al.*, 2008) (Figure 2.1). The nonstructural loop containing the JNK1 phosphorylation sites is absent in the Kaposi's sarcoma-associated herpesvirus. This would explain why the viral Bcl-2 is an effective inhibitor of starvation-induced

autophagy. The JNK1-dependent stimulation of starvation-induced autophagy is restored in cells expressing a chimeric viral/cellular Bcl-2 expressing the cellular Bcl-2 nonstructural loop (Wei *et al.*, 2008). The mechanism that contributes to the JNK1 activation in starved cells remains to be investigated. During starvation, JNK1 and upstream activating kinases are recruited to microtubules via its interaction with the scaffold protein JNK-interacting-protein-1 (JIP-1) (Geeraert *et al.*, 2010). This recruitment contributes to the activation of JNK1. The recruitment depends on the interaction of JIP-1 and the motor protein kinesin-1. The starvation-dependent increase in acetylation of microtubules is important to recruit proteins to microtubules and to activate JNK1. These results suggest that microtubules play a role in the regulation of the early stage of autophagy. In fact, the Beclin-1–PI3K CIII core complex is recruited to microtubules via the dynein light chain 1 (LC8) by two nonexclusive mechanisms (Di Bartolomeo *et al.*, 2010; Luo *et al.*, 2012). The protein Beclin-1 interacts with the BH3-only protein Bim that bridges Beclin-1–LC8 interaction. In starvation conditions, the phosphorylation of Bim by JNK abolished its interaction with LC8 and dissociates the Bim–Beclin-1 interaction (Luo *et al.*, 2012). LC8 also recruits the Beclin-1–Vps34 via its interaction with the Beclin-1 interacting protein AMBRA1 (Di Bartolomeo *et al.*, 2010). Upon starvation of nutrients, ULK1 phosphorylates AMBRA1 to release its interaction from LC8 and it is translocated to the ER together with Beclin-1–Vps34. The starvation-dependent activation of Beclin-1 complexes is downstream of the ULK1/MTORC1 signaling cassette. Moreover, the ULK1-dependent phosphorylation of AMBRA1 indicates a direct molecular coordination between two complexes involved in the early stage of autophagosome formation. However, how activation of the different Beclin-1 complexes is integrated during the stimulation of autophagy by starvation remains to be clarified.

CONCLUSION

This chapter was mostly focused on the regulation of autophagy by amino acids. However, other nutrients such as carbohydrates and lipids are also able to regulate autophagy. Among lipids, fatty acids (palmitate) stimulate autophagy by controlling the cytoplasmic association of the transcription factor STAT3 and the kinase eIF kinase PKR (Shen *et al.*, 2012). The palmitate-induced dissociation of the complex activates the PKR-JNK1 pathway upstream of the Beclin-1 complex. Other lipids, such as the sphingolipid ceramide, stimulate autophagy by both inhibiting the MTORC1 pathway and activating the dissociation of the Beclin-1–Bcl-2 complex (Pattingre *et al.*, 2009). This regulation is reminiscent of the stimulation of autophagy by a fall in amino acids probably because ceramide downregulates the membrane expression of amino acid transporters (Guenther *et al.*, 2008). Stimulation of the AMPK-ULK1/2 pathway plays a pivotal role in the stimulation of autophagy by glucose deprivation (Inoki *et al.*, 2012). In contrast, growth factor deprivation stimulates autophagy by eliciting acetylation of ULK1 by the KAT5/TIP60 acetyltransferase, which is activated by glycogen synthase kinase-3 (Lin *et al.*, 2012). It is interesting to note that the growth factor-dependent signaling of autophagy is probably unique to metazoans whereas glucose and amino acid autophagy signaling are conserved in all eukaryotic cells. Lastly, the regulation of autophagy by nutrients is probably not only cell autonomous: some byproducts of nutrients can control autophagy distant from their production site

because of the diffusion in the cell environment. This is the case for ammonia which is produced during the degradation of glutamine to glutamate (Eng *et al.*, 2010). The stimulation of autophagy by ammonia is independent of the ULK1 complex but depends on the ATG complexes acting downstream of ULK1 (Cheong *et al.*, 2011). The regulation of autophagy by ammonia is probably important to consider in tumors because cancer cells depend on the use of glutamate for energetic needs. Ammonia is perhaps important to regulate autophagy in stromal cells and immune cells. In addition, metabolites can contribute to the cross-talk between different forms of autophagy, as ketone bodies, formed by hepatic fatty acid oxidation, are potent activators of chaperone-mediated autophagy (Finn and Dice, 2005).

References

Blommaart, E.F., Luiken, J.J., Blommaart, P.J., et al., 1995. Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. J. Biol. Chem. 270, 2320–2326.

Cheong, H., Lindsten, T., Wu, J., et al., 2011. Ammonia-induced autophagy is independent of ULK1/ULK2 kinases. Proc. Natl Acad. Sci. USA 108, 11121–11126.

Di Bartolomeo, S., Corazzari, M., Nazio, F., et al., 2010. The dynamic interaction of AMBRA1 with the dynein motor complex regulates mammalian autophagy. J. Cell Biol. 191, 155–168.

Duran, A., Amanchy, R., Linares, J.F., et al., 2011. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. Mol. Cell 44, 134–146.

Durán, R.V., MacKenzie, E.D., Boulahbel, H., et al., 2013. HIF-independent role of prolyl hydroxylases in the cellular response to amino acids. Oncogene 32 (38), 4549–4556.

Durán, R.V., Oppliger, W., Robitaille, A.M., et al., 2012. Glutaminolysis activates Rag-mTORC1 signaling. Mol. Cell 47, 349–358.

Eng, C.H., Yu, K., Lucas, J., et al., 2010. Ammonia derived from glutaminolysis is a diffusible regulator of autophagy. Sci. Signal. 3, ra31.

Fetalvero, K.M., Yu, Y., Goetschkes, M., et al., 2013. Defective autophagy and mTORC1 signaling in myotubularin null mice. Mol. Cell Biol. 33, 98–110.

Finn, P.F., Dice, J.F., 2005. Ketone bodies stimulate chaperone-mediated autophagy. J. Biol. Chem. 280, 25864–25870.

Funderburk, S.F., Wang, Q.J., Yue, Z., 2010. The Beclin 1-VPS34 complex – at the crossroads of autophagy and beyond. Trends Cell Biol. 20, 355–362.

Geeraert, C., Ratier, A., Pfisterer, S.G., et al., 2010. Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. J. Biol. Chem. 285, 24184–24194.

Ghislat, G., Patron, M., Rizzuto, R., et al., 2012. Withdrawal of essential amino acids increases autophagy by a pathway involving Ca2+/calmodulin-dependent kinase kinase-beta (CaMKK-beta). J. Biol. Chem. 287, 38625–38636.

Guenther, G.G., Peralta, E.R., Rosales, K.R., et al., 2008. Ceramide starves cells to death by downregulating nutrient transporter proteins. Proc. Natl Acad. Sci. USA 105, 17402–17407.

Gulati, P., Gaspers, L.D., Dann, S.G., et al., 2008. Amino acids activate mTOR complex 1 via Ca^{2+}/CaM signaling to hVps34. Cell Metab. 7, 456–465.

Han, J.M., Jeong, S.J., Park, M.C., et al., 2012. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. Cell 149, 410–424.

Inoki, K., Kim, J., Guan, K.L., 2012. AMPK and mTOR in cellular energy homeostasis and drug targets. Annu. Rev. Pharmacol. Toxicol. 52, 381–400.

Kim, J., Guan, K.L., 2011. Amino acid signaling in TOR activation. Annu. Rev. Biochem. 80, 1001–1032.

Kim, J., Kundu, M., Viollet, B., et al., 2011a. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.

Kim, S., Kim, S.F., Maag, D., et al., 2011b. Amino acid signaling to mTOR mediated by inositol polyphosphate multikinase. Cell Metab. 13, 215–221.

Kroemer, G., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell 40, 280–293.

Ktistakis, N.T., Manifava, M., Schoenfelder, P., et al., 2012. How phosphoinositide 3-phosphate controls growth downstream of amino acids and autophagy downstream of amino acid withdrawal. Biochem. Soc. Trans. 40, 37–43.

Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149, 274–293.

Li, L., Chen, Y., Gibson, S.B., 2013. Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation. Cell Signal. 25, 50–65.
Li, M., Li, C., Allen, A., et al., 2012. The structure and allosteric regulation of mammalian glutamate dehydrogenase. Arch. Biochem. Biophys. 519, 69–80.
Lin, S.Y., Li, T.Y., Liu, Q., et al., 2012. GSK3-TIP60-ULK1 signaling pathway links growth factor deprivation to autophagy. Science 336, 477–481.
Liu, J., Xia, H., Kim, M., et al., 2011. Beclin1 controls the levels of p53 by regulating the deubiquitination activity of USP10 and USP13. Cell 147, 223–234.
Lorin, S., Tol, M.J., Bauvy, C., et al., 2013. Glutamate dehydrogenase contributes to leucine sensing in the regulation of autophagy. Autophagy 9, 850–860.
Luo, S., Garcia-Arencibia, M., Zhao, R., et al., 2012. Bim inhibits autophagy by recruiting beclin 1 to microtubules. Mol. Cell 47, 359–370.
Maiuri, M.C., Le Toumelin, G., Criollo, A., et al., 2007. Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. EMBO J. 26, 2527–2539.
Meijer, A.J., 2008. Amino acid regulation of autophagosome formation. Methods Mol. Biol. 445, 89–109.
Meijer, A.J., Codogno, P., 2009. Autophagy: regulation and role in disease. Crit. Rev. Clin. Lab. Sci. 46, 210–240.
Mizushima, N., Komatsu, M., 2011. Autophagy: renovation of cells and tissues. Cell 147, 728–741.
Nicklin, P., Bergman, P., Zhang, B., et al., 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 136, 521–534.
Pattingre, S., Bauvy, C., Carpentier, S., et al., 2009. Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. J. Biol. Chem. 284, 2719–2728.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Rubinsztein, D.C., Marino, G., Kroemer, G., 2011. Autophagy and aging. Cell 146, 682–695.
Sancak, Y., Bar-Peled, L., Zoncu, R., et al., 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 290–303.
Schriever, S.C., Deutsch, M.J., Adamski, J., et al., 2013. Cellular signaling of amino acids towards mTORC1 activation in impaired human leucine catabolism. J. Nutr. Biochem. 24, 824–831.
Settembre, C., Zoncu, R., Medina, D.L., et al., 2012. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J. 31, 1095–1108.
Shen, S., Niso-Santano, M., Adjemian, S., et al., 2012. Cytoplasmic STAT3 represses autophagy by inhibiting PKR activity. Mol. Cell 48, 667–680.
Sinha, S., Levine, B., 2008. The autophagy effector Beclin 1: a novel BH3-only protein. Oncogene 27 (Suppl. 1), S137–S148.
van der Vos, K.E., Eliasson, P., Proikas-Cezanne, T., et al., 2012. Modulation of glutamine metabolism by the PI(3)K-PKB-FOXO network regulates autophagy. Nat. Cell Biol. 14, 829–837.
Wang, R.C., Wei, Y., An, Z., et al., 2012. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. Science 338, 956–959.
Wauson, E.M., Zaganjor, E., Lee, A.Y., et al., 2012. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol. Cell 47, 851–862.
Wei, Y., Pattingre, S., Sinha, S., et al., 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell 30, 678–688.
Wiczer, B.M., Thomas, G., 2012. Phospholipase D and mTORC1: nutrients are what bring them together. Sci. Signal. 5, pe13.
Xu, G., Kwon, G., Cruz, W.S., et al., 2001. Metabolic regulation by leucine of translation initiation through the mTOR-signaling pathway by pancreatic beta-cells. Diabetes 50, 353–360.
Yu, L., McPhee, C.K., Zheng, L., et al., 2010. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 465, 942–946.
Zalckvar, E., Berissi, H., Mizrachy, L., et al., 2009. DAP-kinase-mediated phosphorylation on the BH3 domain of beclin 1 promotes dissociation of beclin 1 from Bcl-XL and induction of autophagy. EMBO Rep. 10, 285–292.
Zoncu, R., Bar-Peled, L., Efeyan, A., et al., 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 334, 678–683.

CHAPTER

3

Regulation of Autophagy by Amino Acid Starvation Involving Ca^{2+}

Ghita Ghislat and Erwin Knecht

OUTLINE

Abstract

Macroautophagy is an evolutionarily conserved catabolic process whose main role is to degrade intracellular cargo for the supply of metabolites and energy to the cells. It consists of various vesicle trafficking events, generally triggered by stress conditions such as starvation. Macroautophagy is strictly regulated and the second messenger Ca^{2+} has been recently shown to regulate starvation-induced macroautophagy. Withdrawal of essential amino acids increases intracellular Ca^{2+}, which is provided from both the extracellular medium and intracellular stores. This leads to the activation, via Ca^{2+}/calmodulin-dependent kinase kinase-β, of adenosine monophosphate-activated protein kinase and the inhibition of the mammalian target of rapamycin complex 1. Downstream of these kinases, UNC-51-like kinase, a mammalian autophagy-initiating

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protein, is activated, which leads to an enhanced formation of autophagosomes. We discuss here the importance of this pathway within the autophagy signaling network activated under amino acid starvation and pay attention to energy considerations in the induction of autophagy by intracellular Ca^{2+} and its sensor Ca^{2+}/calmodulin-dependent kinase kinase-β.

INTRODUCTION

Autophagy is a cellular self-digestion system that involves lysosomes for the degradation of the cell constituents. At least three different forms of autophagy have been described: microautophagy, chaperone-mediated autophagy and macroautophagy.

Microautophagy leads to lysosomal internalization of cytosolic components by invaginations of the lysosomal membranes, forming intralysosomal vesicles that are subsequently degraded together with their content (Li *et al.*, 2012). Chaperone-mediated autophagy is responsible for the specific lysosomal degradation of soluble proteins whose amino acid sequence holds KFERQ-like motifs. These proteins are translocated to the lysosomal lumen after recognition by a chaperone, HSC70, and the subsequent association to isoform A of the lysosome-associated membrane protein type 2 (Kaushik and Cuervo, 2012). Finally, macroautophagy is the best-known and more extensive autophagic process and, hence, it will be here simply referred to as autophagy. It initiates with the formation of a double lipid bilayer, termed the isolation membrane or phagophore, which subsequently closes into a double membrane vesicle called the autophagosome, sequestering in this way cytosolic macromolecules and even entire organelles. This structure fuses then with late endosomes and lysosomes to form autolysosomes, where the clearance of the autophagic cargo takes place.

The main role of autophagy is to remove superfluous, damaged or toxic components and to recycle cellular material in case of energy needs. Thus, the levels of autophagy in full nutrient and healthy cellular states are low and this autophagy is used to remove the small amount of needless or virulent material and to maintain cellular proteostasis. Under conditions of cellular stress such as nutrient starvation, autophagy quickly reaches much higher levels to supply the cell with energy and building blocks that ensure cell survival. Therefore, autophagy is a physiological and highly regulated cell process.

Nutritional and hormonal factors are the main regulators of autophagy in mammalian cells. For instance, autophagy is induced by glucagon and, under certain conditions, by glucose, and it is inhibited by vitamins, osmotic stress, various growth factors, insulin and some amino acids (Knecht *et al.*, 2009). The best studied hormonal and nutritional regulators of autophagy in mammalian cells are, respectively, insulin and amino acids (Lavallard *et al.*, 2012). Insulin restrains autophagy by a well-known signaling pathway involving phosphatidylinositol 3-kinase (PI_3K) class I and AKT/PKB, which inhibits the Tuberous Sclerosis Complex 1/2 (TSC1/2 complex) and activates a serine-threonine kinase, the mammalian target of rapamycin complex 1 (mTORC1), via the RAS-family GTP-binding protein RHEB (Figure 3.1). By contrast, the signaling pathways by which amino acids inhibit autophagy remain less well defined and even the nature of a cellular sensor for amino acids is still a matter of conjecture. Nevertheless, it is well established now that both regulators can inhibit autophagy via activation of mTORC1.

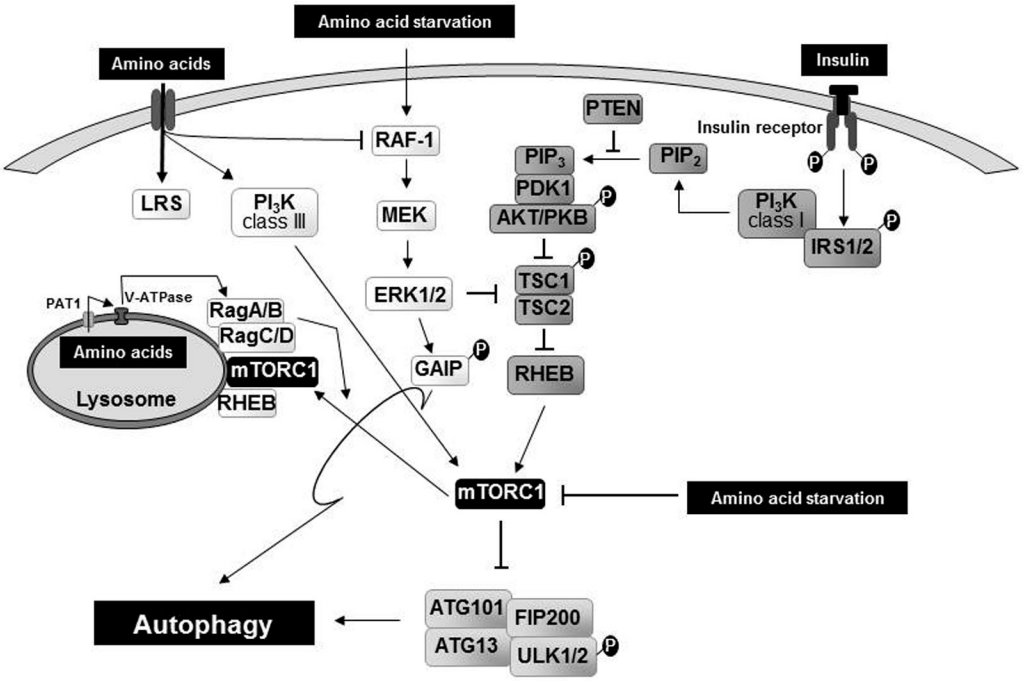

FIGURE 3.1 Main signaling pathways regulating autophagy in response to insulin and to the presence and absence of essential amino acids. The binding of insulin to its tyrosine kinase receptor produces its auto-phosphorylation and subsequently the phosphorylation of insulin receptor substrate 1 and 2 (IRS1/2). Phosphatidylinositol 3-kinase (PI_3K) class I is then activated and generates phosphatidylinositol 3,4,5 trisphosphate (PIP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2), which leads to the activations of AKT/PKB via phosphorylation by the phosphoinositide-dependent kinase 1 (PDK1). In contrast, the phosphatase and tensin homologue (PTEN) hydrolyzes PIP_3 to PIP_2, inhibiting in this way the downstream pathway induced by insulin. Activation of AKT/PKB results in the inhibition of the Tuberous Sclerosis Complex 1/2 (TSC1/2) complex, the activation of mTORC1 by RHEB and the consequent inhibition of autophagy. Amino acids restrain autophagy by activating mTORC1 through PI_3K class III and the RAG complex. Withdrawal of amino acids induces autophagy by activating the extracellular signaling-regulated kinase (ERK) 1/2 through RAF-1 and MEK, leading to a high phosphorylation/activation of the regulator of the cytoplasmic heterotrimeric Gi3 protein, the Gα-interacting protein (GAIP). However, the inhibition of ERK 1/2 by amino acids is controversial (Settembre et al., 2012). Amino acid starvation also triggers autophagy through the inhibition of mTORC1 that represses a complex containing ATG13, ATG 101, focal adhesion kinase-interacting protein 200 (FIP200), and UNC-51 like kinases 1 and 2 (ULK1/2). See text for further details.

Two distinct complexes constitute the kinase mTOR: the already mentioned rapamycin-sensitive mTORC1 and the less rapamycin-sensitive mTORC2. Both complexes contain mTOR and other proteins. The complex mTORC1 is the main sensor of nutrients in the control of autophagy and comprises, in addition to mTOR, the regulatory associated protein of mTOR (RAPTOR), the G-protein β-subunit-like protein (GβL) and the inhibitory protein proline-rich AKT substrate of 40 kDa (PRAS40). When it is active, mTORC1 interacts with the UNC-51-like kinase 1 (ULK1, the homologue of the yeast protein ATG1) and inactivates it by phosphorylation at a specific site, which leads to the inhibition of autophagy.

The complex mTORC2 is not considered to be directly involved in the regulation of autophagy, except in isolated reports that describe an activation of autophagy (Renna *et al.*, 2013). It comprises, apart from mTOR, the rapamycin-insensitive companion of mTOR (RICTOR), GβL, the SAPK-interacting protein 1 (SIN1) and the protein observed with RICTOR (PROTOR).

As indicated above and in contrast to insulin, the regulation of autophagy by amino acids involves various signaling pathways, most of them mTORC1-dependent (Lavallard *et al.*, 2012). This complexity is probably due to the diversity of amino acids and to the variety of their metabolisms in different cells. Here we will specifically concentrate on the pathways involving Ca^{2+} by which amino acids regulate autophagy.

Ca^{2+} is a major intracellular second messenger that participates in the regulation of several physiological cell functions, such as gene transcription, metabolism, secretion and apoptosis. In addition, perturbations in the homeostasis of Ca^{2+} have been associated with various pathological processes such as disorders of the nervous system, cardiac and vascular pathologies and diabetes mellitus (Berridge *et al.*, 2003). During the last few years, some of the mechanisms that link Ca^{2+} and Ca^{2+}-binding proteins with signaling and trafficking steps related with autophagy have been deciphered (see Ghislat and Knecht, 2013, for a review) and a novel Ca^{2+}-dependent pathway activated by the withdrawal of amino acids leading to the induction of autophagy was identified (Ghislat *et al.*, 2012). In the following sections, we will first briefly describe the different signaling pathways triggered by either amino acid availability or starvation in the regulation of autophagy and afterwards we will discuss the role and physiological relevance in this context of intracellular Ca^{2+} and of its sensor Ca^{2+}/calmodulin-dependent kinase kinase-β (CaMKK-β).

REGULATION OF AUTOPHAGY BY AMINO ACIDS

Inhibition of Autophagy by Amino Acids

Essential amino acids are the best-known nutritional stimulators of mTORC1 for the inhibition of autophagy. In particular, branched amino acids such as leucine activate mTORC1 in most, but not in all (Esteban *et al.*, 2007), cell types (Lavallard *et al.*, 2012). In the last years, both extracellular and intracellular sensors of amino acids have been proposed. Thus, a receptor of leucine and of other amino acids was identified on the plasma membrane (see Wauson *et al.*, 2012 and references cited therein). Also on the cell surface, an antiporter that exports glutamine and imports some branched amino acids, including leucine, was proposed as a candidate receptor for amino acids (Nicklin *et al.*, 2009).

As for intracellular sensors, glutamate dehydrogenase was suggested to contribute to leucine sensing in the inhibition of autophagy (see Lorin *et al.*, 2013 and references cited therein). Similarly, leucyl-tRNA synthetase (LRS) was shown to have this role in mammals (Han *et al.*, 2012) and also in yeast (Bonfils *et al.*, 2012). After binding intracellular leucine, LRS activates mTORC1 via RAG, which is a GTPase that functions as a heterodimer and is localized in the vicinity of lysosomes (Han *et al.*, 2012). RAG binds to mTORC1 through RAPTOR in a perilysosomal area where RHEB, an activator of mTORC1, is buried (Sancak *et al.*, 2010). The amino acids released from lysosomes, as a product of lysosomal

degradation, have also been recently shown to have an effect on mTORC1 activity. Their release through the lysosomal proton-assisted amino acid transporter PAT1 was reported to activate mTORC1 via RAG by a process that requires the hydrolysis of ATP by the vacuolar H^+-ATPase (V-ATPase) (Zoncu *et al.*, 2011). Since lysosomes should release more amino acids to the cytosol when autophagy is active, it is difficult to understand why, under these conditions, mTORC1, which inhibits autophagy, should become activated. However, although mTORC1 is inhibited during the initiation of starvation, it has been shown to be activated through the V-ATPase after a more prolonged starvation of 6–12 h by the excess of amino acids released in the autolysosomal degradation of proteins (Yu *et al.*, 2010). Thus, LRS and the V-ATPase are considered as intracellular sensors of cytosolic and intralysosomal amino acids, respectively. Both sensors recruit mTORC1 to the lysosomal surface and activate it through RAG GTPases, but the details of these processes are not yet fully elucidated and remain a matter of active investigation.

The activation of mTORC1 by amino acids was also proposed to occur via PI_3K class III. However, it is unlikely that this is associated with the inhibition of autophagy, since PI_3K class III is required for the initiation of autophagy and it has been also recently reported that under nutrient starvation PI_3K class III is activated by adenosine monophosphate-activated protein kinase (AMPK) to induce autophagy (Kim *et al.*, 2013). Therefore, there is not a consensus on the involvement of PI_3K class III in the inhibition of autophagy by mTORC1, presumably and most probably because it is part of different protein complexes with distinct functions.

Induction of Autophagy by Amino Acid Starvation

Whereas the inhibition of autophagy by amino acids requires mTORC1, amino acid starvation activates autophagy through both mTORC1-dependent and -independent mechanisms. Inactivation of mTORC1 is involved in the induction of autophagy by amino acid starvation via repression of an autophagy-related complex containing at least four different proteins: ATG13, ATG 101, focal adhesion kinase-interacting protein 200 (FIP200) and ULK1/2. In addition, mTORC1-independent signaling pathways have also been described in the activation of autophagy by amino acids. For instance, in the RAF kinase signaling triggered by amino acid starvation, RAF-1 activates MEK1/2 (MAPK/ERK kinase), which leads to the activation of ERK1/2 (extracellular signal-regulated kinase 1 and 2) and the subsequent promotion of autophagy (Shaw and Cantley, 2006). Moreover, the eukaryotic translation initiation factor-2, subunit α (eIF2α) and an endoplasmic reticulum stress were also reported to be involved in the activation of autophagy in response to amino acid starvation (Ron and Walter, 2007).

Overall, although amino acid starvation is a well-known inducer of autophagy, this effect seems to be due to a variety of signaling pathways that form a complex network whose molecular details are still not well defined. Despite the identification of some mTORC1-independent pathways for the induction of autophagy, the inhibition of this kinase appears to be crucial in the signaling network triggered by amino acid starvation that leads to the activation of autophagy. In this regard, a Ca^{2+}-dependent pathway has been recently shown to be operative in the absence of amino acids for the inhibition of mTORC1 and the subsequent activation of autophagy (Ghislat *et al.*, 2012). Here, we will discuss this pathway and its physiological relevance.

CA^{2+}-DEPENDENT ACTIVATION OF AUTOPHAGY BY AMINO ACID STARVATION

The spatial and temporal distribution of intracellular Ca^{2+} represents one of the most important signals regulating several physiological processes in the cells. When essential amino acids were removed from the extracellular milieu in mouse and human fibroblasts, human embryonic kidney and Hela cells, the levels of intracellular Ca^{2+} were found to increase (Ghislat *et al.*, 2012). An increase in cytosolic Ca^{2+} has been frequently associated with stress conditions, but both positive and negative roles of this ion in the regulation of autophagy have been reported under these conditions (Ghislat and Knecht, 2013). Whereas rises in intracellular Ca^{2+} were reported to inactivate autophagy in excitable cells through the modulation of voltage-dependent Ca^{2+} channels (Williams *et al.*, 2008), increases in the intracellular levels of this cation activate autophagy in nonexcitable cells that do not express these channels (Ghislat and Knecht, 2013). These opposite responses in both groups may be due to differences in the compartments where the Ca^{2+} comes from and this probably determines the activation of distinct Ca^{2+}-sensor proteins in the proximity of each specific compartment and, thus, variations in their signaling routes. In fact, cytosolic Ca^{2+} in excitable cells is mainly provided from the extracellular space by voltage-activated channels, whereas in nonexcitable cells it is mainly released from intracellular stores, most probably via second messengers such as inositol 1,4,5-trisphosphate (Pfisterer *et al.*, 2011).

As indicated previously, the rise of cytosolic Ca^{2+} observed under amino acid starvation is provided by extracellular and especially intracellular stores. Lysosomes are a plausible intracellular source, given that several experimental evidences associated lysosomal Ca^{2+} with autophagy. Lysosomes have been reported to release Ca^{2+} to facilitate their fusion with autophagosomes (Coen *et al.*, 2012). Moreover, this later step of autophagy has been shown to require three Ca^{2+}-dependent phospholipid binding proteins, annexin A1, annexin A5 and copine 1, which translocate to lysosomal membranes under starvation (Ghislat and Knecht, 2012). At least for the case of annexin A5 this translocation is Ca^{2+} dependent (Ghislat *et al.*, 2012) and this Ca^{2+} may be derived from a lysosomal source. Thus, although these reports suggest that lysosomal Ca^{2+} induces late steps of autophagy, it is possible that it also triggers the Ca^{2+}-dependent pathway activated by amino acid starvation. However, the contribution of other Ca^{2+} sources much more prominent than the lysosomes, such as the endoplasmic reticulum, cannot be excluded. Thus, a pioneer study demonstrated a role of Ca^{2+} storage within the endoplasmic reticulum for autophagy stimulation (Gordon *et al.*, 1993). Later, amino acid starvation was shown to disrupt the association with the endoplasmic reticulum of B-cell lymphoma/leukemia 2 (BCL-2), which decreases the exit of Ca^{2+} from this source, leading to Ca^{2+} release from the endoplasmic reticulum and autophagy activation (Pattingre *et al.*, 2005). Therefore, further investigations are required to identify the compartment/s responsible for Ca^{2+} release under starvation and its/their mechanism/s.

Subsequent to this Ca^{2+} rise observed under amino acid starvation, a signaling pathway involving Ca^{2+}/calmodulin-dependent kinase kinase-β (CaMKK-β) was activated (Ghislat *et al.*, 2012). CaMKK-β has already been shown to induce autophagy through AMPK activation when cytosolic Ca^{2+} levels were increased using pharmacological agents (Hoyer-Hansen *et al.*, 2007). It is known that this kinase is activated when it is bound to

Ca^{2+}/calmodulin. We found that one of its downstream effectors, AMPK, is required for the Ca^{2+}-dependent activation of autophagy by amino acid starvation. AMPK is known to inhibit mTORC1, both directly through phosphorylation of RAPTOR in the mTORC1 complex (Gwinn *et al.*, 2008), or indirectly via tuberous sclerosis complex (TSC1/2) and its substrate RHEB (Sarbassov *et al.*, 2005). We described that under amino acid starvation, AMPK inhibited mTORC1 in a Ca^{2+}-dependent way, a finding supported by more recent results showing that the activation of autophagy by mTOR inhibition using rapamycin requires intracellular Ca^{2+} (Decuypere *et al.*, 2013). The autophagy-related protein, ULK1, appeared to be a key element in the Ca^{2+}-dependent pathway, which linked together essential amino acid starvation, intracellular Ca^{2+}, CaMKK-β, AMPK, mTORC1 and the autophagic machinery. It has been shown that the coordinated positive and negative regulation of ULK1 activity by, respectively, AMPK and mTORC1 occurs through a multisite phosphorylation of this protein (Egan *et al.*, 2011; Kim *et al.*, 2011). In the case of the Ca^{2+}-dependent activation of autophagy by amino acid starvation, ULK1 was shown to be activated via AMPK-mediated phosphorylation (at least at the Ser-555) and a decrease of mTORC1-mediated phosphorylation at the Ser-757. This effect on ULK1 was fully dependent on the activation of CaMKK-β (Figure 3.2).

It is important to note that this previously described Ca^{2+}- and CaMKK-β-dependent pathway is part of a more complex signaling network by which autophagy is induced in response to amino acid starvation and, therefore, additional pathways should be involved in the Ca^{2+}-dependent activation of autophagy by amino acid starvation. In fact, it has been reported that Ca^{2+} can also induce autophagy by an alternative pathway downstream of CaMKK-β that bypasses AMPK and activates Ca^{2+}/calmodulin-dependent protein kinase I (Pfisterer *et al.*, 2011).

In any case, intracellular Ca^{2+} levels represent an important signal in the cell response to the availability of amino acids and it would be important to identify the specific amino acids that change the intracellular Ca^{2+} levels and the molecular details of the mechanism by which amino acid withdrawal increases intracellular Ca^{2+}.

CA^{2+}/CAMKK-β-DEPENDENT AUTOPHAGY AND ENERGY

Physiological concentrations of amino acids are required for several cellular and tissue functions. Unlike dietary protein digestion, in intracellular protein degradation the amino acids are released from endogenous proteins. Equilibrium between these two processes, digestion of dietary proteins and intracellular protein degradation, is essential to maintain a pool of synthesized tissue proteins within a range that is appropriate for the homeostasis of the organism. Thus, intracellular protein degradation, and more especially autophagy, gains importance when the supply of dietary amino acids to the organism is limited. Autophagy requires energy, which may appear, at a first glance, inconvenient for a cell under starvation. However, only a low amount of ATP is required for autophagy activation (Moruno-Manchon *et al.*, 2013). Therefore, it is crucial for cell homeostasis to maintain a good management of energy consumption under starvation. The previously described Ca^{2+}-dependent pathway activated under amino acid starvation links an intracellular Ca^{2+} signal with a well-established energy sensor, AMPK, via CaMKK-β. This Ca^{2+}- and

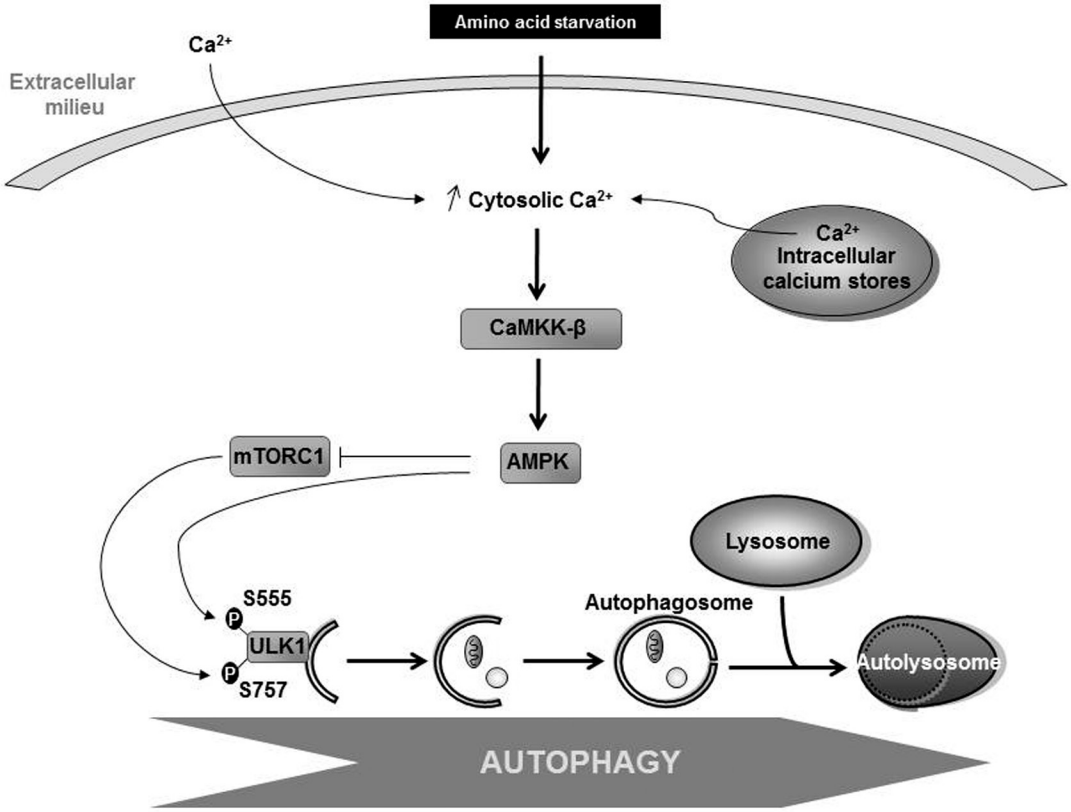

FIGURE 3.2 Ca^{2+}-dependent activation of autophagy under amino acid starvation in nonexcitable cells. A rise of cytosolic Ca^{2+} from both extracellular and intracellular stores is produced by amino acid starvation in nonexcitable cells. Subsequently, CaMKK-β activates AMPK, which restrains mTORC1 activity. This brings out a global activation of ULK1, which can be positively and negatively regulated by AMPK and mTORC1, respectively, through phosphorylation at least on the indicated two different sites. See text for further details.

calmodulin-dependent kinase is one of the most prominent sensors of intracellular Ca^{2+} and brings information to signaling cascades regulating important cell processes by reversible binding to this cation. It is appealing that under starvation the second messenger Ca^{2+} uses CaMKK-β to induce autophagy through the activation of the energy sensor AMPK, which canonically becomes activated when the amount of energy available for the cells is low. Although it is unknown if the rise of intracellular Ca^{2+} under amino acid starvation consumes energy and how much it uses, the Ca^{2+}-dependent pathway through CaMKK-β appears to be a useful alternative to activate AMPK and autophagy without the need of a large ATP consumption by the cells, especially when CaMKK-β levels are high in the cell. CaMKK-β is expressed at low levels in a wide range of tissues, such as testis, spleen, and lung (Anderson *et al.*, 1998), but higher levels of this protein are detected in various areas of the brain, including the hypothalamus, hippocampus and cerebellum (Anderson *et al.*,

1998; Vinet *et al.*, 2003). Moreover, the CaMKK-β–AMPK complex was shown to regulate the energy balance in the hypothalamus by maintaining high levels of glucose in this organ (Anderson *et al.*, 2008). Given that the brain, especially the hypothalamus, is the master regulator of energy intake and expenditure of the organism, and that autophagy impairment in this organ leads to serious damage, a signaling pathway to autophagy without the need for a strong energy decrease in the cells is necessary for brain homeostasis. Thus, the activation of AMPK by CaMKK-β under amino acid starvation for autophagy induction may represent in these cells a better alternative, in terms of energy levels in the cell, to the classical stimulation of AMPK by the increase in the AMP/ATP ratio. Although the Ca^{2+}-dependent pathway triggered by amino acid starvation has not been studied in neurons, and autophagy can be activated by amino acid starvation via a complex signaling network that also includes AMPK-independent pathways, the high levels of CaMKK-β in the brain may facilitate the activation of AMPK for autophagy induction without the need for a strong decrease in the ATP levels of the cells.

Moreover, a physiological role of CaMKK-β in the maintenance of energy homeostasis in the liver has been demonstrated in various reports. Although this kinase was not initially found in the liver (Anderson *et al.*, 1998), its presence was later shown in hepatocytes (Anderson *et al.*, 2012). In these cells, depletion of CaMKK-β decreased blood glucose, especially in the fasted state, after treatment with noradrenaline, a Ca^{2+} agonist, by downregulating key enzymes of the gluconeogenesis pathway such as glucose-6-phosphate dehydrogenase and phosphoenolpyruvate carboxykinase (Anderson *et al.*, 2012). Thus, CaMKK-β emerges as an energy controller in intracellular Ca^{2+} signaling in the liver, and this role may be involved in autophagy activation since it gains importance under starvation.

In summary, CaMKK-β requires less energy decrease for autophagy induction in neurons and in other cells to maintain high energy levels under starvation. Therefore, its role in autophagy and in other cell functions deserves further investigation in the tissues where its expression is high.

CONCLUSION

Growing evidence supports that Ca^{2+} plays a role in the complex network by which amino acids control autophagy. The distinction between excitable and nonexcitable cells is important for the role of this second messenger in the regulation of autophagy, because it exerts opposite effects in each of both groups of cells. In nonexcitable cells, a rise of cytosolic Ca^{2+} triggered by amino acid starvation originates from both intracellular and extracellular stores. Consequently, the Ca^{2+}-sensor proteins calmodulin and CaMKK-β switch this signal to the energy-related kinase AMPK, which induces autophagy via the inhibition of mTORC1 and the activation of ULK1. These Ca^{2+}-sensor proteins gain prominence for the activation of this pathway in nonexcitable cells, while in excitable cells the intracellular distribution of the Ca^{2+} signal and its sensors is different. The link of Ca^{2+} and CaMKK-β to the energy sensor AMPK may not be casual in the cell. It is likely that the absence of amino acids triggers this Ca^{2+}-dependent pathway to activate autophagy without the need of a low energy decrease, since CaMKK-β is an alternative to the necessity of an increased AMP/ATP ratio for AMPK activation. This is supported by the fact that the CaMKK-β–AMPK complex

was shown to have a role in energy balance, at least in some tissues. Therefore, exploring the involvement of this Ca^{2+}-dependent pathway in the activation of autophagy in different tissues may be useful to understand how the organism deals with energy challenges during stress conditions, such as amino acid starvation.

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References

Anderson, K.A., Lin, F., Ribar, T.J., et al., 2012. Deletion of CaMKK2 from the liver lowers blood glucose and improves whole-body glucose tolerance in the mouse. Mol. Endocrinol. 26, 281–291.

Anderson, K.A., Means, R.L., Huang, Q.H., et al., 1998. Components of a calmodulin-dependent protein kinase cascade. Molecular cloning, functional characterization and cellular localization of Ca^{2+}/calmodulin-dependent protein kinase kinase beta. J. Biol. Chem. 273, 31880–31889.

Anderson, K.A., Ribar, T.J., Lin, F., et al., 2008. Hypothalamic CaMKK2 contributes to the regulation of energy balance. Cell Metab. 7, 377–388.

Berridge, M.J., Bootman, M.D., Roderick, H.L., 2003. Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. 4, 517–529.

Bonfils, G., Jaquenoud, M., Bontron, S., et al., 2012. Leucyl-tRNA synthetase controls TORC1 via the EGO complex. Mol. Cell 46, 105–110.

Coen, K., Flannagan, R.S., Baron, S., et al., 2012. Lysosomal calcium homeostasis defects, not proton pump defects, cause endo-lysosomal dysfunction in PSEN-deficient cells. J. Cell Biol. 198, 23–35.

Decuypere, J.P., Kindt, D., Luyten, T., et al., 2013. mTOR-controlled autophagy requires intracellular Ca(2+) signaling. PLoS One 8, e61020.

Egan, D.F., Shackelford, D.B., Mihaylova, M.M., et al., 2011. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456–461.

Esteban, I., Aguado, C., Sanchez, M., et al., 2007. Regulation of various proteolytic pathways by insulin and amino acids in human fibroblasts. FEBS Lett. 581, 3415–3421.

Ghislat, G., Knecht, E., 2012. New Ca(2+)-dependent regulators of autophagosome maturation. Commun. Integr. Biol. 5, 308–311.

Ghislat, G., Knecht, E., 2013. Ca(2+)-sensor proteins in the autophagic and endocytic traffic. Curr. Protein Pept. Sci. 14, 97–110.

Ghislat, G., Patron, M., Rizzuto, R., et al., 2012. Withdrawal of essential amino acids increases autophagy by a pathway involving Ca2+/calmodulin-dependent kinase kinase-beta (CaMKK-beta). J. Biol. Chem. 287, 38625–38636.

Gordon, P.B., Holen, I., Fosse, M., et al., 1993. Dependence of hepatocytic autophagy on intracellularly sequestered calcium. J. Biol. Chem. 268, 26107–26112.

Gwinn, D.M., Shackelford, D.B., Egan, D.F., et al., 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol. Cell 30, 214–226.

Han, J.M., Jeong, S.J., Park, M.C., et al., 2012. Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway. Cell 149, 410–424.

Hoyer-Hansen, M., Bastholm, L., Szyniarowski, P., et al., 2007. Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol. Cell 25, 193–205.

Kaushik, S., Cuervo, A.M., 2012. Chaperone-mediated autophagy: a unique way to enter the lysosome world. Trends Cell Biol. 22, 407–417.

Kim, J., Kim, Y.C., Fang, C., et al., 2013. Differential regulation of distinct Vps34 complexes by AMPK in nutrient stress and autophagy. Cell 152, 290–303.

Kim, J., Kundu, M., Viollet, B., et al., 2011. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141.

Knecht, E., Aguado, C., Carcel, J., et al., 2009. Intracellular protein degradation in mammalian cells: recent developments. Cell. Mol. Life Sci. 66, 2427–2443.
Lavallard, V.J., Meijer, A.J., Codogno, P., et al., 2012. Autophagy, signaling and obesity. Pharmacol. Res. 66, 513–525.
Li, W.W., Li, J., Bao, J.K., 2012. Microautophagy: lesser-known self-eating. Cell. Mol. Life Sci. 69, 1125–1136.
Lorin, S., Tol, M.J., Bauvy, C., et al., 2013. Glutamate dehydrogenase contributes to leucine sensing in the regulation of autophagy. Autophagy 9, 850–860.
Moruno-Manchon, J.F., Perez-Jimenez, E., Knecht, E., 2013. Glucose induces autophagy under starvation conditions by a p38 MAPK-dependent pathway. Biochem. J. 449, 497–506.
Nicklin, P., Bergman, P., Zhang, B., et al., 2009. Bidirectional transport of amino acids regulates mTOR and autophagy. Cell 136, 521–534.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122, 927–939.
Pfisterer, S.G., Mauthe, M., Codogno, P., et al., 2011. Ca^{2+}/calmodulin-dependent kinase (CaMK) signaling via CaMKI and AMP-activated protein kinase contributes to the regulation of WIPI-1 at the onset of autophagy. Mol. Pharmacol. 80, 1066–1075.
Renna, M., Figueira-Bento, C., Fleming, A., et al., 2013. IGF-1 receptor antagonism inhibits autophagy. Hum. Mol. Genet. 22 (22), 4528–4544.
Ron, D., Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nat. Rev. Mol. Cell Biol. 8, 519–529.
Sancak, Y., Bar-Peled, L., Zoncu, R., et al., 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141, 290–303.
Sarbassov, D.D., Ali, S.M., Sabatini, D.M., 2005. Growing roles for the mTOR pathway. Curr. Opin. Cell Biol. 17, 596–603.
Settembre, C., Zoncu, R., Medina, D.L., et al., 2012. A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J. 31, 1095–1108.
Shaw, R.J., Cantley, L.C., 2006. Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature 441, 424–430.
Vinet, J., Carra, S., Blom, J.M., et al., 2003. Cloning of mouse Ca^{2+}/calmodulin-dependent protein kinase kinase beta (CaMKKbeta) and characterization of CaMKKbeta and CaMKKalpha distribution in the adult mouse brain. Brain Res. Mol. Brain Res. 111, 216–221.
Wauson, E.M., Zaganjor, E., Lee, A.Y., et al., 2012. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Mol. Cell 47, 851–862.
Williams, A., Sarkar, S., Cuddon, P., et al., 2008. Novel targets for Huntington's disease in an mTOR-independent autophagy pathway. Nat. Chem. Biol. 4, 295–305.
Yu, L., McPhee, C.K., Zheng, L., et al., 2010. Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 465, 942–946.
Zoncu, R., Bar-Peled, L., Efeyan, A., et al., 2011. mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 334, 678–683.

CHAPTER

4

Regulation of Autophagy by microRNAs

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OUTLINE

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Abstract

Autophagy is a cellular survival pathway that is responsible for the degradation of cellular constituents such as long-lived proteins and organelles. Autophagy is highly regulated by various signaling pathways including the mTOR, AKT and AMPK pathways. Moreover, conditions resulting in cellular stress such as hypoxia or pathogen entry might activate autophagy. Being at the crossroads of various cellular response pathways, dysregulation of autophagy might result in pathological states including cancer, myopathies or neurodegenerative diseases. Therefore, discovery of novel proteins and pathways regulating autophagy is important for both basic and clinical scientists. Recently, microRNAs were introduced as novel regulators of autophagy. microRNAs are non-protein-coding small RNAs that control cellular levels of transcripts and proteins through post-trancriptional mechanisms. This chapter summarizes the current knowledge of microRNA regulation of autophagy and attempts to integrate this novel layer of regulation into the known autophagy pathways.

INTRODUCTION

Anabolic and catabolic processes are key events that are important for cellular homeostasis. Hence, synthetic and degradative pathways are highly regulated in cells. The two major catabolic mechanisms in cells are the ubiquitin-proteasome system (UPS) and autophagy. The UPS is responsible for the degradation of ubiquitin-conjugated and short-lived proteins in the multimeric protease complex called "proteasome." On the other hand, autophagy is a lysosomal degradation mechanism, through which long-lived proteins and organelles such as mitochondria are engulfed by double membrane autophagic vesicles (autophagosomes) and delivered to and degraded by lysosomes, allowing recycling of cellular building blocks (Mizushima *et al.*, 2011). There are at least three subtypes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. In this chapter, we will mainly focus on macroautophagy (called autophagy herein). Autophagy is active at a basal level in all eukaryotic cells from yeast to man, but it can be upregulated under various conditions leading to stress, including nutrient and/or growth factor deprivation, disease-related aggregate-prone protein accumulation, oxidative stress, toxins, etc. Stress-responsive autophagy generally acts as a survival mechanism allowing the cell to cope with stress. But under certain circumstances autophagy may have an impact on cell death through a special caspase-independent pathway called "autophagic cell death" or through its cross-talk with apoptotic and necrotic death pathways (Gozuacik and Kimchi, 2007). Therefore, autophagy is an essential and vital cellular mechanism on the crossroads of several cellular pathways, and requires a high level of regulation. In this chapter, we will focus on microRNA control of autophagy. Before providing a detailed discussion of the role of microRNAs as new players in autophagy regulation, we will first introduce general autophagy proteins and pathways, and then summarize major signaling events controlling the autophagic activity.

MOLECULAR MECHANISMS OF AUTOPHAGY

Autophagy is a cellular recycling pathway degrading waste material in cells and recycling some cellular organelles, including mitochondria and peroxisomes (Mizushima *et al.*, 2011). The term itself was derived from Greek words for self(auto)-eating (phagos)

by Christian de Duve, the Nobel Laureate of 1960 for his work on lysosomes. During autophagy, the cargo is engulfed by and delivered to lysosomes by unique vesicles composed of double membrane bilayers called "autophagic vesicles or autophagosomes" (Ravikumar *et al.*, 2010). Fusion of the outer bilayer with the membrane of the lysosomes releases the cargo in the inner autophagosomal membrane layer to the lumen of the organelle and results in the formation of the so-called "autolysosomes." Together with the autophagy components, the cargo is then degraded as a result of the activity of lysosomal hydrolases. Products of degradation, for example amino acids, are produced from whole proteins and are recycled back to the cytoplasm in order to allow the reuse of the components by the cell. In this way, autophagy provides nutrients and energy through the use of cells' internal resources, allowing them to survive unfavorable conditions such as starvation, growth factor deprivation and detachment from the natural environment, etc. Autophagy is also the only way to clear and recycle bulky cellular components, including organelles, aggresomes or intracellular parasites, destruction of which is important for cellular health (Ravikumar *et al.*, 2010). For example, depolarized and damaged mitochondria are sources of reactive oxygen radicals that might be detrimental to the cell. By a specialized autophagy process called "mitophagy," those damaged mitochondria are cleared and further damage to the cell is avoided. As such, autophagy is a cellular stress response and a mechanism protecting cellular homeostasis and well-being.

More than 30 *ATG* genes (autophagy-related genes) were identified from baker's yeast and plants to man, in all organisms that were analyzed, revealing the conservation of this process during evolution and the essential nature of it for life (Mizushima *et al.*, 2011). ATG gene protein products were shown to serve at different steps or stages of autophagy, namely initiation, autophagosome formation and elongation, and maturation and fusion with the lysosomes. We will now summarize these stages and explain roles and functions of major ATG proteins in these events.

Initiation and Formation of the Autophagosome

The origin of the autophagosome membrane is still not clear, yet a number of recent studies provided evidence that autophagosome formation is related to preexisting membranous compartments such as endoplasmic reticulum, mitochondria or plasma membrane. Whatever might be the origin, several upstream signals leading to autophagosome formation (see the following sections) converge at the signaling complex TORC1 (mTORC1 in mammals). This protein complex possesses serine/threonine kinase activity due to its central kinase component mTOR. TORC1 was shown to play a role in cellular growth, cell cycle progression and protein synthesis. When cellular and organismal conditions are favorable, mTOR complex is active allowing protein synthesis and cellular growth. Since autophagic activity above basal levels is not required under favorable conditions, TORC1 directly blocks autophagy (Laplante and Sabatini, 2012). In fact, mTOR kinase regulates the activity of the autophagy-related ATG1 kinase (or ULK1/2 in mammals) complex. ATG1 kinase complex consists of ATG1-13-17-29-31 in yeast, and its mammalian counterpart, ULK1/2 complex, is composed of ULK1/2-ATG13-ATG101-FIP200 proteins (Mizushima *et al.*, 2011). This multimeric complex is responsible for initiation of the autophagic activity. mTOR phosphorylation of ATG13 regulates ULK1/2-ATG1 activity. Under stress conditions, mTORC1 is

blocked leading to ATG13 hypophosphorylation. ATG13 binds to ULK1/2 in its hypophosphorylated state and mediates the interaction with FIP200, leading to the phosphorylation of FIP200 by ULK1/2. Under these circumstances, FIP200-ATG1-ATG13 complex triggers cascades that result in autophagosome initiation and nucleation.

The class III phosphatidylinositol 3-kinase (PI3K) complex consists of VPS34 (the PI3K), VPS30, ATG14/Barkor, VPS15 and ATG6/BECN1 (Beclin-1). AMBRA1 was also shown as one of the regulators of the complex in the mammalian system (Mizushima *et al.*, 2011). The VPS34 PI3K complex is responsible for the formation of phosphatidylinositol 3-phosphate (PI3P) from phosphatidylinositols found on cellular membranes. This lipid decoration serves as a landing path for the recruitment of the other ATG proteins to the site of autophagosome formation (PAS, preautophagosomal structure) in the yeast or omegasome/cradle in mammals.

ATG18 or mammalian counterparts WIPI 1–4 are PI3P-binding and WD-repeat containing proteins that localize to PAS or omegasomes and regulate the autophagic activity (Mizushima *et al.*, 2011). ATG2 protein is also another component that interacts with ATG18 and it is important for ATG18 localization to PI3P-rich membranes. Although the exact role is not yet clear, ATG2–ATG18 complex is believed to play a role in formation of autophagosomes. In line with this, the mammalian WIPI 1 and 2 were shown to co-localize with proteins ATG14 and ATG16L1, which are involved in the initiation and elongation stages. Another important protein, ATG9 (mammalian homologue: ATG9L1), is a multipass transmembrane protein that is present on endosomes, Golgi and also autophagic membranes. ATG9 is believed to be involved in lipid delivery to the autophagosome formation centers.

Elongation of the Autophagosome

Following priming of PAS or omegasomes with appropriate protein complexes, already mentioned, autophagic membrane elongation begins. During this step, two ubiquitination-like conjugation systems, namely the ATG12-ATG5-ATG16 and ATG8 (MAP1LC3, or briefly LC3 in mammals) systems, are involved (Mizushima *et al.*, 2011).

ATG12-ATG5-ATG16 is the system where ATG12 is conjugated to ATG5 through activation by ATG7 (E1-like enyzme) and followed by transfer to the E2-like enzyme ATG10. Then, ATG10 triggers ATG12 conjugation to a central lysine residue of ATG5. Formation of a large multimeric complex (300 kDa complex in the yeast and an 800 kDa complex in mammals) requires the coiled-coil protein ATG16 (ATG16L1 in mammals). Resulting ATG12-ATG5-ATG16 complex possesses an E3-like enzyme activity for the second conjugation system.

The second system involves the conjugation of LC3/ATG8 to a lipid molecule, phosphatidylethanolamine (PE). After cleavage of the carboxyl-terminus of LC3 by the cysteine protease ATG4, a glycine residue is exposed, resulting in the formation of so-called LC3-I cytosolic form. LC3-I-lipid conjugation requires the activity of ATG7 (E1-like) and ATG3 (E2-like), and leads to the formation of the lipid-conjugated and autophagic membrane-bound form LC3-II. Consequently, detection of LC3-I conversion into LC3-II is commonly used as a marker of autophagy activation. There are several mammalian LC3 orthologues with overlapping but somewhat different functions in autophagy and other vesicular events, including LC3A-D, GABARAP (GABA-A receptor associated protein) and GATE-16 (Golgi associated ATPase enhancer of 16 kDa). As autophagosome biogenesis and clearance is a dynamic process, LC3-II formation and recycling is regulated on a tight schedule, where

the same ATG4 enzymes cleave the lipid bond to allow detachment and recycling of LC3 from mature autophagosomes.

Until recently, autophagy was believed to be a nonselective phenomenon. In the last few years, several target-specific autophagy receptors were revealed, including p62/SQSTM1, NBR1, NIX, NDP52 and OPTN. They are similar to other receptors; for example, p62/SQSTM1 binds to LC3 on autophagic membranes and targets to be degraded (e.g., ubiquitylated aggregate proteins or depolarized mitochondria) in the same time, facilitating the segregation and concentration of the cargo in the vesicles.

Maturation and Fusion with the Lysosomes

Fully mature autophagosomes move within the cell to meet late endosomes or lysosomes (vacuole in the yeast) for delivering their cargo to be degraded (Mizushima *et al.*, 2011). Homotypic fusion events play an important role in the autophagosome and lysosome fusion process, and proteins such as vacuolar syntaxin homologue Vam3, SNAP-25 homologue Vam7, the Rab family GTP-binding protein Ypt7 and Sec18 are required for the process in the yeast. In mammals, together with the integral lysosome membrane protein LAMP2 and the SNARE machinery, Rab7, Rab22 and Rab24 were shown to play important roles in fusion. Moreover, dyneins are necessary for the transport of autophagosomes along microtubules to allow them to meet acidic compartments. Following fusion, the cargo is degraded through the action of lysosomal enzymes including cathepsins, and the monomers that are generated such as amino acids are recycled to cytosol and reused by the cell in various synthetic processes.

MAJOR SIGNALING PATHWAYS REGULATING AUTOPHAGY

mTOR Pathway

The mTOR pathway integrates several signals that modulate autophagy activation. Under fed condition and in the absence of stress signals, mTOR coordinates growth-related cellular events, including initiation of translation, ribosome biogenesis, protein synthesis, and consequently cell size. The mTOR pathway involves two complexes: mTOR complex 1 (mTORC1) that controls autophagy, and the mTORC2 complex that mainly regulates cytoskeletal reorganization and migration (Laplante and Sabatini, 2012).

The subunits of the mTORC1 complex are Raptor (regulatory associated protein of mTOR), GβL (G-protein β-subunit-like protein) and PRAS40 (proline-rich Akt substrate of 40 kDa). The second complex, the mTOR complex 2 (mTORC2), consists of mTOR, Rictor (rapamycin-insensitive companion of mTOR), GβL, SIN1 (SAPK-interacting protein 1) and PROTOR (protein observed with rictor). Rapamycin, a chemical inducer of autophagy, was only shown to inhibit the activity of mTORC1 but not that of mTORC2. Rapamycin does so by forming an mTOR-inhibitory complex with FKBP12 (immunophilin FK506-binding protein of 12 kDa) blocking the activity of kinase, and hence activating autophagy. Phosphorylation and activation of downstream targets of mTORC1, including S6K1 (P70S6K) and 4E-BP1 (translation initiation factor 4E binding protein-1), are also decreased when the complex is inhibited by rapamycin. Under physiological conditions, nutrient or

growth factor deprivation leads to the stabilization of the raptor-mTOR complex and results in the inhibition of mTORC1 and activation of autophagy.

Tuberous sclerosis complex 1 and 2 proteins (TSC1/2) function as upstream regulators of the mTOR complex. TSC1/2 act as GTPase activating protein (GAP) complex and inactivate the small GTPase RHEB (Ras homologue enriched in brain) protein, a key activator of mTORC1. As mentioned previously, the ATG1/ULK1-2 complex is placed downstream to the mTOR complex and it translates mTORC1 inhibition to autophagy activation.

AKT/PKB and Growth Factors

The AKT/PKB pathway is mainly controlled by growth factors, hormones (e.g., insulin-like growth factors) and transmits signals affecting autophagy activation (He and Klionsky, 2009). In this pathway, stimulation of cell surface receptors activates Class I PI3K proteins that are responsible for the conversion of lipid phosphatidylinositol-4,5-biphosphate to phosphatidylinositol-3,4,5-triphosphate. Accumulation of these lipid messengers leads to the recruitment of AKT/PKB to the plasma membrane. AKT/PKB is an upstream activator of mTORC1 through direct phosphorylation of TSC2 (a terminator of the mTOR activator RHEB activity) and PRAS40 (an inhibitor of the RHEB activity), events that result in autophagy inhibition. Conversely, PTEN (phosphatase and tensin homologue deleted from chromosome 10), which is responsible for dephosphorylating inositol lipids and leading to AKT signal termination, was shown to activate autophagy.

FoxO Regulation of Autophagy

FoxO3, a stress-responsive transcription factor that is downstream to the AKT/PKB pathway, was shown to control the transcription of various autophagy genes such as LC3B, ATG12, ATG4b and BNIP3 (He and Klionsky, 2009). Especially, LC3 is recycled and consumed during long-lasting autophagic activity, and its accumulation through transcription and protein synthesis is critical to sustain the activity. Therefore, in many cases, transcriptional upregulation of the autophagy genes through FoxO3 is an important event in the control of autophagic activity (He and Klionsky, 2009).

AMPK Pathway

Change in intracellular energy levels might also activate or inhibit autophagy through mTORC1 (He and Klionsky, 2009). AMPK is the sensor for the ATP/AMP ratio, hence the energy status in cells. AMPK phosphorylates TSC2 and glycogen synthase β (GSK3β), to inhibit mTORC1 signaling and to activate autophagy. LKB1 is an upstream kinase to AMPK, and some other kinases such as RSK1 and ERK1/2 influence the mTORC1 pathway through their effects on TSC1/2, by phosphorylating and inhibiting its activity. The Ras pathway also conveys signals to these pathways through AKT, ERK1/2 and RSK1 pathways.

Inositol Pathway

Apart from the mTOR pathway, autophagy is regulated through mTOR-independent pathways as well, including the inositol signaling pathway (Sarkar, 2013). Upon a stimulus, phospholipase C is activated and hydrolyzes PtdIns (4, 5) *P2* to form Ins (1, 4, 5) *P3* (IP3)

and DAG (diacylglycerol). IP3 results in Ca^{+2} release from ER and acts as a secondary messenger. Increase in IP3 levels in the cytoplasm inhibits autophagic activity and autophagosome formation. Drugs such as lithium or L-690 330 that lower cytoplasmic levels of IP3 through inhibition of IMP (inositol monophosphatase) activate autophagy and facilitate clearance of aggregate proteins independently from the mTOR activity.

Stress-Responsive BECN1/BCL2 Complex

BECN1 was shown to be subject to stress-related regulation through its interaction with antiapoptotic BCL2/BCL2L1/MCL1 BCL2 family proteins (Eisenberg-Lerner *et al.*, 2009). These proteins can bind to BECN1, sequester it and inhibit its contribution into autophagy pathways. A number of different kinases were shown to regulate binding, hence autophagic activity. It is shown that upon nutrient deprivation, phosphorylation of BCL2 by JNK1 resulted in its dissociation from BECN1 and resulting in autophagy activation. Tumor suppressor and cell death-related kinase DAPk was also shown to interrupt BCL-XL-BECN1 interaction, and activate autophagy through direct phosphorylation of BECN1.

Hypoxia, ROS and Autophagy

Hypoxia is one of the activators of autophagy. One of the major responses to hypoxia-related stress is the stabilization of the hypoxia inducible factor-1a (HIF-1a) (He and Klionsky, 2009). HIF-1a induces transcription of a Bcl-2 family member BNIP3 (Bcl-2 adenovirus E1a 19 kDa interacting protein 3) that can bind to Bcl-2 and release BECN1 resulting in the activation of autophagy.

Controlled reactive oxygen species (ROS) release serves as an intracellular signal modulating autophagy. Yet, imbalanced production of ROS might result in oxidative damage and cell death. Mitochondria are responsible for the generation of the ROS in cells. Hence, in order to reduce cellular ROS levels, damaged mitochondria should be degraded. A special type of autophagy, called "mitophagy," is the major recycling mechanism for this organelle (Chen and Chan, 2009). A key step in mitophagy is the recruitment of the E3 ubiquitin ligase Parkin to mitochondria. Parkin binds Pink1 (PTEN-induced putative protein kinase-1) protein that accumulates on the outer membrane of damaged mitochondria and ubiquitinates several mitochondrial outer membrane proteins, leading to changes in fusion–fission dynamics and triggering mitophagy.

P53 Pathway

The p53 pathway is the guardian of genome integrity during stress conditions including DNA damage and oncogene activation; p53 was proposed to play a dual role in the regulation of autophagy. Cytoplasmic p53 was shown to repress autophagy; however, the nuclear protein was reported to stimulate autophagy through activation of DRAM and Sestrin2 (Tasdemir *et al.*, 2008). DRAM isoforms were localized to various vesicular compartments including autophagosomes and lysosomes and they might play a role in autophagosome maturation (Mah *et al.*, 2012).

Genes of several proteins that were mentioned here were found to be regulated by microRNAs resulting in changes in autophagic activity of cells. In the next sections, we will

briefly introduce microRNA pathways and then discuss the emerging role of miRNAs and their networks in autophagy regulation.

SMALL REGULATORS: microRNAs, THEIR BIOGENESIS AND BIOLOGICAL FUNCTIONS

Discovery of the RNA interference mechanism led to a breakthrough in the understanding of post-transcriptional gene regulation and brought the Nobel Prize in Physiology and Medicine to Andrew Z. Fire and Craig C. Mello in the year 2006. There are different types of small RNAs in the mammalian system and small interfering RNAs (siRNAs) and microRNAs (miRNAs) are the best characterized members of the small RNA world. Against the central dogma in molecular biology, noncoding small RNAs are gene regulators and they are not translated into proteins. They mainly act on post-transcriptional regulation of genes by affecting messenger RNA (mRNA) stability and/or by blocking protein translation.

microRNAs

microRNAs, endogenous regulators of gene expression, are coded by the genome of various organisms, including plants and mammals (Kim, 2005). They regulate important biological processes, including differentiation, proliferation, cell death and cell cycle. It has been estimated that miRNAs regulate nearly 30% of all human genes. Deregulation of miRNA expression might result in pathologies such as cancer, neurodegenerative diseases or developmental abnormalities. In cancer, miRNAs can generally act as either tumor suppressors or oncogenes, and even sometimes in both ways depending on tumor types (Shenouda and Alahari, 2009).

Names of miRNAs are denoted as a combination of letters and numbers (Kim, 2005). Prefixes (3–4 letters) in miRNA names indicate the species (e.g., *Homo sapiens*: hsa-miR-376a). Mature miRNA sequences are denoted with "miR"; however, primary-miRNAs are denoted with "mir." miRNAs conserved between species are usually given the same number (e.g., rno-miR-101 and mmu-miR-101). miRNAs with closely related mature sequences are marked with letter suffixes (e.g., hsa-miR-376a and hsa-miR-376b) and they usually belong to the same miRNA family. In the mature miRNA/miRNA* duplex, the complementary arm of the predominant strand is indicated with a star (*), and it may sometimes be functionally relevant. Yet, in line with the HUGO Gene Nomenclature Committee rules, several authors prefer to briefly use the mature miRNA names in italic capital letters (e.g., *MIR376B*).

microRNA Biogenesis

microRNA genes might be located within introns as well as in intergenic regions (Kim, 2005). Intronic miRNAs are transcribed together with the protein coding precursor mRNA, whereas intergenic miRNAs are transcribed from their own promoters as a gene cluster or as a single unit. Sometimes, several miRNAs belonging to the same family can be transcribed as long transcripts called polycistronic clusters.

miRNAs are transcribed by the RNA polymerase II as primary-miRNAs (pri-miRNAs) (Figure 4.1). They are processed by a Drosha-DGCR8 complex in the nucleus to produce

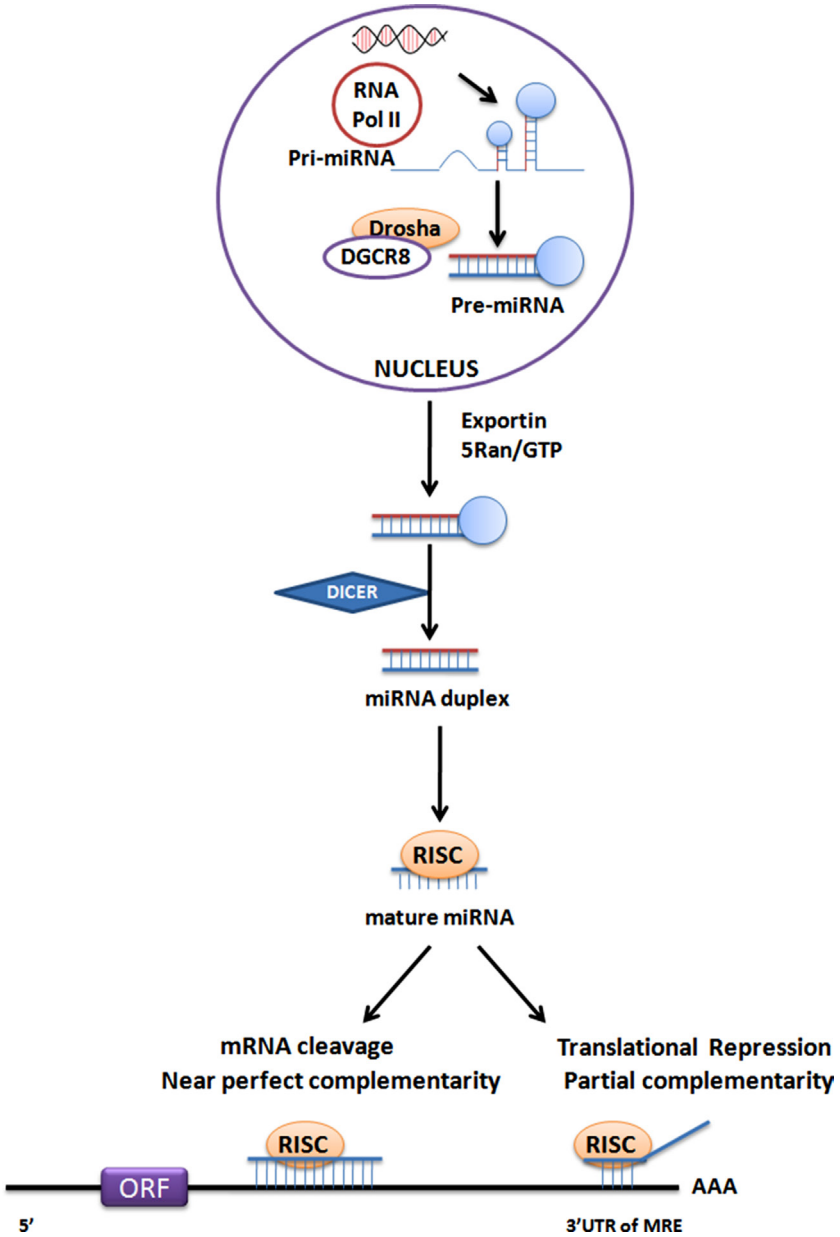

FIGURE 4.1 Biogenesis and mechanism of action of microRNAs (miRNA) in mammals. miRNAs are transcribed by RNA polymerase II as primary-miRNAs (pri-miRNAs) and processed by a Drosha-DGCR8 complex in the nucleus to produce precursor miRNAs (pre-miRNAs). Then pre-miRNAs are transported from nucleus to cytoplasm, and Dicer complex cleaves the hairpin of pre-miRNA to form the miRNA duplex. One of the mature miRNA strands is loaded onto the RNA-induced silencing complex (RISC). The fate of the target is determined by the degree of complementarity between the mature miRNA strand and the target mRNA sequences (mainly in the 3′UTR regions) and the end result is downregulation of target protein levels either by a translational repression (partial complementarity) or by cleavage of the target mRNAs (near perfect complementarity).

hairpin-shaped premature-miRNAs (pre-miRNA) (Kim, 2005). After their transport from the nucleus to the cytoplasm, DICER protein further cleaves the hairpin of pre-miRNA leading to the formation of ~21–22 nt long miRNA/miRNA* duplexes. Then, one of the mature miRNA strands is loaded onto the RNA-induced silencing complex (RISC) containing an Argonaute protein (AGO), which guides the mature miRNA strand to its target messenger RNAs (mRNAs). The degree of complementarity between the mature miRNA strand and the target mRNA sequences (mainly in the 3′UTR regions) determines target specificity and the fate of the target. In case of a perfect match between the mature miRNA and target sequences, the mRNA is generally degraded. Additionally, deadenylation of the mRNA and decapping are important for degradation. While a perfect match is believed to lead to mRNA destabilization, in case of a partial complementarity between the miRNA and its target mRNA, translation inhibition through blockage of the translation machinery seems to be the dominant mechanism. In the latter case, miRNA-mRNA complexes are mainly sequestered in the P-bodies (Figure 4.1).

microRNAs: NOVEL REGULATORS OF AUTOPHAGY

Studies in the last 3–4 years introduced miRNAs as new players in the regulation of autophagy. mRNA of proteins playing a role in various steps of autophagy, from proteins functioning in the upstream stimulatory or inhibitory pathways to the final stages of autophagic degradation, were reported to be miRNA targets. microRNA studies so far reveal that we are only beginning to understand stress-related miRNA networks controlling cellular responses, including autophagy. Several autophagy-related miRNAs were also shown to affect other biological responses, such as apoptosis, growth or proliferation. Moreover, while only one autophagy-related target was reported for some miRNAs, others affected intracellular levels of proteins playing key roles in more than one stage of the autophagy pathway. In this chapter, we will summarize accumulating data on how miRNAs control and modulate autophagy in various contexts. (See Table 4.1 for a list of autophagy-regulating microRNAs that are explained in the following sections.)

miRNA Regulation of Signals Upstream to Autophagy Pathways

The mTOR complex is an important regulator of autophagy and a number of miRNAs were shown to target it. For example, *MIR7A* was shown to regulate mTOR and inhibition of the miRNA resulted in an increase in mTOR activity, resulting in increased cellular proliferation in adult pancreatic β-cells (Wang, Y. *et al.*, 2013). Another study showed a similar role for *MIR7* in a hepatocellular carcinoma (HCC) model, where *MIR7* inhibited tumor growth and metastasis through targeting of mTOR and p70S6K (Fang *et al.*, 2012). Therefore, *MIR7* seems to act as a tumor suppressor, inhibiting tumorigenesis and cell proliferation in different tissue types and possibly activating autophagy in this context. Various other miRNAs were shown to target mTOR in different cell types. *MIR199-3P* and *MIR101* targeted mTOR in hepatocellular carcinoma and vascular endothelial cells, respectively. Direct binding of *MIR1993P* to mTOR 3′UTR was demonstrated and the miRNA was shown to potentiate

TABLE 4.1 Anticancer agents inducing cell death and autophagy in cancer cell lines

Drug class	Anticancer agent	Pathways involved/ targets	Autophagy inhibition	Cancer cell type	Reference
Antimetabolites	Pemetrexed	AMPK, mTOR	3-MA, siRNA Beclin1	Breast cancer cells (BT474), metastatic breast cancer cells (4T1), and hepatocellular carcinoma cells (HuH7), lung cancer cells (H460)	Bareford *et al.* (2011)
	3-Bromopyruvate + glutamine starvation	ATP, ROS	3-MA, siRNA Atg5	Cervical cancer cells (HeLa)	Cardaci *et al.* (2012)
Tyrosine kinase inhibitors	Lapatinib + obatoclax	Akt/mTOR, BCLXL/ MCL1, AIF	siRNA Atg5, siRNA Beclin1	Colon cancer cells (HCT116), breast cancer cells (MCF-7, SKBR3, BT474, MMTV-HER2, 4TI-luc-I2B, HCC1954)	Martin *et al.* (2009)
	Cetuximab + rapamycin	PI3K, mTOR	Cloroquine, siRNA Atg5	Vulvar squamous carcinoma cells (A431), head and neck cancer cells (HN5, FaDu)	Li *et al.* (2010)
	Imatinib	Akt/mTOR, ERK1/2 pathway	3-MA, siRNA Atg5, siRNA Atg10, siRNA Atg12	Glioblastoma cells (U87-MG, LN229, U373-MG, LNZ308)	Shingu *et al.* (2009)
	Sorafenib + pemetrexed	mTOR, Mcl-1 and Bcl-XL	3-MA, siRNA Beclin1	Breast cancer cells (BT474), metastatic breast cancer cells (4T1), hepatocellular carcinoma cells (HuH7), lung cancer cells (H460)	Bareford *et al.* (2011)
Histone deacetylase inhibitors	SAHA	n.s.	3-MA	Chondrosarcoma cells (SW1353, RCS, OUMS-27)	Yamamoto *et al.* (2008)
	SAHA	Akt/mTOR	siRNA Beclin1, siRNA Atg7, siRNA TSC2	Cervical carcinoma cells (HeLaS3)	Cao *et al.* (2008)
	OSU-HDAC42, SAHA	Akt/mTOR, ER stress	3-MA, siRNA Atg5	Hepatocellular carcinoma cells (Huh7, HepG2, Hep3B)	Liu *et al.* (2010)

(Continued)

TABLE 4.1 (Continued)

Drug class	Anticancer agent	Pathways involved/ targets	Autophagy inhibition	Cancer cell type	Reference
Alkalyting agent	Temozolomide	n.s.	3-MA	Glioblastoma cell lines (U373-MG, T98G, U251-MG, GB-1, U87-MG, A172)	Kanzawa *et al.* (2004)
mTOR inhibitors	RAD001 + doxorubicin or radiation	Met dephosphorylation	siRNA Atg5	Papillary thyroid cancer cells (TPC-1, 8505-C)	Lin *et al.* (2010)
	RAD001 + Z-DEVD + radiation	n.s.	siRNA Atg5, siRNA Beclin1	Lung cancer cells (H460)	Kim *et al.* (2008)
Proteasome inhibitors	Bortezomib	Caspase-8 activation	3-MA, siRNA Atg5	Cervical carcinoma cells (HeLa Bcl-2 overexpressing), NSCLC cells (H460 Bcl-2 overexpressing)	Laussmann *et al.* (2011)
	Bortezomib	Lysosomal degradation of TRAF6 3-MA		Acute myeloid leukemia cells (TF-1, THP1, HL60), primary myelodysplastic syndrome cells (MDS-01, MDS-02)	Fang *et al.* (2012)
BH3 mimetics	(-)-Gossypol + temozolomide	mTOR	siRNA Atg5, siRNA Beclin1	Glioblastoma cells (U87, U343, MZ-54)	Voss *et al.* (2010)
	Obatoclax + dexamethasone	mTOR, dissociation of Beclin-1/MCL-1 interaction	3-MA, bafilomycin A, siRNA Atg7, siRNA Beclin1	GC-resistant acute lymphoblastic leukemia cells (CEM-C1, Jurkat)	Bonapace *et al.* (2010)
	Obatoclax + lapatinib	Akt/mTOR, AIF	siRNA Atg5, siRNA Beclin1	Colon cancer cells (HCT116)	Martin *et al.* (2009)
Glucocorticoids	Dexamethasone	PMLi, Akt inhibition	3-MA, LY294002, siRNA Beclin1	Acute lymphoblastic leukemia cells (RS4;11, SUP-B15)	Laane *et al.* (2009)
	Dexamethasone + obatoclax or rapamycin	mTOR, dissociation of Beclin-1/MCL-1 interaction	3-MA, bafilomycin A, siRNA Atg7, siRNA Beclin1	GC-resistant acute lymphoblastic leukemia cells (CEM-C1, Jurkat)	Bonapace *et al.* (2010)

Others	STF-62247	Golgi trafficking and PI3K	3-MA, siRNA Atg5, siRNA Atg7, siRNA Atg9	Renal cell carcinoma cells (VHL deficient; RCC4, RCC10)	Turcotte *et al.* (2008)
Sphingolipids	CERS1 and C18-ceramide	Lethal mitophagy	siRNA Atg3, siRNA Atg5, siRNA Atg7	Head and neck squamous cell carcinoma cells (UM-SCC-22A)	Sentelle *et al.* (2012)
Others	Fenretinide	n.s.	3-MA	Breast cancer cells (MCF-7)	Fazi *et al.* (2008)
Vitamin D analogue	EB1089	n.s.	3-MA	Breast cancer cells (MCF-7)	Hoyer-Hansen *et al.* (2005)
Plant-based chemotherapeutics	Resveratrol	Akt/mTOR	si RNA Atg7	Breast cancer (MCF-7)	Scarletti *et al.* (2008)
	Resveratrol	mTOR, JNK and AMPK	Bafilomycin A, siRNA Atg5,	Chronic myeloid leukemia cells (K562)	Puissant *et al.* (2010)
	Cannabinoids	ER stress, mTOR	siRNA Atg1/Ulk1, siRNA Atg5,	Glioblastoma cells (U87MG)	Salazar *et al.* (2009)
	Cannabinoids	ER stress, Akt/mTOR	3-MA, E64d + pepstatin A, siRNA	Hepatocellular carcinoma cells (Huh7, HepG2)	Vara *et al.* (2011)
	β-Lapachone	ROS induction	3-MA, siRNA Atg6, siRNA Atg7,	Glioblastoma cells (U87MG)	Park *et al.* (2011)
	Avicin D	ATPl, AMPK, mTOR	siRNA Atg5, siRNA Atg7, siRNA	Osteosarcoma cells (U2OS)	Xu *et al.* (2007)
	B10 betulinic acid derivative	Caspase-3, LMP	siRNA Atg5, siRNA Atg7, siRNA	Glioblastoma cells (U87MG)	Gonzalez *et al.* (2012)
	Polygonatum cyrtonema lectin	ROS, p38–p53 activation	3-MA	Melanoma cells (A375)	Liu *et al.* (2009)

n.s. = not studied.

doxorubicin-induced apoptosis in HCC (Fornari *et al.*, 2010). *MIR101* overexpression in response to laminar shear stress faced by endothelial cells resulted in decreased mTOR expression and proliferation (Chen *et al.*, 2012a). Two recent studies again showed a role for *MIR199A* in mTOR inhibition. miRNA expression resulted in selective resistance to cisplatin-induced apoptosis in human ovarian cancer cells (Wang, Z. *et al.*, 2013) and regulated endometrial cancer cell proliferation (Wu *et al.*, 2013).

Pathways upstream to mTOR, including the PI3K/AKT pathway, were shown to be regulated by miRNAs as well. *MIR30A* was shown to target PIK3CD in colorectal carcinoma (Zhong *et al.*, 2013) and *MIR376A*, which was downregulated in HCC, regulated PIK3R1 (P85α) directly (Zheng *et al.*, 2012). Another study showed that upregulation of *MIR122* in renal cell carcinoma resulted in the activation of the AKT/PKB pathway, leading to phosphorylation of mTOR itself and its targets, and possibly inhibiting autophagy in this context (Lian *et al.*, 2013). RHEB protein, an activator of mTOR and a negative regulator of autophagy, was targeted by *MIR155*. miRNA-mediated downregulation of RHEB resulted in the activation of autophagy and led to the clearance of intracellular mycobacteria (Wang, J. *et al.*, 2013). Also, very recently, *MIR155* was shown to have a role in hypoxia-induced autophagy through inhibition of mTOR pathway components (Wan *et al.*, 2014). Direct targets of *MIR155* were described as RHEB, RICTOR and RPS6KB2, and dysregulation of the mTOR pathway by miRNA resulted in autophagic activation.

miRNA Regulation of Autophagosome Initiation and Formation

Several microRNAs were shown to directly target ULK1/2 complexes that play a key role in autophagosome initiation. Huang and coworkers (2011) showed that ULK2 was a direct target of *MIR885*. This microRNA is also a regulator of genes important for cell viability and apoptosis, hence possibly integrating them with the autophagy pathway. Another study showed that members of the *MIR7* cluster, *MIR20A* and *MIR106B*, were regulating leucine deprivation-induced autophagy in the C2C12 myoblast cell line through targeting of ULK1. Direct interaction of these miRNAs with the ULK1 3′UTR was demonstrated in this functional study (Wu *et al.*, 2012). The *MIR290-295* cluster codes for a set of miRNAs regulating ULK1. In melanoma cells, these miRNAs inhibited autophagic cell death during glucose deprivation, and gave selective survival advantage to tumor cells through targeting of ULK1 and ATG7 (Chen *et al.*, 2012b). A recent study showed that MIR130A targeted ATG2B and DICER1 and thus inhibited autophagy and triggered death of chronic lymphocytic leukemia cells (Kovaleva *et al.*, 2012). Therefore, ATG1/ULK complex is a critical step in autophagic vesicle formation and, consequently, several independent miRNAs target this step to modulate and fine-tune autophagy.

Regulation of autophagosome formation by *MIR30A* was investigated in several studies. Initially, a paper by Zhu *et al.* (2009) first revealed a role for *MIR30A* in the regulation of rapamycin-induced autophagy. They showed that the miRNA targeted BECN1 and inhibited autphagy in MCF-7 cells. Two other studies confirmed the role of *MIR30A* in autophagy regulation. Zou *et al.* (2012) showed that *MIR30A* decreased cisplatin-induced autophagy in a BECN1-dependent manner, sensitized tumor cells to chemotherapy and resulted in decreased tumor size in *in vivo* experiments. On the other hand, Yu *et al.* (2012) showed

that combined treatment of chronic myeloid leukemia cells with imatinib and *MIR30A* increased cytotoxicity through regulation of ATG5 and BECN1. Other studies suggested that *MIR30A* was important in the regulation of autophagy induced by alcohol or angiotensin-II in cardiomyocytes (Guo *et al.*, 2012; Pan *et al.*, 2013). In both cases, treatments resulted in a decreased expression of *MIR30A*, and therefore increased cardioprotective autophagy in relation to increased expression of BECN1. Another member of the *MIR30* family, *MIR30D*, was shown to block starvation- and rapamycin-induced autophagy by regulating the expression of several autophagy-related genes including BECN1, BNIP3L, ATG12, ATG5 and ATG2 (Yang *et al.*, 2013). All studies cited here showed that *MIR30* family members might play important roles in the regulation of autophagy, mainly through their effects on BECN1 and ATG5 levels.

Indeed, BECN1 is an important hub and a recurrent target in autophagy regulation: members of the *MIR376* family, *MIR376A* and *MIR376B*, were shown to regulate autophagy through their effect on BECN1 and ATG4C in breast and liver cancer cells (Korkmaz *et al.*, 2012, 2013). *MIR376A* and *B* directly targeted the 3′UTR sequences of BECN1 and ATG4C. In addition, *MIR1995P* was shown to regulate irradiation-induced autophagy by targeting BECN1 and DRAM1 in two different breast cancer cell lines (Yi *et al.*, 2013).

miRNA Regulation of the Autophagosome Elongation Step

Ubiquitin-like conjugation system components that are responsible for autophagic vesicle elongation were also shown to be miRNA targets. Both *MIR181A* and *MIR30A* were shown to regulate cellular levels of the Atg5 protein. mTOR-dependent autophagy was blocked by the overexpression of *MIR181A* in different cell lines, including breast cancer, hepatocellular carcinoma and leukemia cells (Tekirdag *et al.*, 2013). *MIR181A* was directly targeting the ATG5 3′UTR. *MIR30A* was also reported to target ATG5, and overexpression of the miRNA increased imatinib-induced cytotoxicity through inhibition of autophagy, leading to cell death by apoptosis in leukemia cells (Yu *et al.*, 2012). Huang *et al.* (2011) showed that a number of genes important in autophagosome formation were targeted by microRNAs. ATG5, BECN1, ATG10, ATG12, ATG16L2 and UVRAG were the targets of *MIR181A, MIR519A, MIR374A* and *MIR630*. ATG7 was targeted by several different miRNAs as well. *MIR375* was shown to regulate the expression of ATG7 under hypoxic conditions. Impairment of autophagic activity through targeting of ATG7 reduced viability of hepatocellular carcinoma cells during hypoxia (Chang *et al.*, 2012). In another study, Xu *et al.* (2012) reported that a cisplatin-induced downregulation of *MIR1995P* in human hepatocellular carcinoma cells enhanced autophagy by increasing the expression of ATG7. Lastly, ATG7 was also a target of the *MIR290/295* cluster that was previously mentioned to affect ULK1 levels. Expression of *MIR290/295* cluster members resulted in the inhibition of autophagic cell death in melanoma cells, giving a selective survival advantage to tumor cells (Chen *et al.*, 2012b). *MIR17* is another miRNA regulating autophagy through ATG7 and sensitizing glioblastoma cells to radiation and chemotherapy (Comincini *et al.*, 2013). ATG16L1, a key component of the first ubiquitin-like conjugation system, was targeted by *MIR106B* in intestinal epithelial cells (Zhai *et al.*, 2013b). Additionally, a decrease in *MIR23* expression was shown to correlate with increased ATG12 expression in a pancreatic cancer cell model (Wang, P. *et al.*, 2013b).

Overexpression of the miRNA resulted in a decrease in radiation-induced autophagy; hence, therapeutic modulation of *MIR23* might be used to increase the sensitivity of pancreatic cancer to radiation (Wang, P. *et al.*, 2013a). The *LC3* gene itself was reported to be targeted by *MIR204* and might be affecting hypoxia-induced autophagy in cardiomyocytes (Xiao *et al.*, 2011).

ATG4 family members were shown to be regulated by several miRNAs including *MIR376A*, *MIR376B* and *MIR101*. *MIR376A* and *MIR376B* overexpression downregulated, and their inhibition by antagomirs increased, ATG4C levels in cancer cells (Korkmaz *et al.*, 2012, 2013). In another study, a luciferase-based functional microRNA screen revealed ATG4D gene as a target of *MIR101* (Frankel *et al.*, 2011). The miRNA blocked autophagy and sensitized breast cancer cells to the cytotoxic effects of tamoxifen. Moreover, the autophagic adaptor protein p62/SQSTM1 was reported to be targeted by *MIR17*, *MIR 20*, *MIR93* and *MIR106* (Meenhuis *et al.*, 2011).

miRNA Regulation of Vesicular Transport Events, Autophagosome Maturation and Fusion with Lysosomes

Several independent studies revealed a number of miRNAs targeting the Rab family. *MIR101* mentioned previously was also shown to target Rab5a in MCF-7 cells (Frankel *et al.*, 2011). *MIR502* targeted RAB1B and overexpression of this miRNA inhibited autophagy and blocked tumor progression in colon cancer (Zhai *et al.*, 2013a). Other RAB proteins regulating endocytic pathways were shown to be targeted by microRNAs as well. For example, *MIR373* targeted Rab22A and *MIR451* targeted Rab14 in cancer cells but consequences of their miRNA-dependent regulation on autophagy were not studied (Wang *et al.*, 2011). Very recently, lysosomal proteins RAB27A and LAMP3 were shown to be targeted by MIR205 in a prostate cancer cellular model (Pennati *et al.*, 2013). UVRAG, a BECN1-binding protein, was revealed to be functional in endosomal trafficking through its interaction with Vps tethering complexes, and it was shown to be a target of *MIR374A* and *MIR630* (Huang *et al.*, 2011).

microRNA REGULATION OF AUTOPHAGY-RELATED SIGNALING PATHWAYS

Besides microRNAs that target genes related to autophagosome formation and maturation, genes of proteins that directly or indirectly affect the autophagy pathways were also reported to be regulated by miRNAs.

A study showed that autophagic cell death might be induced through an *MIR7*-epidermal growth factor receptor (EGFR) axis. In this context, virus-mediated upregulation of *MIR7* resulted in a decrease in EGF expression and activated autophagic cell death in human cancer cells (Tazawa *et al.*, 2012). In a tumor stroma model, autophagy was proposed to be a stress adaptation mechanism in caveolin-1 null cells, and upregulation of *MIR31* and *MIR34C* correlated with hypoxia-related oxidative stress, and possibly autophagy and/or mitophagy (Pavlides *et al.*, 2010). In another study, *MIR212* and *MIR132* were found to negatively modulate the expression of the transcription factor FoxO3, an important regulator of autophagy genes (Ucar *et al.*, 2012).

Polymorphism in the genes of autophagy proteins ATG16L1 and IRGM was associated with the inflammatory bowel disease called Crohn's disease (CD). A study showed that *MIR196* was overexpressed in the intestines of individuals with CD and downregulated IRGM protective allele but not the risk-associated allele (Brest *et al.*, 2011). They suggested that autophagic clearance of bacteria (xenophagy) halted as a result of miRNA overexpression, leading to an increase in the intracellular bacterial load and contributing to CD pathogenesis. Similarly, targeting of ATG16L1 by *MIR93* and *MIR106B* led to xenophagy abnormalities in a CD context (Lu *et al.*, 2014).

miRNAs were also involved in the regulation of autophagy through modulation of histone acetylation. Waldenström macroglobulinemia (a type of B cell lymphoma) cells showed altered expression on *MIR9** and *MIR206* (decreased *MIR9** and increased *MIR206* expression) (Roccaro *et al.*, 2010). Predicted targets of the miRNAs were histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), indicating that miRNAs modulated histone acetylation, leading to decreased acetylation and increased deacetylation of important cellular genes. In this context, increasing *MIR9** levels inhibited HDAC activity and resulted in cell death by apoptosis and autophagy.

Direct functional analyses using experimental approaches are essential to prove interaction and regulation of genes by miRNAs; however, systems biology-based integrative computational analyses might also give an insight into complex miRNA networks regulating gene expression. Jegga *et al.* (2011) followed a systems biology approach to reveal miRNAs that regulate autophagy-lysosomal networks and revealed that several miRNAs including *MIR130*, *MIR98*, *MIR124*, *MIR204* and *MIR142* might play a role in this network. Experimental verifications should reveal the importance of these miRNAs in the regulation of the pathways.

DNA damage was shown to stimulate autophagy and a major genotoxic stress response component, ATM, was regulated by miRNAs. Indeed, radiation-induced *MIR18A* resulted in increased ATM expression and in the suppression of mTOR activity, stimulating autophagy in colon cancer cells (Qased *et al.*, 2013).

CONCLUSION

microRNAs play important roles in various basic cellular events and autophagy seems no exception. Studies in recent years revealed the importance of miRNAs in the regulation of autophagy. Various stages of the autophagy pathways, from upstream signals to autophagosome completion and maturation, were shown to be controlled by miRNAs. Complex signaling pathways, including physiological signals and stress-related pathways, control and modulate autophagy. Until recently, most of the studies performed on autophagy signaling pathways concentrated on transcriptional or post-translational events. Yet, miRNAs are powerful regulators of mRNA stability and translation, and discovery of miRNAs playing a role in autophagy regulation revealed another neglected but equally important layer of biological control mechanisms of autophagic activity. Considering that each miRNA determines cellular levels of tens to hundreds of genes and controls sometimes several related pathways, an miRNA network connecting autophagy regulation to other important cellular pathways and coordinating major cellular events should soon emerge. In addition to

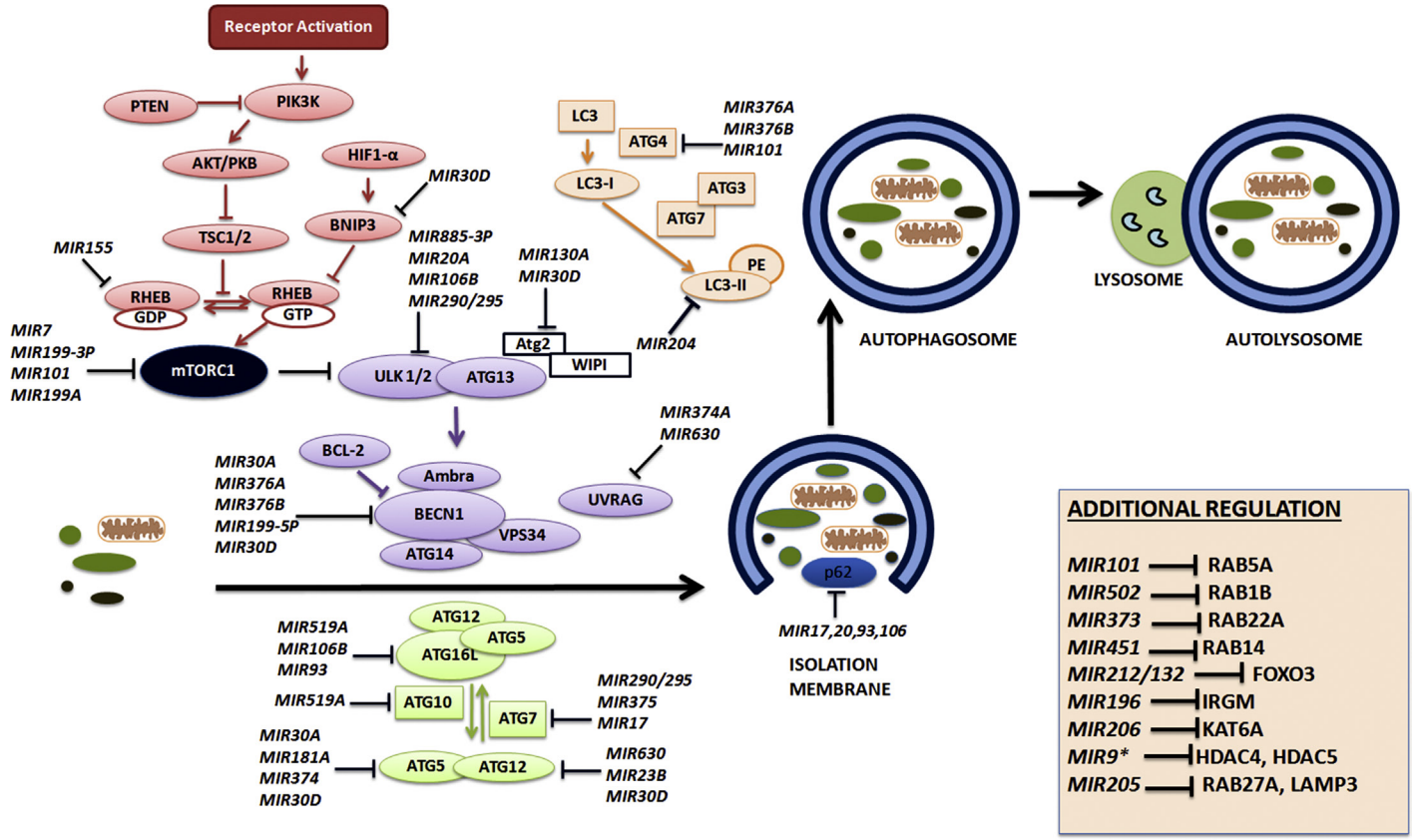

FIGURE 4.2 Overview of the mammalian autophagy pathway and its regulation by miRNAs. miRNAs are involved in regulating the expression of key autophagy-related proteins, from proteins functioning in the upstream stimulatory or inhibitory pathways to the final stages of autophagic degradation.

experimental studies unraveling molecular details of each interaction and connection, high-throughput screens and bioinformatic analyses are also being performed, accelerating the process.

A general picture of autophagy regulating miRNAs and miRNA networks has started to emerge (Table 4.1 and Figure 4.2). Several miRNAs target more than one autophagy-related protein and even proteins playing roles in separate stages of the autophagy pathways. Moreover, some autophagy proteins that were previously shown to be subject to strict transcriptional and post-translational regulation, such as BECN1 or ATG5, were discovered to be recurrent targets of different miRNAs. For example, *MIR376A* and *MIR376B* targeted both BECN1, which functions in the PIP3 kinase regulation and autophagosome nucleation, and ATG4C, which is responsible for LC3 maturation and autophagosome membrane elongation. *MIR30A, MIR376A* and *MIR376B* were all shown to target BECN1, but they used different target sequences in the 3′ UTR of the *BECN1* mRNA, indicating that these miRNAs might be conveying different signals to the same target. Indeed, while cellular endogenous levels of *MIR30A* were decreased following starvation or mTORC1 inhibition by rapamycin, *MIR376A* and *MIR376B* levels were increased in response to similar stimuli.

Several studies showed a correlation between cell death, survival and proliferation-related effects of miRNAs and autophagy. In fact, variations in miRNA expression levels were observed in a spectrum of diseases such as cancer, and some of these changes were demonstrated to be important for disease formation and/or progression. Therefore, considering the emerging role of autophagy abnormalities in cancer and other diseases, determination of the contribution of autophagy regulation problems arising from miRNA dysregulation under disease-related conditions might contribute to a better understanding of the mechanisms of major health problems, and provide new disease markers and/or drug targets. Indeed, miRNAs might be used as sensitive diagnosis and follow-up markers, and disease classification tools. Moreover, recent developments in nanotechnology-based drug carriers give us hope about the possible use of nucleic acids and even miRNAs as targeted gene therapy drugs in the near future.

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References

Brest, P., Lapaquette, P., Souidi, M., et al., 2011. A synonymous variant in IRGM alters a binding site for miR-196 and causes deregulation of IRGM-dependent xenophagy in Crohn's disease. Nat. Genet. 43 (3), 242–245.

Chang, Y., Yan, W., He, X., et al., 2012. miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. Gastroenterology 143 (1), 177–187. e8.

Chen, H., Chan, D.C., 2009. Mitochondrial dynamics: fusion, fission, movement, and mitophagy in neurodegenerative diseases. Hum. Mol. Genet. 18, 169–176.

Chen, K., Fan, W., Wang, X., et al., 2012a. microRNA-101 mediates the suppressive effect of laminar shear stress on mTOR expression in vascular endothelial cells. Biochem. Biophys. Res. Commun. 427 (1), 138–142.

Chen, Y., Liersch, R., Detmar, M., 2012b. The miR-290-295 cluster suppresses autophagic cell death of melanoma cells. Sci. Rep. 2, 808.
Comincini, S., Allavena, G., Palumbo, S., et al., 2013. microRNA-17 regulates the expression of ATG7 and modulates the autophagy process, improving the sensitivity to temozolomide and low-dose ionizing radiation treatments in human glioblastoma cells. Cancer Biol. Ther. 14 (7), 574–586.
Eisenberg-Lerner, A., Bialik, S., Simon, H.U., et al., 2009. Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death Differ. 16 (7), 966–975.
Fang, Y., Xue, J.L., Shen, Q., et al., 2012. microRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma. Hepatology 55 (6), 1852–1862.
Fornari, F., Milazzo, M., Chieco, P., et al., 2010. MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells. Cancer Res. 70 (12), 5184–5193.
Frankel, L.B., Wen, J., Lees, M., et al., 2011. microRNA-101 is a potent inhibitor of autophagy. EMBO J. 30 (22), 4628–4641.
Gozuacik, D., Kimchi, A., 2007. Autophagy and cell death. Curr. Top. Dev. Biol. 78, 217–245.
Guo, R., Hu, N., Kandadi, M.R., et al., 2012. Facilitated ethanol metabolism promotes cardiomyocyte contractile dysfunction through autophagy in murine hearts. Autophagy 8 (4), 593–608.
He, C., Klionsky, D.J., 2009. Regulation mechanisms and signaling pathways of autophagy. Annu. Rev. Genet. 43, 67–93.
Huang, Y., Chuang, A.Y., Ratovitski, E.A., 2011. Phospho-DeltaNp63alpha/miR-885-3p axis in tumor cell life and cell death upon cisplatin exposure. Cell Cycle 10 (22), 3938–3947.
Jegga, A.G., Schneider, L., Ouyang, X., et al., 2011. Systems biology of the autophagy-lysosomal pathway. Autophagy 7 (5), 477–489.
Kim, V.N., 2005. microRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. 6 (5), 376–385.
Korkmaz, G., le Sage, C., Tekirdag, K.A., et al., 2012. miR-376b controls starvation and mTOR inhibition-related autophagy by targeting ATG4C and BECN1. Autophagy 8 (2), 165–176.
Korkmaz, G., Tekirdag, K.A., Ozturk, D.G., et al., 2013. MIR376A is a regulator of starvation-induced autophagy. PLoS One 8 (12), e82556.
Kovaleva, V., Mora, R., Park, Y.J., et al., 2012. miRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells. Cancer Res. 72 (7), 1763–1772.
Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149 (2), 274–293.
Lian, J.H., Wang, W.H., Wang, J.Q., et al., 2013. microRNA-122 promotes proliferation, invasion and migration of renal cell carcinoma cells through the PI3K/Akt signaling pathway. Asian Pac. J. Cancer Prev. 14 (9), 5017–5021.
Lu, C., Chen, J., Xu, H.G., et al., 2014. MIR106B and MIR93 prevent removal of bacteria from epithelial cells by disrupting atg16l1-mediated autophagy. Gastroenterology 146 (1), 188–199.
Mah, L.Y., O'Prey, J., Baudot, A.D., et al., 2012. DRAM-1 encodes multiple isoforms that regulate autophagy. Autophagy 8 (1), 18–28.
Meenhuis, A., van Veelen, P.A., de Looper, H., et al., 2011. MiR-17/20/93/106 promote hematopoietic cell expansion by targeting sequestosome 1-regulated pathways in mice. Blood 118 (4), 916–925.
Mizushima, N., Yoshimori, T., Ohsumi, Y., 2011. The role of Atg proteins in autophagosome formation. Annu. Rev. Cell Dev. Biol. 27, 107–132.
Pan, W., Zhong, Y., Cheng, C., et al., 2013. MiR-30-regulated autophagy mediates angiotensin II-induced myocardial hypertrophy. PLoS One 8 (1), e53950.
Pavlides, S., Tsirigos, A., Migneco, G., et al., 2010. The autophagic tumor stroma model of cancer: role of oxidative stress and ketone production in fueling tumor cell metabolism. Cell Cycle 9 (17), 3485–3505.
Pennati, M., Lopergolo, A., Profumo, V., et al., 2014. miR-205 impairs the autophagic flux and enhances cisplatin cytotoxicity in castration-resistant prostate cancer cells. Biochem. Pharmacol. 87 (4), 579–597.
Qased, A.B., Yi, H., Liang, N., et al., 2013. microRNA-18a upregulates autophagy and ataxia telangiectasia mutated gene expression in HCT116 colon cancer cells. Mol. Med. Rep. 7 (2), 559–564.
Ravikumar, B., Sarkar, S., Davies, J.E., et al., 2010. Regulation of mammalian autophagy in physiology and pathophysiology. Physiol. Rev. 90 (4), 1383–1435.
Roccaro, A.M., Sacco, A., Jia, X., et al., 2010. microRNA-dependent modulation of histone acetylation in Waldenstrom macroglobulinemia. Blood 116 (9), 1506–1514.
Sarkar, S., 2013. Regulation of autophagy by mTOR-dependent and mTOR-independent pathways: autophagy dysfunction in neurodegenerative diseases and therapeutic application of autophagy enhancers. Biochem. Soc. Trans. 41 (5), 1103–1130.

Shenouda, S.K., Alahari, S.K., 2009. microRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev. 28 (3–4), 369–378.
Tasdemir, E., Chiara Maiuri, M., Morselli, E., et al., 2008. A dual role of p53 in the control of autophagy. Autophagy 4 (6), 810–814.
Tazawa, H., Yano, S., Yoshida, R., et al., 2012. Genetically engineered oncolytic adenovirus induces autophagic cell death through an E2F1-microRNA-7-epidermal growth factor receptor axis. Int. J. Cancer 131 (12), 2939–2950.
Tekirdag, K.A., Korkmaz, G., Ozturk, D.G., et al., 2013. MIR181A regulates starvation- and rapamycin-induced autophagy through targeting of ATG5. Autophagy 9 (3), 374–385.
Ucar, A., Gupta, S.K., Fiedler, J., et al., 2012. The miRNA-212/132 family regulates both cardiac hypertrophy and cardiomyocyte autophagy. Nat. Commun. 3, 1078.
Wan, G., Xie, W., Liu, Z., et al., 2014. Hypoxia-induced MIR155 is a potent autophagy inducer by targeting multiple players in the MTOR pathway. Autophagy 10 (1), 70–79.
Wang, J., Yang, K., Zhou, L., et al., 2013. microRNA-155 promotes autophagy to eliminate intracellular mycobacteria by targeting Rheb. PLoS Pathog. 9 (10), e1003697.
Wang, P., Zhang, J., Zhang, L., et al., 2013a. microRNA 23b regulates autophagy associated with radioresistance of pancreatic cancer cells. Gastroenterology 145 (5), 1133–1143. e12.
Wang, P., Zhang, L., Chen, Z., et al., 2013b. microRNA targets autophagy in pancreatic cancer cells during cancer therapy. Autophagy 9 (12), 2171–2172.
Wang, R., Wang, Z.X., Yang, J.S., et al., 2011. microRNA-451 functions as a tumor suppressor in human non-small cell lung cancer by targeting Ras-related protein 14 (RAB14). Oncogene 30 (23), 2644–2658.
Wang, Y., Liu, J., Liu, C., et al., 2013. microRNA-7 regulates the mTOR pathway and proliferation in adult pancreatic beta-cells. Diabetes 62 (3), 887–895.
Wang, Z., Ting, Z., Li, Y., et al., 2013. microRNA-199a is able to reverse cisplatin resistance in human ovarian cancer cells through the inhibition of mammalian target of rapamycin. Oncol. Lett. 6 (3), 789–794.
Wu, D., Huang, H.J., He, C.N., et al., 2013. microRNA-199a-3p regulates endometrial cancer cell proliferation by targeting mammalian target of rapamycin (mTOR). Int. J. Gynecol. Cancer 23 (7), 1191–1197.
Wu, H., Wang, F., Hu, S., et al., 2012. MiR-20a and miR-106b negatively regulate autophagy induced by leucine deprivation via suppression of ULK1 expression in C2C12 myoblasts. Cell Signal. 24 (11), 2179–2186.
Xiao, J., Zhu, X., He, B., et al., 2011. MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II. J. Biomed. Sci. 18, 35.
Xu, N., Zhang, J., Shen, C., et al., 2012. Cisplatin-induced downregulation of miR-199a-5p increases drug resistance by activating autophagy in HCC cell. Biochem. Biophys. Res. Commun. 423 (4), 826–831.
Yang, X., Zhong, X., Tanyi, J.L., et al., 2013. mir-30d regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells. Biochem. Biophys. Res. Commun. 431 (3), 617–622.
Yi, H., Liang, B., Jia, J., et al., 2013. Differential roles of miR-199a-5p in radiation-induced autophagy in breast cancer cells. FEBS Lett. 587 (5), 436–443.
Yu, Y., Yang, L., Zhao, M., et al., 2012. Targeting microRNA-30a-mediated autophagy enhances imatinib activity against human chronic myeloid leukemia cells. Leukemia 26 (8), 1752–1760.
Zhai, H., Song, B., Xu, X., et al., 2013a. Inhibition of autophagy and tumor growth in colon cancer by miR-502. Oncogene 32 (12), 1570–1579.
Zhai, Z., Wu, F., Chuang, A.Y., et al., 2013b. miR-106b fine tunes ATG16L1 expression and autophagic activity in intestinal epithelial HCT116 cells. Inflamm. Bowel Dis. 9 (11), 2295–2301.
Zheng, Y., Yin, L., Chen, H., et al., 2012. miR-376a suppresses proliferation and induces apoptosis in hepatocellular carcinoma. FEBS Lett. 586 (16), 2396–2403.
Zhong, M., Bian, Z., Wu, Z., 2013. miR-30a suppresses cell migration and invasion through downregulation of PIK3CD in colorectal carcinoma. Cell. Physiol. Biochem. 31 (2–3), 209–218.
Zhu, H., Wu, H., Liu, X., et al., 2009. Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. Autophagy 5 (6), 816–823.
Zou, Z., Wu, L., Ding, H., et al., 2012. microRNA-30a sensitizes tumor cells to cis-platinum via suppressing beclin 1-mediated autophagy. J. Biol. Chem. 287 (6), 4148–4156.

CHAPTER

5

Mechanisms of Cross-Talk between Intracellular Protein Degradation Pathways

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OUTLINE

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Abstract

Tight spatial and temporal regulation of protein turnover is essential to ensure maintenance of cellular health and survival. Cells employ several degradative mechanisms to execute the clearance of damaged or superfluous proteins, as well as of other cellular components including lipids and entire organelles. Autophagy is the umbrella term under which three distinct modes of lysosome-dependent protein degradation occur, namely macroautophagy, microautophagy and chaperone-mediated autophagy. In addition to autophagy, another major protein degradation mechanism is the ubiquitin-proteasome system whereby selectively targeted proteins are degraded into their constitutive peptides and amino acids aided by catalytic activities of a macromolecular complex named proteasome. Degradation of intracellular components through these catabolic pathways allows deliberation of basic building blocks required to maintain cellular energy and homeostasis. The extent to which different protein degradation pathways interact, collaborate or antagonize each other is under intense research scrutiny. Human pathologies arising from perturbed protein turnover, including aging, neurodegeneration and cancer, make further understanding of cross-talk between these homeostasis regulators an important research avenue medically, socially and economically.

INTRODUCTION

Cells are extremely dynamic structures and a careful balance between anabolic (building) and catabolic (breaking down) processes is required to ensure maintenance of cellular health, function and survival. Catabolism is important not only to clear the cell of potentially toxic aggregates, proteins or damaged organelles but also to selectively remove integral regulatory proteins in a temporal manner. In this respect, catabolic processes contribute to the control of every aspect of cellular homeostasis, including cell cycle progression, DNA transcription, cell signaling and cellular repair. The subsequent liberation of cellular building blocks such as amino acids via these pathways contributes to the recycling and, therefore, preservation of valuable resources.

There are two major catabolic systems in the cell, the ubiquitin-proteasome system (UPS) and autophagy (Figure 5.1). Both systems participate in the degradation of proteins, albeit with varying selectivity, while autophagy is further able to regulate the degradation of other macromolecules including lipids and entire organelles. The UPS and autophagy were traditionally considered to be independent processes; despite the fact that both systems occur in the cytoplasm, they employ a variety of distinct regulators, show differing kinetics and appeared to target different substrates. More recently, however, this view has been challenged by the identification of a number of cross-talk mechanisms most notably the realization that the conjugation of the small protein ubiquitin (or polyubiquitin chains) can integrate signals between different degradative pathways.

In this chapter we review the mechanisms and regulation of cross-talk between protein degradation pathways. Furthermore, we will provide an insight into how disruption of these interactions can have serious detrimental effects on cellular protein and energy homeostasis with particular focus on aging and age-related diseases.

THE UBIQUITIN-PROTEASOME SYSTEM: SELECTIVE DEGRADATION OF CYTOPLASMIC PROTEINS

The ubiquitin-proteasome system is primarily responsible for the degradation of short-lived, soluble proteins and is active in both the cytoplasm and nucleus. The UPS is highly

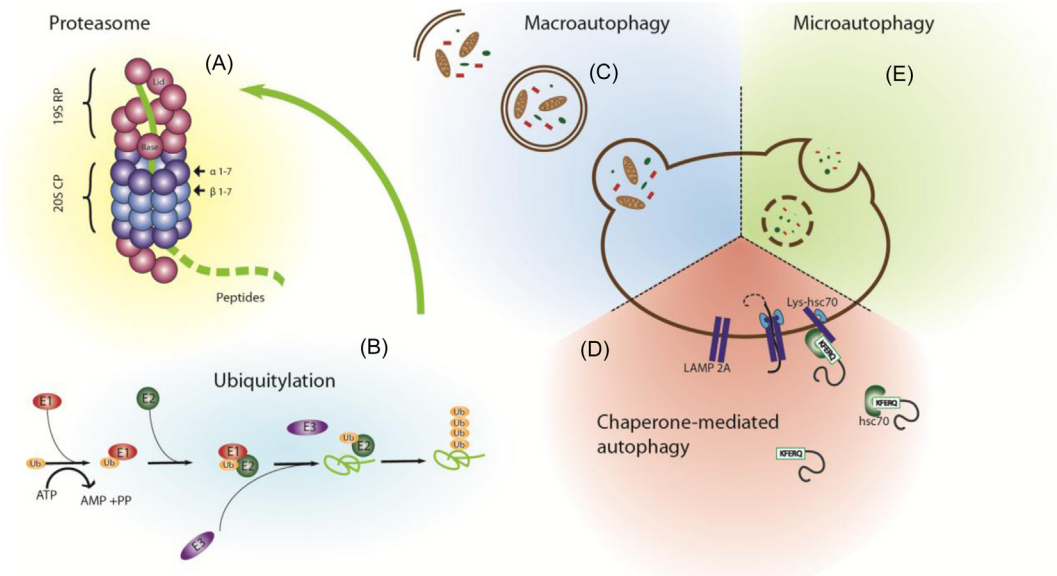

FIGURE 5.1 Schematic diagram of the major intracellular protein degradation pathways. (A) The proteasome is formed by the catalytic 20S core particle (CP) and the 19S regulatory particle (RP). (B) The general scheme of the ubiquitylation cascade which involves enzymes E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), E3 (ubiquitin-ligase). A series of ubiquitylation events targets the substrates to the proteasome for degradation. (C) Macroautophagy involves the formation of double membrane-bound vesicles that are transported along microtubules and fuse with lysosomes; both nonspecific (bulk) and selective recruitment of substrates to autophagosomes have been reported. (D) Chaperone-mediated autophagy allows for selective uptake of proteins containing a consensus KFERQ motif across the lysosomal membrane via LAMP-2A; the process requires the chaperone activity of Hsc70 and LysHsc-70. (E) Microautophagy refers to a process whereby portions of the cytoplasm containing substrates are directly engulfed by invagination of the lysosomal membrane. Microautophagy is still poorly characterized in mammalian cells. Ub, ubiquitin.

selective and the majority of proteins require to be specifically tagged with a small protein called ubiquitin in order to be recruited to the proteasome and degraded (Figure 5.1A,B). The core machinery involved in the UPS is the 26S proteasome, a multicatalytic, ATP-dependent protease complex. The UPS is able to rapidly process a wide variety of cellular proteins in a highly specific manner and thus contributes to the tight regulation of the cell cycle, DNA repair, signal transduction quality control and much more (Glickman and Ciechanover, 2002).

Ubiquitin-Dependent Protein Targeting

Proteins are targeted for degradation by the UPS via a series of enzymatic reactions involving E1, E2 and E3 enzymes that activate, transfer and conjugate, respectively, ubiquitin to lysine residues on target protein substrates (Glickman and Ciechanover, 2002). The labeling of a protein substrate with one ubiquitin molecule at one or many lysine residues

(monoubiquitylation) is not sufficient to target the substrate for degradation via UPS. Rather, multiple rounds of ubiquitylation of the same protein substrate occur, leading to a polyubiquitylated substrate (Figure 5.1A,B).

The process of polyubiquitylation confers an extremely high level of specificity to the UPS; the numerous E1 enzymes expressed in mammalian cells are able to interact with all known E2 enzymes. The E2 enzymes, however, are only capable of interacting with a subset of E3 enzymes. Furthermore, E3 enzymes must directly interact with protein substrates to facilitate their role in conjugating ubiquitin and each E3 enzyme can only interact with a limited number of substrates. Fascinatingly, ubiquitin itself contains a number of specific lysine residues, located at positions 6, 11, 27, 31, 33, 48, and 63, that are susceptible to self-ubiquitylation reactions. The ability of ubiquitin to self-oligomerize via different linkages (i.e., a polyubiquitin chain whereby the ubiquitin monomers are conjugated at K48 are often referred to as K48-linkages) further contributes to the complexity and diversity of polyubiquitin chains. A polyubiquitin chain containing at least four ubiquitin molecules joined via K48 residues has been identified as a particularly robust proteasome-targeting signal (Glickman and Ciechanover, 2002). Polyubiquitylation mediated by K11, K29 and more controversially K63 linkages also participates in proteasomal targeting of protein substrates (Figure 5.2A). K11 linkages appear to be particularly important for regulatory turnover of proteins involved in the cell cycle (Jin *et al.*, 2008).

The Molecular Architecture of the Proteasome

Polyubiquitylated protein substrates are transported to the proteasome via a poorly understood mechanism. The 26S proteasome is a large ATP-dependent protease complex comprised of two main components, the catalytic 20S core particle (CP) and the 19S regulatory particle (RP). Ubiquitylated proteins enter the CP via the RP and are exposed to catalytic cleavage reactions that degrade them to oligopeptides before releasing them into the cyto- or nucleoplasm.

The CP consists of a total of 28 subunits arranged to form a barrel-like structure. This barrel-like structure is made up of four rings of subunits, seven subunits per ring. The outer two rings consist of α-subunits while the two inner rings are formed from β-subunits (Figure 5.1A). The outer α-rings are thought to act as a gate for protein substrate entry into the inner chamber formed by the β-rings. It is within this chamber that substrates are degraded by a number of proteolytic reactions. Specifically, β-rings have been observed to possess trypsin, caspase and chymotrypsin-like activities. Further specificity of proteolytic activity is conferred to proteasomes via different compositions of β-ring subunits (Glickman and Ciechanover, 2002).

Entry of ubiquitylated proteins into the CP is regulated by the RP which controls the opening and closing of the α-ring structures of the CP. The RP is composed of 19 subunits that are organized to form lid and base structures. The base is made up of six ATPase and four non-ATPase subunits (referred to as Rpt 1–6 and Rpn 1, 2, 10 and 12, respectively). The ATPase activity provides energy for de-ubiquitylation and protein unfolding, both of which are prerequisites for their entrance into the 20S CP (Glickman and Ciechanover, 2002).

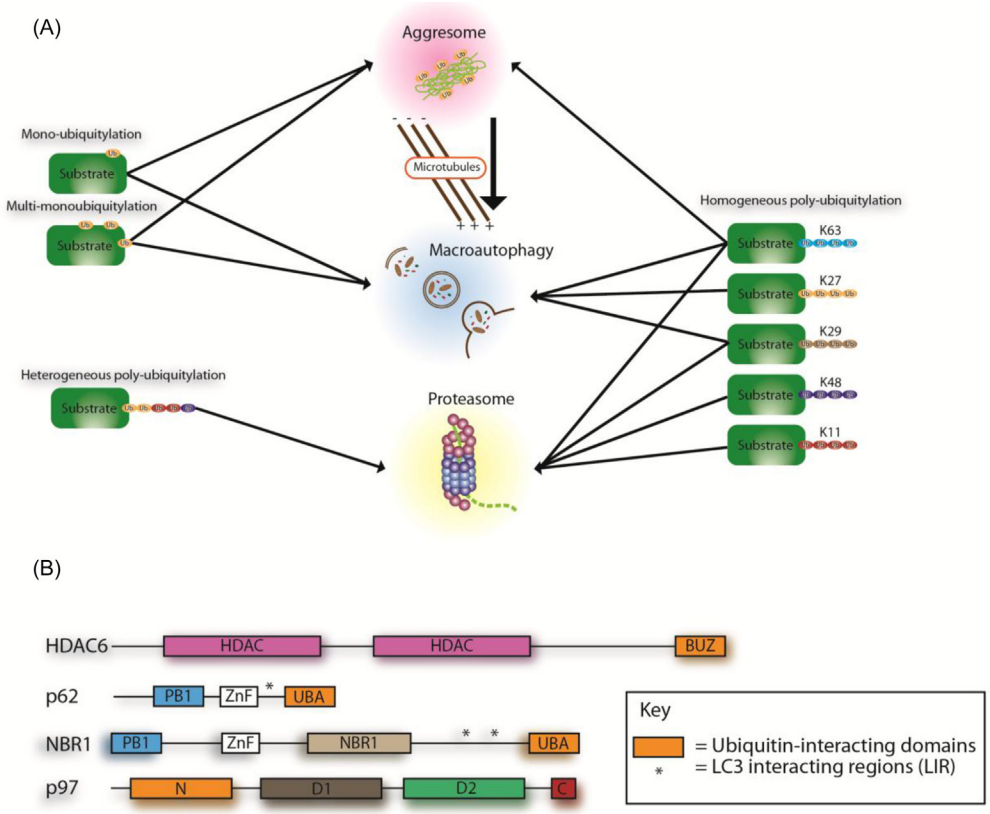

FIGURE 5.2 The ubiquitin code at a glance. (A) The diagram summarizes the current knowledge on different types of ubiquitylation and their role in targeting substrates to proteolytic pathways. Ubiquitin molecules can be attached to substrates as monomers or polyubiquitin chains. Polyubiquitin is formed through the conjugation of additional monomers to one of seven lysine residues in the ubiquitin molecule. This allows the formation of ubiquitin chains with different topologies. The large number of ubiquitin-binding combinations/possibilities confers extremely tight regulation to the targeting and degradation of substrates via the UPS and autophagy. See text for further explanations. (B) Domain structure of several Ub-binding adaptor molecules implicated in targeting of ubiquitylated substrates for degradation by the proteasome and macroautophagy. Ubiquitin-binding domains are marked in orange (UBA, BUZ, N-terminal domain of p97). Positions of LIR motifs are marked with asterisks: PB1, Zinc-finger (ZnF), deacetylase (HDAC) and p97 domains are also indicated. Ub, ubiquitin.

THE THREE BRANCHES OF AUTOPHAGY: DIVERSE REGULATION OF LYSOSOME-DEPENDENT DEGRADATION

Macroautophagy

Macroautophagy is responsible for what is often referred to as bulk degradation; it has the broadest range of substrates and is by far the best studied of all three autophagic pathways, often being referred to simply as autophagy. Macroautophagy occurs in all cells at

basal, albeit varying, levels and these basal levels are often reflective of cellular function and energy demands. The most well-known regulator of macroautophagy is the serine/threonine kinase target of rapamycin (TOR) as part of the macromolecular complex (in mammals called mTORC1), which is activated in nutrient-rich, low cellular stress conditions and promotes protein translation and cell growth. In these growth-promoting conditions macroautophagy is largely suppressed. Cellular starvation (amino acids and growth factors), in addition to many other cellular stressors such as hypoxia and DNA damage, can lead to the inactivation of mTORC1 and subsequently activate macroautophagy.

Macroautophagy is characterized by the formation of a double membrane-bound vesicle called an autophagosome (Figure 5.1C). Autophagosomes form in the cytoplasm from precursors referred to as phagophores that eventually engulf regions of the cytoplasm containing protein and lipid substrates. The origin of this growing membrane is currently debatable; there is evidence that a variety of sources, including the plasma membrane, endoplasmic reticulum, Golgi and mitochondria, can provide lipids to growing autophagosomal membranes. The relative contribution of each source of the lipid and the mechanisms of recruitment are currently unknown. The subsequent maturation of autophagosomes involves their transport along the microtubule network toward the lysosome-rich perinuclear region of the cell where they fuse with lysosomes to facilitate degradation of the autophagosome contents. A large and ever-growing array of proteins and lipids participate in the tight spatial and temporal regulation of autophagosome initiation, elongation, closure and maturation, discussion of which is beyond the scope of this chapter.

Macroautophagy was historically considered to be a nonspecific process whereby in sensing stress or starvation, a cell would indiscriminately target cytoplasmic contents for degradation to liberate nutrient and energy catabolites. More recent evidence, however, suggests that macroautophagy can in fact be highly specific (Reggiori *et al.*, 2012). In this capacity macroautophagy participates in the selective turnover of many organelles including protein aggregates (aggrephagy), the ER (reticulophagy), peroxisomes (pexophagy) and mitochondria (mitophagy), to name a few (Ravikumar *et al.*, 2010).

The identification that macroautophagy is, at least in part, a selective process raises the question of how functional and obsolete substrates can be distinguished. As is the case for chaperone-mediated autophagy (CMA – see the following), it is reasonable to assume that chaperones participate in specific recognition of damaged or misfolded proteins. Indeed, a process referred to as chaperone-assisted selective autophagy (CASA) has been identified. Interestingly, both adaptor and co-chaperone proteins participate in regulating this process of degradation. Co-chaperones such as those of the BAG family, specifically BAG-3, associate with hsc70 and hspB8 and help to facilitate the binding of these chaperones to adaptor molecules such as p62. This allows a wide range of chaperone clients to be bound to adaptor molecules and specifically targeted for degradation via the macroautophagy machinery and adds a further level of complexity to the network of proteolytic pathways (Kettern *et al.*, 2010). CASA is an emerging area of interest; as yet there has been no direct evidence of cross-talk between it and other protein degradation pathways. However, with the involvement of chaperones in other pathways such as CMA and the UPS it would not be surprising if these pathways were extensively interlinked.

Chaperone-Mediated Autophagy

Unlike the other forms of autophagy, CMA is an exclusively selective degradation process. As the name may suggest, CMA is aided by a protein chaperone called heat shock cognate protein (hsc70). The interaction of hsc70 with cytosolic protein substrates is mediated by a consensus pentapeptide motif KFERQ found in all CMA-targeted proteins (Figure 5.1D). Hsc70 alone or in complex with a protein substrate is able to bind to a plethora of co-chaperones through interactions that are thought to participate in specific substrate recognition, substrate delivery, protein unfolding and the final protein translocation across the lysosomal membrane. Delivery of the protein substrate to the lysosome is facilitated by binding to the cytosolic tail of the transmembrane protein, lysosomal-associated membrane protein 2A (Lamp-2A). The substrate protein is then unfolded and transported into the lumen of the lysosome via a poorly understood mechanism. Following translocation the substrate is rapidly degraded by hydrolytic enzymes (Ravikumar *et al.*, 2010).

The mechanisms that regulate CMA activation, substrate recognition, transport and translocation are not well understood. Evidence suggests, however, that regulation of LAMP-2A expression levels is particularly important for efficient CMA and indeed the level of lysosome-associated LAMP-2A directly correlates with CMA activity. Thus, induction of CMA by oxidative stress has been shown to induce LAMP-2A transcription and in CMA-activating conditions the protein half-life of LAMP-2A increases, thus enhancing the CMA response. In addition, during prolonged CMA activation, LAMP-2A can be transported from lysosomal membrane into the matrix but can seemingly be retrieved from an intact pool of LAMP-2A and reinserted back into the lysosomal membrane, again ensuring a robust maintenance of CMA response (Cuervo and Dice, 2000).

Interestingly, while only approximately 25 proteins have been identified as substrates for CMA, the targeting motif, KFERQ, is relatively common in cytosolic proteins (approximately 30%). Proteins that have been confirmed to be CMA substrates participate in a range of cellular processes including glycolysis, transcription and proteasome-based protein degradation. CMA is therefore an important participant in the general turnover of proteins required to maintain cellular homeostasis.

Microautophagy

Direct delivery of cytoplasmic contents, either by lysosomal membrane invagination or protrusion is referred to as microautophagy (Figure 5.1E). The specific regulatory mechanisms are poorly understood in mammalian cells but seminal work in yeast indicates that microautophagy can mediate degradation of cytoplasmic contents via both nonselective and selective mechanisms (reviewed in Mijaljica *et al.*, 2011). Indeed, direct lysosomal engulfment of mitochondria and nucleus fragments has been observed in yeast and is referred to as micromitophagy and micronucleophagy, respectively. Since little is known about any specific regulators of microautophagy, the primary tool for investigating this process is electron microscopy, limiting the scope for experimentation. Despite this, the process of microautophagy was characterized to occur via five main steps: (1) invagination of the lysosomal

membrane; (2) vesicle formation; (3) vesicle expansion; (4) vesicle scission; and (5) vesicle degradation (Li *et al.*, 2012). Due to the poorly defined molecular mechanisms in mammalian cells we will not focus on microautophagy further in this chapter except to say that, in yeast models at least, it shares some key upstream regulators with macroautophagy including the autophagy-related Atg proteins and the potent negative regulator TOR (Li *et al.*, 2012). Future work will undoubtedly unravel the current mystery that is microautophagy and of particular interest will be to identify how common upstream regulators are able to influence multiple protein degradation pathways.

REGULATION OF INTRACELLULAR PROTEOLYSIS BY CROSS-TALK BETWEEN DEGRADATION PATHWAYS

Interplay between Autophagy Pathways

Despite sharing an ultimate endpoint of lysosomal degradation, the three autophagy pathways differ in their kinetics, selectivity and regulatory mechanisms. Interestingly, while micro- and macroautophagy appear to be evolutionary conserved, CMA has only been noted in higher organisms. Coupled with the fact that CMA is the only pathway that is entirely selective suggests that this mechanism evolved to cope with the complex cellular homeostasis mechanisms required to meet sufficient energy demands.

The coordinated activation of macroautophagy and CMA has been noted in response to environmental stimuli such as starvation. The kinetics of each pathway, however, differs in response to the same stimulus. Macroautophagy, for example, reaches a maximum level of activation after six hours of starvation before slowly declining even where the stimulus persists. This decline in activity is likely to be the result of a feed-forward mechanism whereby the catabolites released from autophagosome-lysosome fusion reach sufficient cytoplasmic concentrations to "re-active" mTOR signaling. It has been suggested that there is a mutual inhibition between macroautophagy and CMA as concomitant to the observed decline in macroautophagy there is an activation of CMA. CMA activity peaks after 24 hours of nutrient deprivation but can remain elevated for extended periods of time during starvation. These temporal differences in activation may be due to the higher selectivity CMA has for its substrates; it is therefore able to have tighter control over the choice of proteins being degraded. Maintaining high levels of the less selective macroautophagy could quickly tip the balance and become detrimental to cell growth and survival.

Despite apparent coordination between the two autophagy pathways they cannot fully compensate for each other; CMA cannot degrade organelles while, as mentioned previously, macroautophagy does not have the high selectivity of CMA. Indeed, inactivation of CMA in cultured cells results in increased macroautophagy, but not to sufficient levels to alleviate the increased sensitivity of CMA-deficient cells to stress (Massey *et al.*, 2006). The ability, however, of the two pathways to seemingly interact suggests that there are indeed underlying molecular mechanisms coordinating their action. It has been suggested, for example, that macroautophagy may degrade endogenous CMA inhibitors, or that macroautophagy machinery could become CMA substrates, which would explain the transition from the early activation of macroautophagy to later onset of CMA. These and other potential

mechanisms remain to be investigated. Similarly, little is known about microautophagy in metazoans and about its integration with other branches of autophagy.

Ubiquitin: A Small Protein with a Big Job

We have demonstrated how protein turnover can be regulated in both a selective and nonselective manner. The complexity of substrate recognition and targeting required for proteasomal degradation is contrasted by the seemingly indiscriminate, bulk degradation of macroautophagy. Increasing evidence, however, suggests that not only can macroautophagy occur in an equally selective manner but that adaptors, chaperones and mechanisms involved in conferring specificity to the UPS and CMA may be able to participate in multiple proteolytic pathways, providing mechanisms of cross-talk and helping to orchestrate protein turnover and energy homeostasis.

Polyubiquitylation via K11, K27, K29 and K63 linkages has been implicated as targeting signals for lysosomal degradation in addition to its role in UPS-dependent proteolysis (Figure 5.2A). This, however, still remains controversial as genetic ablation of macroautophagy by the knockout of essential autophagic genes (Atg5 or Atg7) results in the accumulation and aggregation of proteins carrying ubiquitylated chains of all types (Riley *et al.*, 2010). One explanation for this complexity is that substrates may be shared by autophagic and proteasomal pathways. For example, the neuronal protein α-synuclein involved in the pathology of Parkinson's disease can be degraded by the UPS, macroautophagy and CMA. However, the extent to which autophagy contributes to the degradation of the total pool of cellular ubiquitylated proteins currently remains unclear and, therefore, it is still arguable whether the accumulation of ubiquitylated substrates seen in autophagy-deficient mice can be attributed to the lack of autophagy alone.

Among other explanations for the accumulation of ubiquitylated proteins in autophagy-deficient cells is that autophagosomal substrates that initially are not ubiquitylated become so once they are exposed to the ubiquitylation machinery for prolonged periods. The situation is further complicated by findings that autophagy impairment could also lead to the inhibition of the UPS and result in an accumulation of proteasomal substrates (Korolchuk *et al.*, 2009). We will see below how this and other mechanisms of cross-talk between the degradative pathways help to coordinate their activity in different physiological and pathological conditions.

For ubiquitin to serve as a targeting signal it needs to be recognized by adaptor proteins that, on the one hand, bind ubiquitin via one of the several known specialized domains and, on another, can interact with core components of proteolytic machinery. Some adaptor molecules are thought to be specific to one degradative pathway; for example, Rad23 and Dsk2 bind ubiquitylated substrates and shuttle them to the proteasome for degradation. Similarly, targeting of ubiquitylated substrates for autophagic degradation can be assisted by proteins like HDAC6 and NBR1 (Figure 5.2B) (Lamark and Johansen, 2010). Other proteins, such as p62 and p97, can participate in both UPS and autophagy-dependent proteolytic degradation (Figure 5.2B).

The cytosolic protein histone deacetylase 6 (HDAC6) binds preferentially to K63-linked ubiquitylated proteins via a binder of ubiquitin zinc-finger domain (BUZ domain) and facilitates degradation of autophagic substrates by transporting them along microtubules. In the

perinuclear region these ubiquitylated substrates form an inclusion body called aggresome, which is eventually processed by macroautophagy (Figure 5.2A). The adaptor proteins p62 and NBR1 have ubiquitin-associated (UBA) domains in addition to so-called LC3-Interacting Regions (LIR) motifs (Figure 5.2B). This combination of binding motifs allows them to directly and specifically bind and target ubiquitylated cargo to autophagosomes. Interestingly, in addition to its function in selective autophagy, p62 has also been implicated in shuttling of proteasomal substrates (Seibenhener *et al.*, 2004). It is possible that specific physiological conditions dictate the fate of individual p62-substrate complexes to the autophagic or UPS degradation pathways supporting the idea of collaboration between proteolytic pathways. Where autophagy is impaired, p62 may actually have a negative impact on the UPS suggesting a different mechanism of interaction between the pathways (see the following).

The AAA-ATPase p97 (CDC48 in yeast) (Figure 5.2B) can also participate in multiple proteolysis pathways via its ability to bind ubiquitin, both directly and through a myriad of cofactors. It then uses energy generated by ATP hydrolysis to separate ubiquitylated substrates from protein complexes and membranes and facilitates their proteasomal degradation. Interestingly, p97 has also been shown to play a role in autophagy by regulating the formation of autophagic vesicles (Dargemont and Ossareh-Nazari, 2012). In addition, p97 competes for binding of ubiquitylated proteins with p62 (Korolchuk *et al.*, 2009) suggesting that p97 may also contribute to substrate recruitment during selective ubiquitin-assisted autophagy. Indeed, the role for p97 in selective degradation processes such as ribophagy and mitophagy has been demonstrated. The exact mechanisms by which p97 contributes to the integration between proteolytic pathways require further investigation.

FUNCTIONAL IMPLICATIONS OF CROSS-TALK: AUTOPHAGY CAN COMPENSATE FOR UPS IMPAIRMENT BUT NOT VICE VERSA

Macroautophagy Upregulation in Response to UPS Disruption

While the UPS and autophagy share some of their substrates (as outlined previously), this cross-talk in normal physiological conditions appears to be limited. The complex interplay between pathways, however, becomes more apparent when one of the pathways is impaired. In numerous *in vitro* and *in vivo* studies, inhibition of the UPS was shown to activate macroautophagy as a compensatory mechanism (Figure 5.3). Upregulation of macroautophagy in such circumstances is thought to be beneficial as further activation of macroautophagy by rapamycin in cultured cells and mice protects against toxicity induced by proteasomal inhibition. Similarly, macroautophagy upregulation was found to be protective against proteasomal impairment in fruit flies (Pandey *et al.*, 2007).

Several mechanisms explaining upregulation of macroautophagy in response to proteasome inhibition have been suggested. One of the consequences of proteasome inhibition is the formation of perinuclear aggresomes (Kawaguchi *et al.*, 2011). As discussed above, the delivery of misfolded proteins into aggresomes is dependent on autophagy adaptor molecules including HDAC6, p62 and NBR1 and is thought to enhance their degradation by

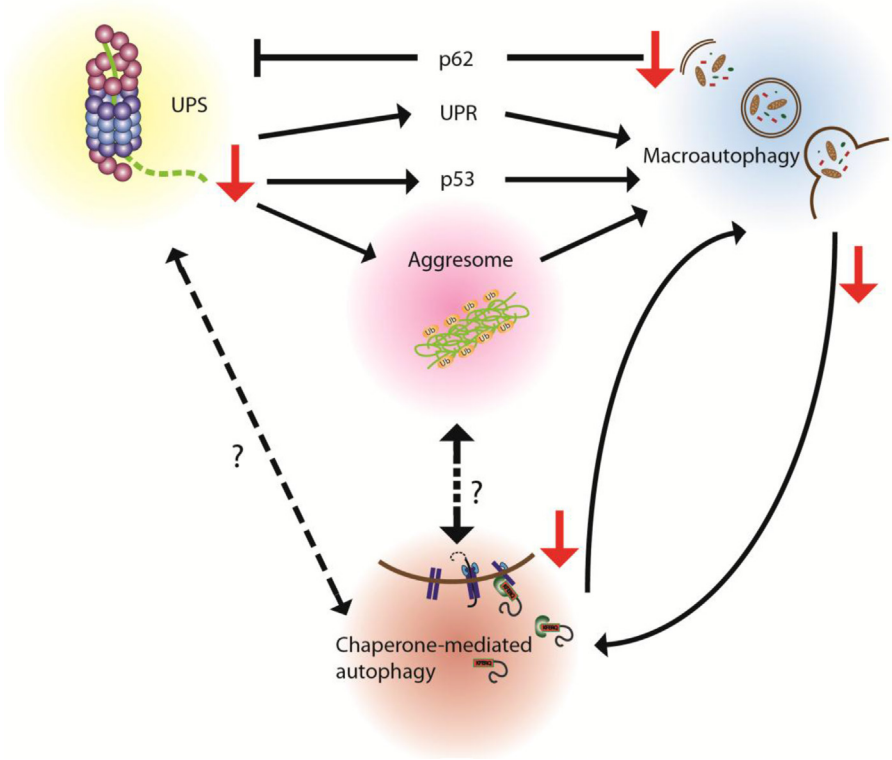

FIGURE 5.3 Summary of proteostasis interactome. Tight regulation of protein turnover is conferred by a number of proteolytic mechanisms. These mechanisms are able to interact and influence their activity states to maintain tight temporal and spatial control of protein degradation and ensure cellular homeostasis. Red downward arrows indicate inhibition of the pathway. Black arrows and black bars indicate activating or inhibitory interactions, respectively, between the pathways. For example, inhibition of the UPS may induce upregulation of macroautophagy mediated by the UPR, p53 or through aggresome formation. Dotted lines are interactions that have not been reported to date. UPR, unfolded protein response.

macroautophagy machinery. This suggests that aggregation of misfolded proteins may be a prerequisite for their efficient degradation by autophagic processes.

Another consequence of proteasome inhibition and the accumulation of misfolded proteins is the induction of the unfolded protein response (UPR), eventually resulting in endoplasmic reticulum (ER) stress. The UPR is a complex signaling pathway which in response to the ER overload not only regulates expression of genes involved in protein synthesis, folding and degradation in the ER itself but also leads to the activation of macroautophagy. The molecular details of the cross-talk between the UPS and autophagy through the UPR are very complex with all three arms of the UPR (ATF4, PERK and IRE1) having been implicated in this response. The transcription factor ATF4 was found to be required for the upregulation of macroautophagy genes following proteasome inhibition with an antitumor drug, bortezomib. The exact mechanism of ATF4 stabilization in response to proteasome

inhibition remains controversial as it was shown to be both dependent and independent of the activation of the PERK arm of the UPR (Milani *et al.*, 2009; Zhu *et al.*, 2010). Irrespective of the mechanism, ATF4 upregulation results in increased expression of several autophagy genes such as ATG5, ATG7 and/or LC3 (Milani *et al.*, 2009; Zhu *et al.*, 2010). The IRE1 arm of the UPR has also been implicated in the compensatory upregulation of autophagy following proteasome inhibition by bortezomib (Ding *et al.*, 2007; Wei *et al.*, 2008). It has been proposed that IRE1 acts through its downstream target c-Jun NH2-terminal kinase (Jnk1) which, in turn, induces autophagy by phosphorylating Bcl-2 in complex with Beclin1. Phosphorylation of Bcl-2 results in the release of pro-autophagic protein Beclin-1, allowing it to take part in the initiation of autophagy (Ding *et al.*, 2007; Wei *et al.*, 2008).

Additionally, proteasome inhibition has been shown to induce autophagy through p53-dependent mechanisms. Several pathways downstream of p53 have been implicated in autophagy regulation. Thus, p53 leads to the inhibition of a negative regulator of autophagy, the protein kinase mTOR through increased expression of several of its negative regulators including TSC2, AMPK and SESN2; p53 also upregulates expression of other autophagy-related proteins such as DRAM, DAPK-1 and PUMA among others. Interestingly, in certain circumstances p53 has also been shown to inhibit rather than activate autophagy, which is mediated by cytoplasmic p53 and is independent of its role in gene expression (reviewed in Ryan, 2011).

The UPS is Impaired upon Autophagy Deregulation

It is evident from the previous sections that autophagic pathways are often activated as a mechanism of compensation for the loss of other protein degradation systems. This, however, is not always the case. For example, while acute inhibition of the UPS results in compensatory upregulation of autophagy, rather chronic low-level proteasome inhibition leads to deregulation of inducible macroautophagy. Similarly, the loss of autophagy is not compensated for by the UPS, which, instead, also becomes impaired (Figure 5.3). Specific mechanisms have been identified that may lead to the inhibition of proteasomal degradation when autophagy is impaired. It has been shown that inhibition of the UPS function can be mediated by the accumulation of an adaptor protein p62. This mechanism is both sufficient and necessary for inhibition of the UPS; overexpression of p62 alone was sufficient to inhibit the UPS while its knockdown rescued the levels of UPS substrates in autophagy-deficient cells. As p62 was shown to compete for ubiquitylated proteins with other proteins involved in proteasomal degradation (such as p97), elevated levels of p62 in autophagy deficient cells may prevent access of ubiquitylated proteasomal substrates to the UPS machinery.

Therefore, p62 may play multiple functions in degradation of the UPS and autophagy substrates. As previously mentioned, p62 may act to recruit ubiquitylated proteins to autophagosomes and proteasomes for degradation (Lamark and Johansen, 2010; Seibenhener *et al.*, 2004). However, p62 may also have a negative impact on degradation of ubiquitylated proteins when autophagy is impaired; such a situation may be relevant to different pathological conditions – for example, lysosomal storage disorders.

Inhibition of the UPS in response to impairment of autophagy was described in a cell culture-based system but was not observed in differentiated cells *in vivo*. Instead, the main consequence of p62 accumulation in autophagy-deficient tissues was found to be not in

binding ubiquitylated proteins and preventing their degradation through the UPS but in the activation of Nrf2-dependent stress-response pathways (Riley *et al.*, 2010). The increased levels of soluble and aggregated ubiquitylated proteins observed in autophagy-deficient mice were interpreted as an indirect consequence of upregulation of these stress pathways. Moreover, aggregation rather than ubiquitylation was suggested to act as a signal for autophagic degradation (Riley *et al.*, 2010).

Irrespective of the mechanism, it is important to highlight the fact that catalytic activities of the proteasome are not affected in cells with compromised autophagy, corroborating the idea that the influence of autophagy on the UPS may be indirect (Korolchuk *et al.*, 2009). Inability of the UPS to compensate for autophagy dysfunction is in agreement with the fact that p62 and ubiquitylated proteins oligomerize and form aggregates, which are predicted to be poor substrates for the proteasome. Macroautophagy and the UPS can also be coordinated at a transcriptional level. For example, both the UPS and autophagy are simultaneously upregulated downstream of transcriptional factor FoxO3. This mechanism was shown to contribute to muscle atrophy in physiological conditions, such as starvation, as well as in diseases characterized by muscle wasting (Zhao *et al.*, 2007). In contrast to macroautophagy, much less is known about cross-talk between the UPS and CMA or microautophagy. One interesting putative mechanism for such integration is that proteasomal subunits can be selectively degraded by CMA. Functional relevance of this process, however, requires further investigation.

INSIGHTS INTO THE PHYSIOLOGICAL CONSEQUENCES OF PERTURBED PROTEOLYSIS: FOCUS ON AGING

Contribution of Protein Homeostasis to Aging

Numerous studies have identified gradual decline in the proteostasis network as an important contributor to aging and age-related diseases. This decline has been proposed to result from multiple factors including an increasing load of damaged and misfolded proteins and an impairment of proteolytic machinery. These changes, in turn, are often associated with an increase in oxidative stress as well as with age-dependent changes in regulatory factors such as signaling and transcriptional pathways.

Age-Associated Changes in the UPS

Many studies have reported an age-associated decline in activity of the UPS, although this decline does not appear to be universal. This reduction in the UPS activity appears to arise from qualitative and quantitative changes in the components of the UPS. For example, a build-up of ubiquitin-conjugated substrates is common in aged tissues. This build-up is thought to be caused by a reduction in the rate of degradation and not by increased ubiquitylation as levels of ubiquitin, ubiquitin mRNA, E1, E2 and E3 enzymes appear to remain unchanged with age (Martinez-Vicente *et al.*, 2005).

The proteasome can experience changes in oxidation state with age: oxidation, lipid peroxidation and glycation all increase with age and are likely to impact on proteasome regulation. In addition, changes to protein substrates themselves can impact on proteasomal

activity. Oxidized, cross-linked proteins and lipids accumulate with age and can have an inhibitory effect on the proteasome. Some studies have also noted a drop in expression of various components of the proteasome with age. This has been reported in mitotic human fibroblasts and postmitotic rat myocytes, both showing a drop in expression of genes encoding 20S and 26S proteasomal subunits. Interestingly, dietary restriction, an intervention shown to increase longevity in laboratory animal models from yeast to mice, reduced this decline in proteasome gene expression, restoring it to the level of a non-aged control (Lee *et al.*, 1999). Rather surprisingly, it has been reported that the total number of the 20S proteasomes is, in fact, increased in a muscle with age. Despite this, however, a marked reduction in the abundance of regulatory proteins has been observed. Therefore, it has been hypothesized that the deficit in regulatory subunits leads to a decreased activation of the 20S proteasome with age despite an apparent increase in its expression (Ferrington *et al.*, 2005).

Age-Related Changes in Autophagy

An age-related decline both in macroautophagy and CMA activity has been reported. It is thought that this decreased activity in both of these degradation pathways could contribute, at least in part, to the accumulation of damaged proteins and organelles and leads to a loss of homeostasis and aberrant stress responses in aging cells.

Changes in macroautophagy with age are accompanied by a number of morphological changes to the lysosomal system. These include an accumulation of autophagic vacuoles, an expansion of the lysosomal compartment, and a build-up of undegraded material known as lipofuscin in the lumen of the lysosome. These morphological changes are not thought to occur in lysosomes that perform CMA. Functional analysis, however, has shown that both substrate binding and translocation across the lysosomal membrane is greatly impaired in lysosomes isolated from organs of aged animals and from cultured senescent cells. These functional changes in the CMA pathway have been attributed to an age-dependent decrease in LAMP-2A on lysosomal membranes. Although this gradual decline in LAMP-2A levels begins at middle age, the decline in function can be offset by an increase in the number of lysosomes recruited to preform CMA. However, this compensatory increase in the number of lysosomes containing hsc70 is only transient and eventually a functional decline in CMA becomes apparent (Martinez-Vicente *et al.*, 2005).

The age-related reduction in the levels of LAMP-2A is not caused by a reduction in transcription, synthesis or the lysosomal trafficking of LAMP-2A. Instead, it is thought to be caused by reduced stability of LAMP-2A in the lysosomal membrane. The exact mechanisms underlying this drop in stability are unclear; however, changes in the lipid composition of the lysosomal membrane have been observed with age. It is possible that this disrupts the dynamics of LAMP-2A within this system and could contribute to the reduced levels of this receptor seen in old lysosomes (Martinez-Vicente *et al.*, 2005).

Cross-Talk between the UPS and Autophagy in Aging and Age-Related Diseases

Surprisingly, more recent data have challenged the earlier conclusions that macroautophagy is declining with age. Instead, the age-dependent reduction of the UPS capacity was suggested to be partially compensated for by an activation of macroautophagy.

Thus, it was found that aged cells have a tendency to accumulate misfolded proteins into p62-positive bodies. It has been suggested that a shift in the expression of co-chaperones of the BAG family could be responsible for the age-related increase in p62-positive protein aggregates. In particular, the expression of BAG3 is upregulated with age relative to that of BAG1. While high expression of BAG1 in young cells correlates with a high activity of the ubiquitin proteasome pathway, BAG3 has been shown to be upregulated in old cells leading to an increased activation of autophagy and to the formation of p62 bodies. BAG3 stimulates autophagy by binding HspB8 and by activating positive regulator of autophagosome synthesis eIF2α. BAG3 has also been identified in protein complexes containing p62 *in vitro* and *in vivo* suggesting that this co-chaperone together with Hsc70 may form a link between misfolded proteins and p62 (Carra *et al.*, 2009; Gamerdinger *et al.*, 2009). Further work is required in order to improve our understanding of the changes in protein degradation pathways during aging. Future studies are also likely to accentuate an important role of proteostasis network in the maintenance of homeostasis throughout the lifespan.

It is important to highlight here that cross-talk between protein degradation systems is not an artifact of pharmacological interventions or *in vitro* systems. Proteasome functional insufficiency (PFI) has been noted in the development of a wide range of heart diseases. Within many of these diseases macroautophagy is upregulated, suggesting that cross-talk between degradation systems may have significant clinical relevance in these pathologies (Zheng *et al.*, 2011). Perturbations of proteostasis network have also been shown to contribute to age-related neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and Huntington's disease, all of which are associated with the formation of intra- and extracellular protein aggregates. Indeed, impairment of both autophagy and the UPS, which can lead to accumulation and aggregation of misfolded proteins, has been detected in neuronal cells during pathology (Rubinsztein, 2006). So far these changes in different degradative pathways in the context of neurodegenerative diseases have been investigated largely independently of each other. However, it is highly likely that the interactions within the proteostasis network identified in model systems will also be of relevance here. It is of great importance to study the mechanisms of interaction between proteolytic pathways in the context of neurodegeneration as this will improve our understanding of the pathology and help to devise therapeutic strategies aiming to facilitate the clearance of disease-causing aggregate-prone proteins.

Potential cross-talk between degradative pathways is particularly relevant in the area of cancer biology. While autophagy is believed to play a tumor-suppressor role due to its positive regulation of cell homeostasis, it can be a double-edged sword due to its ability to promote survival of tumor cells. Indeed, an increased rate of autophagy has been observed in a number of cancer types and is thought to help these tumors survive in conditions of stress such as low nutrients, hypoxia and during anti-tumor drug interventions. In particular, an increase in autophagy has been observed in cells treated with anticancer drugs, hormone antagonists and ionizing irradiation where it may contribute to drug resistance. This is particularly relevant to the antitumor therapies involving proteasome inhibitors. The latter are potent therapeutic agents in the treatment of many forms of cancer due to a broad spectrum of antiproliferative and pro-apoptotic activity (Crawford *et al.*, 2011). Proteasome inhibitors such as bortezomib have been successfully used in combination with existing cancer treatments without overt problems of increased toxicity. However, such treatments are

particularly sensitive to the development of drug resistance problems, which hamper the potential for prolonged use.

Part of this resistance has been shown to arise from mutations in the catalytic subunits of the proteasome, specifically an Ala49Thr mutation in a highly conserved bortezomib-binding pocket of β5 subunit (PSMB5) as well as from overexpression of PSMB5. This has sparked the development of second-generation proteasome inhibitors that intend to overcome these kinds of resistance. Another form of resistance, however, may arise from a compensatory activation of macroautophagy through the mechanisms described in this chapter. It has been shown that suppression of autophagy in transformed cells treated with proteasome inhibitors increased apoptosis. This increase in apoptosis was not seen in nontransformed cells (reviewed in Driscoll and Chowdhury, 2012). This suggests that oncogenic transformation may increase the ability of cells to activate autophagy in response to stressors and indicates that transformed cells have a higher dependence on autophagy for survival. This makes the targeting of both degradation pathways an attractive target in the treatment of some cancers.

CONCLUSION

Protein degradation pathways play a pivotal role in the maintenance of cellular homeostasis and the regulation of many cellular processes. As such they have been the topic of intense research over the past decade. This research has brought a greater understanding of the mechanisms and regulatory processes involved in the complex task of orchestrating protein turnover. It has become clear that intracellular protein degradation pathways, namely the three modes of autophagy and the UPS, are not independent and there is an extensive cross-talk between the deferent catabolic systems. In this chapter we have highlighted some of the potential mechanisms by which these systems influence each other, such as shared substrates, targeting mechanisms and adaptor proteins, compensatory upregulation and mutual inhibition. We discussed how these interactions play a potentially important role in a clinical setting with specific reference to aging and age-related diseases. As our knowledge of proteolytic networks increases, we will undoubtedly unearth further unexpected links between the protein degradation systems in health and disease. The complex interplay between the proteostasis pathways makes developing interventions able to manipulate them a challenging but worthwhile endeavor.

Acknowledgments

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References

Carra, S., Brunsting, J.F., Lambert, H., et al., 2009. HspB8 participates in protein quality control by a non-chaperone-like mechanism that requires eIF2α phosphorylation. J. Biol. Chem. 284, 5523–5532.

Crawford, L.J., Walker, B., Irvine, A.E., 2011. Proteasome inhibitors in cancer therapy. J. Cell Commun. Signal. 5, 101–110.

Cuervo, A.M., Dice, J.F., 2000. Regulation of Lamp2a levels in the lysosomal membrane. Traffic 1, 570–583.

Dargemont, C., Ossareh-Nazari, B., 2012. Cdc48/p97, a key actor in the interplay between autophagy and ubiquitin/proteasome catabolic pathways. Biochim. Biophys. Acta 1823, 138–144.
Ding, W.X., Ni, H.M., Gao, W., et al., 2007. Linking of autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmic reticulum stress and cell viability. Am. J. Pathol. 171, 513–524.
Driscoll, J.J., Chowdhury, R.D., 2012. Molecular crosstalk between the proteasome, aggresomes and autophagy: translational potential and clinical implications. Cancer Lett. 325, 147–154.
Ferrington, D.A., Husom, A.D., Thompson, L.V., 2005. Altered proteasome structure, function, and oxidation in aged muscle. FASEB J. 19, 644–646.
Gamerdinger, M., Hajieva, P., Kaya, A.M., et al., 2009. Protein quality control during aging involves recruitment of the macroautophagy pathway by BAG3. EMBO J. 28, 889–901.
Glickman, M.H., Ciechanover, A., 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. 82, 373–428.
Jin, L., Williamson, A., Banerjee, S., et al., 2008. Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell 133, 653–665.
Kawaguchi, T., Miyazawa, K., Moriya, S., et al., 2011. Combined treatment with bortezomib plus bafilomycin A1 enhances the cytocidal effect and induces endoplasmic reticulum stress in U266 myeloma cells: crosstalk among proteasome, autophagy-lysosome and ER stress. Int. J. Oncol. 38, 643–654.
Kettern, N., Dreiseidler, M., Tawo, R., et al., 2010. Chaperone-assisted degradation: multiple paths to destruction. Biol. Chem. 391, 481–489.
Korolchuk, V.I., Mansilla, A., Menzies, F.M., et al., 2009. Autophagy inhibition compromises degradation of ubiquitin-proteasome pathway substrates. Mol. Cell 33, 517–527.
Lamark, T., Johansen, T., 2010. Autophagy: links with the proteasome. Curr. Opin. Cell Biol. 22, 192–198.
Lee, C.-K., Klopp, R.G., Weindruch, R., et al., 1999. Gene expression profile of aging and its retardation by caloric restriction. Science 285, 1390–1393.
Li, W.-w., Li, J., Bao, J.-k., 2012. Microautophagy: lesser-known self-eating. Cell Mol. Life Sci. 69, 1125–1136.
Martinez-Vicente, M., Sovak, G., Cuervo, A.M., 2005. Protein degradation and aging. Exp. Gerontol. 40, 622–633.
Massey, A.C., Kaushik, S., Sovak, G., et al., 2006. Consequences of the selective blockage of chaperone-mediated autophagy. Proc. Natl Acad. Sci. USA 103, 5805–5810.
Mijaljica, D., Prescott, M., Devenish, R.J., 2011. Microautophagy in mammalian cells: revisiting a 40-year-old conundrum. Autophagy 7, 673–682.
Milani, M., Rzymski, T., Mellor, H.R., et al., 2009. The role of ATF4 stabilization and autophagy in resistance of breast cancer cells treated with bortezomib. Cancer Res. 69, 4415–4423.
Pandey, U.B., Batlevi, Y., Baehrecke, E.H., et al., 2007. HDAC6 at the intersection of autophagy, the ubiquitin-proteasome system and neurodegeneration. Autophagy 3, 643–645.
Ravikumar, B., Sarkar, S., Davies, J.E., et al., 2010. Regulation of mammalian autophagy in physiology and pathophysiology. Physiol. Rev. 90, 1383–1435.
Reggiori, F., Komatsu, M., Finley, K., et al., 2012. Selective types of autophagy. Int. J. Cell Biol. 2012, 1–2.
Riley, B.E., Kaiser, S.E., Shaler, T.A., et al., 2010. Ubiquitin accumulation in autophagy-deficient mice is dependent on the Nrf2-mediated stress response pathway: a potential role for protein aggregation in autophagic substrate selection. J. Cell Biol. 191, 537–552.
Rubinsztein, D.C., 2006. The roles of intracellular protein-degradation pathways in neurodegeneration. Nature 443, 780–786.
Ryan, K.M., 2011. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. Eur. J. Cancer 47, 44–50.
Seibenhener, M.L., Babu, J.R., Geetha, T., et al., 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell Biol. 24, 8055–8068.
Wei, Y., Pattingre, S., Sinha, S., et al., 2008. JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol. Cell 30, 678–688.
Zhao, J., Brault, J.J., Schild, A., et al., 2007. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab. 6, 472–483.
Zheng, Q., Su, H., Tian, Z., et al., 2011. Proteasome malfunction activates macroautophagy in the heart. Am. J. Cardiovasc. Dis. 1, 214–226.
Zhu, K., Dunner Jr., K., McConkey, D.J., 2010. Proteasome inhibitors activate autophagy as a cytoprotective response in human prostate cancer cells. Oncogene 29, 451–462.

CHAPTER

6

Cross-Talk between Autophagy and Apoptosis in Adipose Tissue: Role of Ghrelin

Amaia Rodríguez, Leire Méndez-Giménez and Gema Frühbeck

OUTLINE

Abstract

Excess adiposity contributes to alterations in the molecular mechanisms and cell-intrinsic pathways in the adipose tissue that predispose to the development of obesity-associated metabolic disturbances, such as insulin resistance, type 2 diabetes or the metabolic syndrome. The pathological expansion of the adipose tissue in the onset of obesity contributes to the formation of hypoxic areas, leading to adipocyte apoptosis. Recently, another type of programmed cell death, namely autophagy, has been shown to be activated in fat depots of obese subjects. Autophagy constitutes a major cellular degradation process involving intracellular

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trafficking towards the lysosome. The underlying mechanisms whereby apoptosis and autophagy are regulated in the adipose tissue are not fully understood and the literature is still scarce. We have recently reported that insulin resistance is associated with an aggravation in human adipocytes of the apoptosis and expression of autophagy-related genes *BECN1, ATG5* and *ATG7*, involved in the initiation and elongation of autophagosomes. Our data showed that ghrelin, a gut-derived hormone involved in the regulation of energy balance, operates as a negative regulator of tumor necrosis factor α (TNF-α)-induced apoptosis and autophagy in visceral adipocytes. The imbalance between ghrelin and TNF-α in states of insulin resistance may contribute to the altered apoptosis and autophagy found in adipose tissue of patients with type 2 diabetes. A better understanding of the pathways involved in programmed cell death in adipose tissue is needed for fully disentangling the etiopathology of obesity-associated type 2 diabetes.

INTRODUCTION

Programmed cell death is an evolutionarily conserved phenomenon, which is crucial for several vital functions, including developmental morphogenesis, tissue homeostasis and defense against pathogens. Three different forms of programmed cell death have been classified on the basis of their distinct cell morphology, namely apoptosis, autophagy and necrosis (Gozuacik and Kimchi, 2007). Apoptosis or type I cell death occurs in various physiological and pathophysiological situations, being characterized by morphological and biochemical hallmarks, including cell shrinkage, chromatin condensation (pyknosis), membrane blebbing, nuclear DNA fragmentation (karyorrhexis), and apoptotic body formation (Fulda and Debatin, 2006). The caspases constitute a family of aspartate-specific cistein proteases that play a key role in apoptosis, as effector molecules in the process of cell death. Autophagy or type 2 cell death is characterized by sequestering cytosolic organelles and proteins in double-membrane vesicles, termed autophagosomes, that translocate to lysosomes for fusion and content degradation (Singh, 2012). Autophagy represents a dynamic self-degradative process during nutrient deprivation, but massive cell destruction can lead to an irreversible cellular atrophy and cellular collapse (Gozuacik and Kimchi, 2007). The proteins encoded by autophagy-related genes (ATG) are required for the formation of autophagic vesicles. Necrosis or type 3 cell death represents a caspase-independent process with early plasma membrane rupture and dilatation of cytoplasmic organelles, in particular mitochondria, but without pronounced nuclear chromatin condensation (Fulda and Debatin, 2006).

Adipose tissue mass is determined by competing processes regulating both the volume and the number of adipocytes (Arner and Spalding, 2010). Excess adiposity in obese subjects limits angiogenesis and increases the formation of hypoxic areas, thereby promoting the apoptosis of adipocytes (Cinti *et al.*, 2005). Autophagy has been also described to be altered in adipose tissue in obesity (Kovsan *et al.*, 2011). Apoptotic adipocytes are surrounded by M1-stage macrophages that form crown-like structures in adipose tissue (Cinti *et al.*, 2005). This process is accompanied by a chronic inflammation due to the secretion of proinflammatory cytokines by adipose-tissue embedded immune cells and dysfunctional adipocytes. Reports regarding the regulation of apoptosis and autophagy in the adipose tissue are scarce. In the present chapter, the morphological and molecular basis of apoptosis and autophagy in adipose tissue will be described. Moreover, the role of ghrelin, a gut-derived hormone involved in the homeostasis of energy balance, in the control of programmed cell death in adipose tissue in light of recent reports of our group and others is discussed.

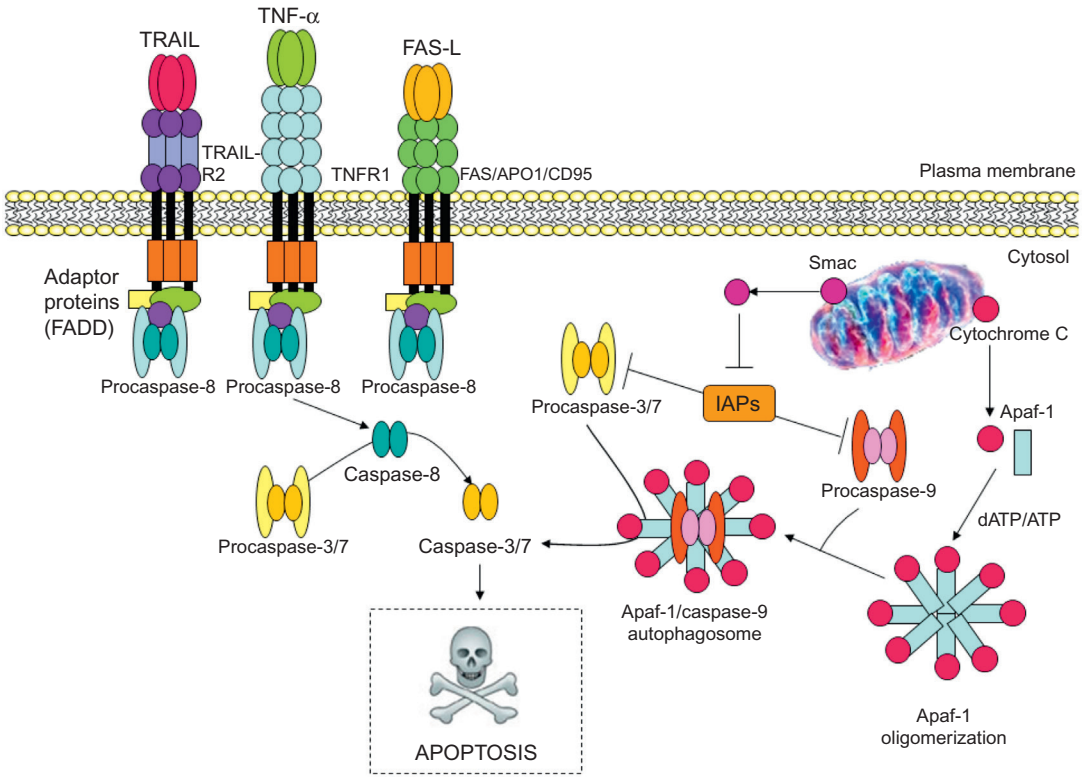

FIGURE 6.1 Apoptosis signaling pathways. Activation of caspases, the effectors of apoptosis, can be triggered at: (i) the plasma membrane by the activation of death receptors (extrinsic pathways); and (ii) the mitochondria by the release of apoptogenic factors, such as cytochrome C or Smac/Diablo proteins (intrinsic pathways). The adipose tissue of obese subjects shows an increased activation of death receptors and mitochondrial pathways, leading to the stimulation of effector caspases and adipocyte apoptosis. FADD, Fas-associated death domain; FAS-L, Fas ligand; IAPs, inhibitor of apoptosis proteins; Smac, second mitochondria-derived activator of caspase; TNF-α, tumor necrosis factor α; TNF-R1, TNF receptor 1; TRAIL, TNF-related apoptosis-induced ligand; TRAIL-R2, TRAIL receptor 2.

APOPTOSIS AND AUTOPHAGY IN ADIPOSE TISSUE

Apoptosis Signaling Pathways

The activation of caspases required for apoptosis can be initiated via two main sites: (i) at the plasma membrane upon ligation of death receptors (extrinsic pathway); and (ii) at the mitochondria, inducing the release of cytocrome C and other mitochondrial proteins (intrinsic pathway) (Fulda and Debatin, 2006) (Figure 6.1).

The extrinsic pathway is activated by the ligation of the pro-apoptotic factors, tumor necrosis factor α (TNF-α), Fas ligand (FAS-L or CD95-L), or TNF-related apoptosis-induced ligand (TRAIL) to their transmembrane death receptors of the TNF receptor superfamily,

such as TNF receptor 1 (TNFR1), CD95 (APO-1/Fas), or death receptor 3–6 (DR3–6), respectively (Fulda and Debatin, 2006). Upon activation, each receptor can form a death-inducing signaling complex (DISC) by the recruitment of the adaptor protein Fas-associated death domain (FADD) and procaspase-8. As a consequence, procaspase-8 is activated and autocatalytically processed. Activated caspase-8 initiates the direct cleavage and activation of the downstream effectors caspase-3 and caspase-7, triggering the apoptosis demise of the cell.

The intrinsic or mitochondrial pathway is initiated by the release of several mitochondrial proteins, such as cytochrome C, Smac (second mitochondria-derived activator of caspase)/Diablo (direct inhibitor of apoptosis protein [IAP]-binding protein with low PI), Omi/HtrA2 or endonuclease G from the mitochondrial intermembrane space (Fulda and Debatin, 2006; Wang and Youle, 2009). Mitochondrial perturbation results in the release of cytochrome C to the cytoplasm, which in conjunction with dATP/ATP, binds and oligomerizes cytosolic protein Apaf-1 and induces the formation of the apoptosome (Fulda and Debatin, 2006). The apoptosome recruits and activates procaspase-9 via Apaf-1, and, in turn, active caspase-9 cleaves and activates the effectors caspase-3 and caspase-7. On the other hand, Smac/Diablo and Omi/HtrA2 promote caspase activation through the neutralization of inhibitor of apoptosis proteins (IAPS) (Fulda and Debatin, 2006; Wang and Youle, 2009).

Adipocyte Apoptosis

Obesity is defined as an excess of adiposity (Frühbeck *et al.*, 2013). The excessive expansion of adipose tissue during the onset of obesity results in the activation of the death receptors CD95, TNFR1 and TRAIL receptors 1 and 2 as well as mitochondrial pathways, leading to the stimulation of effector caspases and adipocyte apoptosis (Herold *et al.*, 2013) (Figure 6.1). In this sense, TNF-α, a proinflammatory, pro-apoptotic cytokine, constitutes a well-known regulator of apoptosis in the adipose tissue. Upon binding to its receptor TNFR1, TNF-α pro-apoptotic signaling results in caspase-8 cleavage and activation, which further activates caspase-3, leading to adipocyte cell death (Rodríguez *et al.*, 2012). The finding that TNF-α is overexpressed in adipose tissue of obese individuals revolutionized the field of obesity research in the late 1990s (Hotamisligil *et al.*, 1997). Nowadays, it is well known that other death receptor ligands, such as TRAIL, are associated with adiposity and related to adipocyte apoptosis (Keuper *et al.*, 2013). By binding to TRAIL-R2, TRAIL activates the cleavage of caspase-8 and -3, ultimately leading to adipocyte cell death.

Lipodystrophies are rare genetic or acquired disorders characterized by the selective loss of adipose tissue, and metabolic complications such as dyslipidemia, type 2 diabetes and fatty liver. Growing evidence suggests that the decrease in adipose tissue mass under pathological conditions, including tumor cachexia, human immunodeficiency virus (HIV) infection-associated lipodystrophy or genetic forms of lipodystrophy, results from loss of fat cells by apoptosis (Tisdale, 2009). Patients with acquired lipodystrophy show an enhanced CD95-DISC formation, resulting in a robust sensitization for CD95-mediated apoptosis (Fischer-Posovszky *et al.*, 2006, 2011). Taken together, adipocyte death appears to be an important component of the inflammatory events associated with obesity and lipodystrophy, and may have common systemic consequences regardless of any consequence in overall fat mass (Villarroya *et al.*, 2007).

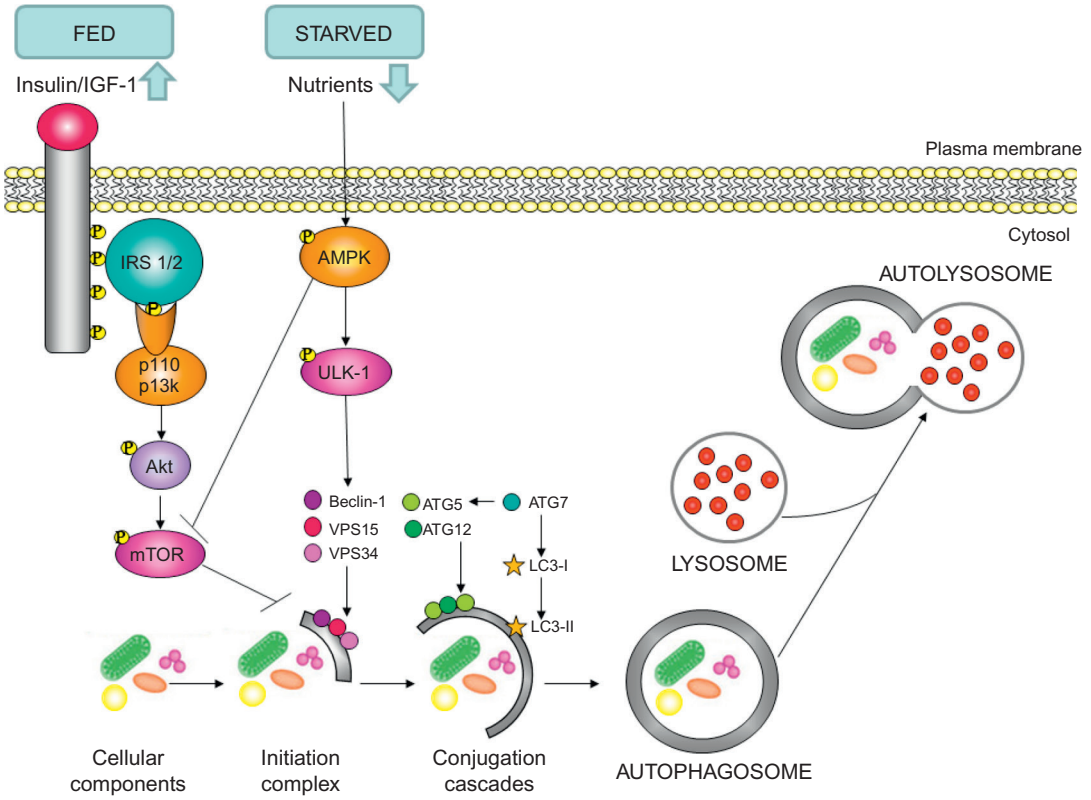

FIGURE 6.2 Regulatory factors governing autophagy. During the fed state, nutrients or insulin stimulation block the autophagosome formation through the mTOR signaling pathway. On the other hand, starvation activates autophagy by mechanisms that involve the activation of AMPK/ULK1 transduction signal. AMPK, AMP-activated protein kinase; Akt, protein kinase B; ATG, autophagy-related gene; IGF-1, insulin growth factor 1; IRS 1/2, insulin receptor substrate 1/2; mTOR, mammalian target of rapamycin; VPS34, class III phosphatidylinositol 3-kinase; ULK-1, unc51-like kinase 1.

Regulatory Elements of Autophagy

Autophagy is activated to degrade damaged cellular components as well as to preserve cellular viability under conditions of starvation or stress (Singh, 2012). Depletion of nutrients activates a second important cellular energy sensor, AMP-activated protein kinase (AMPK), which further activates by phosphorylating the unc51-like kinase 1 (ULK1) (Figure 6.2). Active ULK1 induces autophagy by the phosphorylation of Beclin-1, a protein that recruits regulatory proteins to the VPS34 complex (class III phosphatidylinositol 3-kinase [PI3K]), which is essential for the activity of the phagophore (Russell *et al.*, 2013). During vesicle elongation, ATG7 induces the conjugation of ATG12 to ATG5 as well as the conjugation of cytosolic light chain 3 (LC3)-I to phosphatidylethanolamine to generate LC3-II, one of the best characterized

components of the autophagosomes. Once formed, autophagosomes engulf cytosolic components, including damaged organelles and proteins, and fuse with lysosomes to complete degradation. The hydrolysis of engulfed cargo in the autolysosomes generates amino acids and free fatty acids that are released into the cytoplasm for essential biosynthetic functions.

The nutrient-sensor mammalian target of rapamycin (mTOR) is the best-characterized negative regulator of autophagy through short- and long-term mechanisms (Singh, 2012). Under basal fed conditions, nutrients (particularly amino acids) or insulin and growth factors trigger the activity of class I PI3K which, in turn, activates Akt and mTOR blocking the autophagosome formation (Singh, 2012) (Figure 6.2). Insulin-induced long-term inhibition of autophagy occurs via the transcription factors FoxO1 and FoxO3, which control the transcription of *ATG* genes (Liu *et al.*, 2009). In this regard, insulin-resistance is associated with an increased accumulation of autophagosomes in murine β-pancreatic cells (Fujitani *et al.*, 2009), in human adipocytes and adipose-derived stromovascular cells (Kovsan *et al.*, 2011) as well as in human neuroblastoma SH-SY5Y cells and primary cortical neurons via the inhibition of the PI3K/Akt/mTOR pathway (Son *et al.*, 2012).

Autophagy in the Adipose Tissue

Autophagy actively participates in the regulation of adipocyte differentiation, fat storage and inflammation. Transgenic animals lacking the autophagy-related proteins ATG5 and ATG7 show a reduction in adipose mass, suggesting that autophagy is essential for normal adipogenesis (Singh *et al.*, 2009; Zhang *et al.*, 2009). Analogously, *Atg5* and *Atg7* knockdown in 3T3-L1 adipocytes decreases the intracellular lipid content and gene expression levels of the key adipogenic transcription factors, CCAAT/enhancer-binding protein α and β (C/EBPα and β) and peroxisome proliferator activator receptor γ (PPARγ) (Singh *et al.*, 2009). Interestingly, white adipocytes of *Atg7*-deficient mice acquire some characteristics of brown adipocytes, such as higher mitochondrial content, multilocular lipid droplets and increased levels of brown adipogenic factors PPARγ-coactivator 1α (PGC-1α) and uncoupling protein-1 (UCP-1), triggering adipose tissue fatty acid β oxidation (Zhang *et al.*, 2009). Moreover, autophagy also affects the inflammatory status of adipose tissue, since inhibition of autophagy stimulates proinflammatory gene expression levels and causes endoplasmic reticulum stress in both human and murine adipose tissue (Jansen *et al.*, 2012; Yoshizaki *et al.*, 2012).

Human adipose tissue contains autophagosomes and obesity is associated with an altered expression of the autophagy-related molecules LC3-I, LC3-II, Beclin-1, ATG5 and ATG7 (Kovsan *et al.*, 2011; Nuñez *et al.*, 2013; Rodríguez *et al.*, 2012). Markers of autophagy are correlated with whole-body adiposity, visceral fat distribution and adipocyte hypertrophy. However, the altered expression of autophagy in human obesity appears to be related to the degree of insulin resistance, rather than to excess adiposity (Rodríguez *et al.*, 2012). In this sense, insulin constitutes a major inhibitor of autophagy, with insulin resistance being a potential activator of this process, since patients with type 2 diabetes show elevated formation of autophagosomes in subcutaneous adipose tissue (Ost *et al.*, 2010).

ROLE OF GHRELIN IN THE REGULATION OF APOPTOSIS AND AUTOPHAGY IN ADIPOSE TISSUE

The Ghrelin System

Ghrelin, a gut-derived peptide hormone, was first discovered in 1999 as the endogenous ligand for the growth hormone (GH) secretagogue receptor (GHSR), whereby it stimulates GH release (Kojima *et al.*, 1999). Stomach and intestine represent the two major ghrelin-secreting tissues (Frühbeck *et al.*, 2004), but other tissues synthesize ghrelin, to a lesser extent, including pancreas, kidneys, gonads, heart or adipose tissue (Chen *et al.*, 2009). The human ghrelin gene (*GHRL*), located on chromosome 3p26, encodes a 117-amino acid pre-prohormone, preproghrelin, which is proteolytically processed to yield two peptides: ghrelin and obestatin. Endogenous ghrelin exists in two principal forms, as a pure 28-amino acid peptide (desacyl ghrelin, representing ~95% of total ghrelin) and as an acylated peptide (acylated ghrelin, encompassing ~5% of total ghrelin) that carries an *n*-octanoyl modification at Ser3. The recently discovered ghrelin *O*-acyltransferase (GOAT) enzyme catalyzes the acylation in the endoplasmic reticulum (ER) (Chen *et al.*, 2009; Rodríguez *et al.*, 2012).

Ghrelin stimulates appetite and induces a positive energy balance, leading to body weight gain (Tschöp *et al.*, 2001). Circulating ghrelin levels are characterized by a preprandial rise and a postprandial fall, supporting its role in meal initiation (Chen *et al.*, 2009). In this sense, administration of exogenous ghrelin stimulates appetite and increases food intake by the stimulation of hypothalamic neuropeptide Y/agouti-related peptide neurons expressing its functional receptor, GHS-R 1a (Chen *et al.*, 2009; Theander-Carrillo *et al.*, 2006). In addition to its orexigenic effect, the adipose tissue also constitutes an important target for the adipogenic actions of ghrelin in rodents and humans. The human adipose tissue expresses all the components of the ghrelin system, namely ghrelin, obestatin, GOAT and the receptors of ghrelin-related peptides, GHS-R type 1a and GPR39 (Chen *et al.*, 2009; Rodríguez *et al.*, 2009). Ghrelin expression increases during adipogenesis with the *GHRL* gene knockdown reducing insulin-mediated adipogenesis in 3T3-L1 adipocytes (Gurriarán-Rodríguez *et al.*, 2011). Acylated ghrelin modulates preadipocyte proliferation and differentiation to mature adipocytes by increasing the expression of master adipogenic factors, PPARγ and sterol regulatory element-binding protein (SREBP) (Kim *et al.*, 2004; Rodríguez *et al.*, 2009). In this sense, acylated and desacyl ghrelin directly stimulates the expression of several fat storage-related proteins, including acetyl-CoA carboxylase, fatty acid synthase, lipoprotein lipase and perilipin through both central mechanisms (Theander-Carrillo *et al.*, 2006) and directly acting on human visceral adipocytes (Rodríguez *et al.*, 2009), thereby stimulating intracytoplasmic lipid accumulation.

Paradoxically, despite the orexigenic and adipogenic actions of ghrelin, obesity, insulin resistance, type 2 diabetes or the metabolic syndrome is associated with a paradoxical decrease in circulating total ghrelin levels (Kojima *et al.*, 1999; Tschöp *et al.*, 2001). Nevertheless, these pathologies are associated with a dramatic reduction of plasma desacyl ghrelin levels, the most abundant circulating isoform of the hormone, while plasma concentrations of acylated ghrelin remain unchanged or increased (Rodríguez *et al.*, 2009, 2010).

Ghrelin as a Survival Factor in Adipose Tissue

Ghrelin promotes cell survival by inhibiting apoptosis and stimulating proliferation in several cell types, such as the murine adult cardiomyocyte cell line HL-1, porcine aortic endothelial cells, rat hypothalamic neurons and cortical oligodendrocytes, as well as rat INS-1E pancreatic β-cells and the human adrenal gland carcinoma cell line (Chen *et al.*, 2009).

Ghrelin reportedly prevents the intrinsic apoptotic pathway induced by serum deprivation in murine 3T3-L1 adipocytes (Kim *et al.*, 2004). In human adipocytes, acylated and desacyl ghrelin reduces the activation of caspase-8, caspase-3 and the apoptosis induced by TNF-α (extrinsic pathway) (Figure 6.3) (Rodríguez *et al.*, 2012). Interestingly, ghrelin also inhibits indirectly adipocyte apoptosis through insulin growth factor 1 (IGF-1)-dependent mechanisms. Ghrelin acts on the pituitary and hypothalamus to stimulate GH release, which, in turn, stimulates the hepatic synthesis of its mediator, IGF-1 (Kojima *et al.*, 1999). IGF-1 constitutes an anabolic hormone with an anti-apoptotic activity in the adipose tissue,

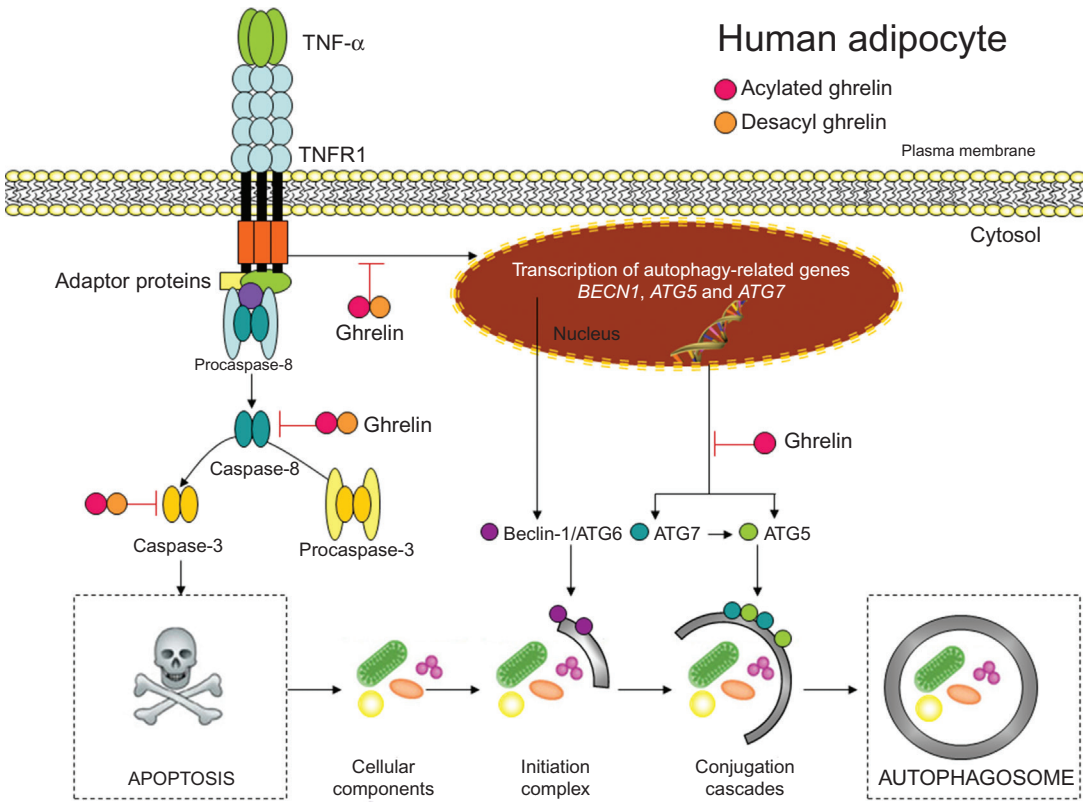

FIGURE 6.3 Cross-talk between apoptosis and autophagy: role of ghrelin. Ghrelin isoforms reduce tumor necrosis factor α (TNF-α) induced apoptosis and expression of autophagy-related genes in human visceral adipocytes (image modified from Rodríguez *et al.*, 2012). ATG, autophagy-related gene; BECN1, Beclin-1; TNF-α, tumor necrosis factor α; TNF-R1, TNF receptor 1.

since it rescues human pre-adipocytes and adipocytes from death receptor-triggered apoptosis. Together, the reduction in the intrinsic and extrinsic pathways of adipocyte apoptosis may contribute to an increase in adipocyte cell number coupled with an increase in adipocyte hypertrophy, thereby favoring adipose tissue expansion.

Ghrelin and Autophagy

Up to now, scarce literature addresses the role of ghrelin on autophagy (Bonfili *et al.*, 2013; Rodríguez *et al.*, 2012; Slupecka *et al.*, 2012; Tong *et al.*, 2012). Energy deficits enhance autophagy to help survival of cells that are under different stressors. Ghrelin activates autophagy in several physiological and pathophysiological processes requiring energy demand. In this sense, ghrelin induces protective autophagy: (i) in cardiomyocytes against hypoxic injury and subsequent energy metabolism dysfunction in an AMPK-dependent manner (Tong *et al.*, 2012); (ii) in epithelial cells of the small intestinal mucosa in neonatal piglets in order to prevent nutrient deficiency under conditions of starvation; and (iii) in colorectal adenocarcinoma cells in order to inhibit apoptosis (Bonfili *et al.*, 2013).

Ghrelin and TNF-α exhibit opposite effects on the regulation of autophagy in human adipocytes (Figure 6.3) (Rodríguez *et al.*, 2012). TNF-α increases the transcript levels of *BECN1*, required to initiate the formation of the autophagosome, and *ATG5* and *ATG7*, the autophagy proteins involved in the conjugation cascades for autophagosome elongation. On the other hand, acylated ghrelin reduces basal *ATG5* and *ATG7*, while desacyl ghrelin inhibits TNF-α-induced expression of *ATG5*, *ATG7* and *BECN1*. Taken together, ghrelin constitutes a negative regulator of basal and TNF-α induced autophagy in human visceral adipocytes (Rodríguez *et al.*, 2012).

DISCUSSION

Visceral adipose tissue of patients with type 2 diabetes is associated with an increased autophagy and apoptosis (Rodríguez *et al.*, 2012). Although apoptosis and autophagy are triggered by independent mechanisms, in some cases, both types of programmed cell death coexist in the same cells (Gozuacik and Kimchi, 2007). During the last decade, several studies have highlighted the mechanisms underlying the cross-talk between autophagy and apoptosis (Gozuacik and Kimchi, 2007). In this regard, ATG5 and Beclin-1 have been proposed as potential links between apoptosis and autophagy, since the cleavage of ATG5 or Beclin-1 by effector caspases switches autophagy to apoptosis (Cho *et al.*, 2009; Yousefi *et al.*, 2006). The activation of autophagy in type 2 diabetes may reflect an underlying apoptosis of hypertrophied adipocytes (Kovsan *et al.*, 2011).

Several signals such as TRAIL, FADD, ceramide or NGF withdrawal can induce cell death either by apoptosis or by autophagy. TNF-α plays a key role in the regulation of programmed cell death in adipose tissue, since it is able to stimulate both the extrinsic pathway of apoptosis as well as the transcription of the autophagy-related genes *ATG5*, *BECN1* and *ATG7* in visceral adipocytes (Rodríguez *et al.*, 2012). Interestingly, we have shown, for the first time, that ghrelin and TNF-α exert opposite effects on the regulation of apoptosis and autophagy in human visceral adipocytes (Figure 6.3).

In summary, ghrelin represents a novel modulator of programmed cell death in adipose tissue. The imbalance of TNF-α and ghrelin isoforms in states of insulin resistance may contribute to the altered apoptosis and autophagy observed in patients with type 2 diabetes. Further studies are needed to further disentangle the molecular mechanisms underlying the cross-talk between apoptosis and autophagy in insulin resistance. Advances in this research field will broaden our knowledge of the biological processes involved in cell survival and death in adipose tissue, and will lead to better understanding of the etiopathology of obesity-associated type 2 diabetes.

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References

Arner, P., Spalding, K.L., 2010. Fat cell turnover in humans. Biochem. Biophys. Res. Commun. 396, 101–104.

Bonfili, L., Cuccioloni, M., Cecarini, V., et al., 2013. Ghrelin induces apoptosis in colon adenocarcinoma cells via proteasome inhibition and autophagy induction. Apoptosis 18 (10), 1188–1200.

Chen, C.Y., Asakawa, A., Fujimiya, M., et al., 2009. Ghrelin gene products and the regulation of food intake and gut motility. Pharmacol. Rev. 61, 430–481.

Cho, D.H., Jo, Y.K., Hwang, J.J., et al., 2009. Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. Cancer Lett. 274, 95–100.

Cinti, S., Mitchell, G., Barbatelli, G., et al., 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J. Lipid Res. 46, 2347–2355.

Fischer-Posovszky, P., Hebestreit, H., Hofmann, A.K., et al., 2006. Role of CD95-mediated adipocyte loss in autoimmune lipodystrophy. J. Clin. Endocrinol. Metab. 91, 1129–1135.

Fischer-Posovszky, P., Keuper, M., Nagel, S., et al., 2011. Downregulation of FLIP by cycloheximide sensitizes human fat cells to CD95-induced apoptosis. Exp. Cell Res. 317, 2200–2209.

Frühbeck, G., Díez Caballero, A., Gil, M.J., 2004. Fundus functionality and ghrelin concentrations after bariatric surgery. N. Engl. J. Med. 350, 308–309.

Frühbeck, G., Toplak, H., Woodward, E., et al., 2013. Obesity: the gateway to ill health – an EASO position statement on a rising public health, clinical and scientific challenge in Europe. Obes. Facts 6, 117–120.

Fujitani, Y., Ebato, C., Uchida, T., et al., 2009. Beta-cell autophagy: a novel mechanism regulating beta-cell function and mass: lessons from beta-cell-specific Atg7-deficient mice. Islets 1, 151–153.

Fulda, S., Debatin, K.M., 2006. Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. Oncogene 25, 4798–4811.

Gozuacik, D., Kimchi, A., 2007. Autophagy and cell death. Curr. Top. Dev. Biol. 78, 217–245.

Gurriarán-Rodríguez, U., Al-Massadi, O., Crujeiras, A.B., et al., 2011. Preproghrelin expression is a key target for insulin action on adipogenesis. J. Endocrinol. 210, R1–R7.

Herold, C., Rennekampff, H.O., Engeli, S., 2013. Apoptotic pathways in adipose tissue. Apoptosis 18, 911–916.

Hotamisligil, G.S., Arner, P., Atkinson, R.L., et al., 1997. Differential regulation of the p80 tumor necrosis factor receptor in human obesity and insulin resistance. Diabetes 46, 451–455.

Jansen, H.J., van Essen, P., Koenen, T., et al., 2012. Autophagy activity is up-regulated in adipose tissue of obese individuals and modulates proinflammatory cytokine expression. Endocrinology 153, 5866–5874.

Keuper, M., Wernstedt Asterholm, I., Scherer, P.E., et al., 2013. TRAIL (TNF-related apoptosis-inducing ligand) regulates adipocyte metabolism by caspase-mediated cleavage of PPARgamma. Cell Death Dis. 4, e474.

Kim, M.S., Yoon, C.Y., Jang, P.G., et al., 2004. The mitogenic and antiapoptotic actions of ghrelin in 3T3-L1 adipocytes. Mol. Endocrinol. 18, 2291–2301.

Kojima, M., Hosoda, H., Date, Y., et al., 1999. Ghrelin is a growth-hormone releasing acylated peptide from stomach. Nature 402, 656–660.
Kovsan, J., Bluher, M., Tarnovscki, T., et al., 2011. Altered autophagy in human adipose tissues in obesity. J. Clin. Endocrinol. Metab. 96, E268–E277.
Liu, H.Y., Han, J., Cao, S.Y., et al., 2009. Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia: inhibition of FoxO1-dependent expression of key autophagy genes by insulin. J. Biol. Chem. 284, 31484–31492.
Nuñez, C.E., Rodrigues, V.S., Gomes, F.S., et al., 2013. Defective regulation of adipose tissue autophagy in obesity. Int. J. Obes. (Lond.) 37 (11), 1473–1480.
Ost, A., Svensson, K., Ruishalme, I., et al., 2010. Attenuated mTOR signaling and enhanced autophagy in adipocytes from obese patients with type 2 diabetes. Mol. Med. 16, 235–246.
Rodríguez, A., Gómez-Ambrosi, J., Catalán, V., et al., 2009. Acylated and desacyl ghrelin stimulate lipid accumulation in human visceral adipocytes. Int. J. Obes. 33, 541–552.
Rodríguez, A., Gómez-Ambrosi, J., Catalán, V., et al., 2010. Association of plasma acylated ghrelin with blood pressure and left ventricular mass in patients with metabolic syndrome. J. Hypertens. 28, 560–567.
Rodríguez, A., Gómez-Ambrosi, J., Catalán, V., et al., 2012. The ghrelin *O*-acyltransferase-ghrelin system reduces TNF-alpha-induced apoptosis and autophagy in human visceral adipocytes. Diabetologia 55, 3038–3050.
Russell, R.C., Tian, Y., Yuan, H., et al., 2013. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. Nat. Cell Biol. 15, 741–750.
Singh, R., 2012. Autophagy in the control of food intake. Adipocyte 1, 75–79.
Singh, R., Xiang, Y., Wang, Y., et al., 2009. Autophagy regulates adipose mass and differentiation in mice. J. Clin. Invest. 119, 3329–3339.
Slupecka, M., Wolinski, J., Pierzynowski, S.G., 2012. The effects of enteral ghrelin administration on the remodeling of the small intestinal mucosa in neonatal piglets. Regul. Pept. 174, 38–45.
Son, S.M., Song, H., Byun, J., et al., 2012. Accumulation of autophagosomes contributes to enhanced amyloidogenic APP processing under insulin-resistant conditions. Autophagy 8, 1842–1844.
Theander-Carrillo, C., Wiedmer, P., Cettour-Rose, P., et al., 2006. Ghrelin action in the brain controls adipocyte metabolism. J. Clin. Invest. 116, 1983–1993.
Tisdale, M.J., 2009. Mechanisms of cancer cachexia. Physiol. Rev. 89, 381–410.
Tong, X.X., Wu, D., Wang, X., et al., 2012. Ghrelin protects against cobalt chloride-induced hypoxic injury in cardiac H9c2 cells by inhibiting oxidative stress and inducing autophagy. Peptides 38, 217–227.
Tschöp, M., Weyer, C., Tataranni, A., et al., 2001. Circulating ghrelin levels are decreased in human obesity. Diabetes 50, 707–709.
Villarroya, F., Domingo, P., Giralt, M., 2007. Lipodystrophy in HIV 1-infected patients: lessons for obesity research. Int. J. Obes. (Lond.) 31, 1763–1776.
Wang, C., Youle, R.J., 2009. The role of mitochondria in apoptosis. Annu. Rev. Genet. 43, 95–118.
Yoshizaki, T., Kusunoki, C., Kondo, M., et al., 2012. Autophagy regulates inflammation in adipocytes. Biochem. Biophys. Res. Commun. 417, 352–357.
Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.
Zhang, Y., Goldman, S., Baerga, R., et al., 2009. Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. Proc. Natl Acad. Sci. USA 106, 19860–19865.

CHAPTER

7

Intracellular Pathogen Invasion of the Host Cells: Role of α-Hemolysin-Induced Autophagy

María Milagros López de Armentia and María I. Colombo

Abstract

Staphylococcus aureus causes a wide range of diseases in humans, from local infection to life-threatening systemic infection, both in health care facilities and the community. Classically it has been considered an extracellular pathogen, but cumulative evidence indicates that it invades cells and replicates intracellularly, leading to staphylococcal persistence and chronic disease. It has been proven that *S. aureus* is able to induce an autophagic response which allows bacteria replication and subsequent escape from autophagosomes into the cytoplasm, leading also to cell death. One of the key features of *S. aureus* infection is the production

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of a series of virulence factors, including secreted enzymes and toxins. It has been shown that the pore-forming toxin α-hemolysin (Hla) injures epithelial cells by interacting with its receptor, the zinc-dependent metalloprotease ADAM10. In addition, we have demonstrated that Hla is a secreted factor that participates in the activation of the autophagic pathway. Once internalized, the toxin is able to activate the autophagic pathway through a PI3K/Beclin-1-independent form. Recently we have reported that EPAC and Rap2b, through calpain activation, are the proteins involved in the regulation of Hla-induced autophagy. This chapter focuses on a pathogen that not only induces an autophagic response in the host cell, localizing in LC3 decorated compartments, but that also seems to benefit from that fate.

INTRODUCTION

Micrococci were first described by Koch in 1878, and the identification of *Staphylococcus aureus* as a human pathogen arose afterwards through the work of Ogston (Ogston, 1881). In the following decades, *S. aureus* was ranked among the most common causes of bacterial infections in humans, producing a wide spectrum of diseases reaching from superficial skin suppurations to life-threatening septicemias. Along with *Escherichia coli*, it also heads the list of agents that are responsible for hospital-acquired infections (Bhakdi and Tranum-Jensen, 1991). Soon after their discovery, *S. aureus* isolates were observed to generate soluble substances that induced inflammatory reactions after inoculation into experimental animals. The production of one or several hemolytic agents could be detected through cultivation on blood agar, in which a clear zone of beta-hemolysis was observed, often taken as a criterion for diagnosis of this bacterium (Burnet, 1930).

The first serious research into α-toxin was initiated by a tragedy in the Australian town of Bundaberg in 1928. Work performed in the following decades led to the identification of α-toxin as a major cause of the observed toxicity. The exotoxin is a secreted protein with hemolytic, cytotoxic, dermonecrotic, and lethal properties (Burnet, 1930). The modern era of α-toxin research was signaled by publications in the mid-1960s describing methods for isolating highly purified toxin. Cassidy *et al.* (1974) confirmed that highly purified toxin interacted with protein-free liposomal membranes and extended these observations by demonstrating that the liposomes became damaged. Today, it is evident that the α-toxin is a pore-forming bacterial cytolysin.

Siegel and Cohen (1964) demonstrated that α-toxin causes the aggregation of human platelets at sublytic concentrations. Since then, α-toxin has been shown to intoxicate a wide range of human cell types, including epithelial cells, endothelial cells, and a group of other hematopoietic cells including T cells, monocytes, macrophages, and neutrophils (Bhakdi and Tranum-Jensen, 1991). Further, multiple studies have investigated the human and animal host response to the toxin, determining how this toxin causes injury and defining salient features of the cellular response to the toxin (Craven *et al.*, 2009).

STAPHYLOCOCCUS AUREUS

Staphylococcus aureus, a Pathogen with a Dual Lifestyle

As mentioned, *Staphylococcus aureus* is a pathogen that may cause serious infectious diseases, eventually leading to septic and toxic shock. Most manifestations of *S. aureus* disease

involve extracellular bacteria or biofilm formation. Aside from this, infections have a second face: there is accumulating evidence that *S. aureus* is able to survive within its host cells and thus it might be considered as a facultative intracellular pathogen (Fraunholz and Sinha, 2012). *S. aureus* is capable of replicating in the phagosome of professional and non-professional phagocytes and then it escapes from the phagosomal compartment, subverts autophagy and induces cell death mechanisms, such as apoptosis. *S. aureus* possesses a vast array of specific virulence factors, like adhesins, toxins and enzymes, whose expression is regulated by a set of global virulence regulators (Schnaith *et al.*, 2007). The most important and best-studied system is the accessory gene regulator (*agr)* system (Novick, 2003). Some *S. aureus* derived factors, which trigger cell death, are under the control of the *agr* system. In fact, it has been shown that loss-of-function *agr* mutants invade mammary bovine epithelial cells with a higher internalization rate, but fail to induce cell death (Haslinger-Löffler *et al.*, 2005).

The ability of *S. aureus* to regulate gene expression in specific host environments is crucial for its success as a pathogen. A number of two-component regulatory systems have been shown to be responsible for the coordinate expression of virulence factors in *S. aureus*. In particular, AgrCA is responsible for the positive regulation of a number of genes encoding secreted factors including α, β, γ and δ toxins, leucotoxins and other secreted enzymes known to be involved in *S. aureus* pathogenesis. One of the major toxins is the cytotoxin α-hemolysin (Hla, α-toxin), secreted as a 34 kDa soluble monomer that functions as a homoheptameric pore-forming toxin with the phospholipid membranes. After secretion the Hla is capable of binding and oligomerization into a heptameric structure on the host cell membrane (Mestre *et al.*, 2010).

S. aureus invades cells and enters the endosome, a vesicle normally destined to intersect with the degradative pathway of the cell. The endosome is a nutrient-poor compartment, and this environment may generate a signal to activate a postexponential phase gene expression controlled by the accessory gene regulator (*agr*) locus, which in turn would enhance expression and secretion of extracellular proteins, particularly the α-hemolysin (Wesson *et al.*, 1998). Formation of the pore allows for hydrogen ions to escape from the lumen of the endocytic vesicle, effectively raising the luminal pH, thus explaining loss of degradation capacity of the phago/lysosomal compartment. Additional factors or enhanced Hla expression may eventually enlarge the pore, allowing the bacterium to escape into the cytoplasm where the nutrient conditions are more favorable to support replication of *S. aureus.* In addition, escaping towards the cytoplasm allows the pathogen to survive avoiding the harmful environment of the lysosome (Jarry *et al.*, 2008).

Interaction of *S. aureus* with the Autophagic Pathway

In the case of an inefficient phagosomal degradation pathway, infected host cells are also capable of combating intracellular pathogens by a process called autophagy. This process is a conserved membrane-traffic pathway present in all eukaryotic cells that sequesters cytoplasmic contents by double membranes and eventually delivers them to lysosomes (Schnaith *et al.*, 2007). Autophagy is crucially involved in physiological processes such as cellular homeostasis, cellular differentiation, tissue remodeling, and antigen presentation but also in response to stress conditions such as nutrient or insulin limitation.

Autophagy can be also induced by pharmacological agents such as rapamycin, an inhibitor of the serine/threonine kinase Tor (target of rapamycin). On the other hand, inhibition of the type III phosphatidylinositol 3-kinase (PI3K) by wortmannin or 3-methyladenine results in autophagy reduction, since the PI3K complex is critical for autophagy activation (Kirkegaard *et al.*, 2004). Genetic studies in yeast have led to the discovery of several Atg (autophagy related genes), many of which have mammalian orthologues. Some of the best-defined markers of autophagy are the Atg5 protein, which is associated with the nascent isolation membrane, and the microtubule-associated protein 1 light chain (LC3-II/Atg8). The presence of Atg5 and its proper conjugation with the ubiquitin-like molecule Atg12 are specifically required for the formation of the autophagic isolation membrane. Disruption of the Atg5 gene in mice resulted in the absence of autophagy. LC3/Atg8, considered a specific marker of the autophagosome, undergoes C-terminal proteolytic processing and conjugation with the lipid phosphatidylethanolamine, subsequently translocating from the cytosol to the autophagosomal membrane (Schnaith *et al.*, 2007).

Recently, it has been increasingly recognized that autophagolysosomes, besides degrading cytosolic components, also degrade organelles and intracellular bacteria, present in a membranous compartment or free in the cytoplasm, in a selective manner. Interactions of the autophagic pathway with pathogenic bacteria have revealed that autophagy may have different roles during different bacterial infections. Indeed, in addition to bacterial clearance, autophagy may coordinate cell autonomous signaling and in some cases may also promote bacterial replication. *S. aureus* diverts from the endosomal pathway to autophagosomes in an *agr*-dependent manner. *S. aureus*-induced autophagy is required for bacteria replication, subsequent escape from autophagosomes into the cytoplasm, and induced host cell death. The *S. aureus* autophagosome appears to be unique in terms of both molecular composition and function. The distinctive characteristic of the autophagic pathway of *S. aureus* seems to be determined already at early stages of the internalization process. *S. aureus*-containing phagosomes become Rab7-positive early after internalization and also stained positive for LC3. The GTPase Rab7 primarily associates with late endosomes, indicating that initial stages of phagosome maturation are not disturbed (Schnaith *et al.*, 2007). Nevertheless, it is important to take into account that Rab7 is also involved in the autophagic pathway being required for the maturation of the autophagic compartment. However, the acidification of the *S. aureus*-containing compartment is perturbed by the piercing of the phagosomal membrane due to the action of the α-hemolysin (Figure 7.1).

In terms of function, the replication of intracellular *S. aureus* is greatly reduced in the presence of wortmannin, a drug that prevents the initial formation of autophagosomes. In addition, *S. aureus* replication is markedly impaired in MEF cells deficient for the autophagy gene Atg5, confirming that certain autophagic proteins are required for pathogen replication. Likewise, treatment of cells with rapamycin, a pharmacological autophagy inducer, restores the replication of Agr deficient mutants. These findings indicate that autophagy is subverted by *S. aureus* for its survival. It is worth emphasizing that *S. aureus* uses the autophagosome not only to multiply but also to escape into the cytoplasm where the host cell apparently loses its control over the infection (Schnaith *et al.*, 2007).

It is interesting that *S. aureus*-induced cell death is not prevented by the use of the pan-caspase inhibitor Z-VAD, thus indicating that it is caspase-independent cell demise. In contrast, in cells overexpressing Bcl-2, *S. aureus*-induced cell death was avoided. It is important

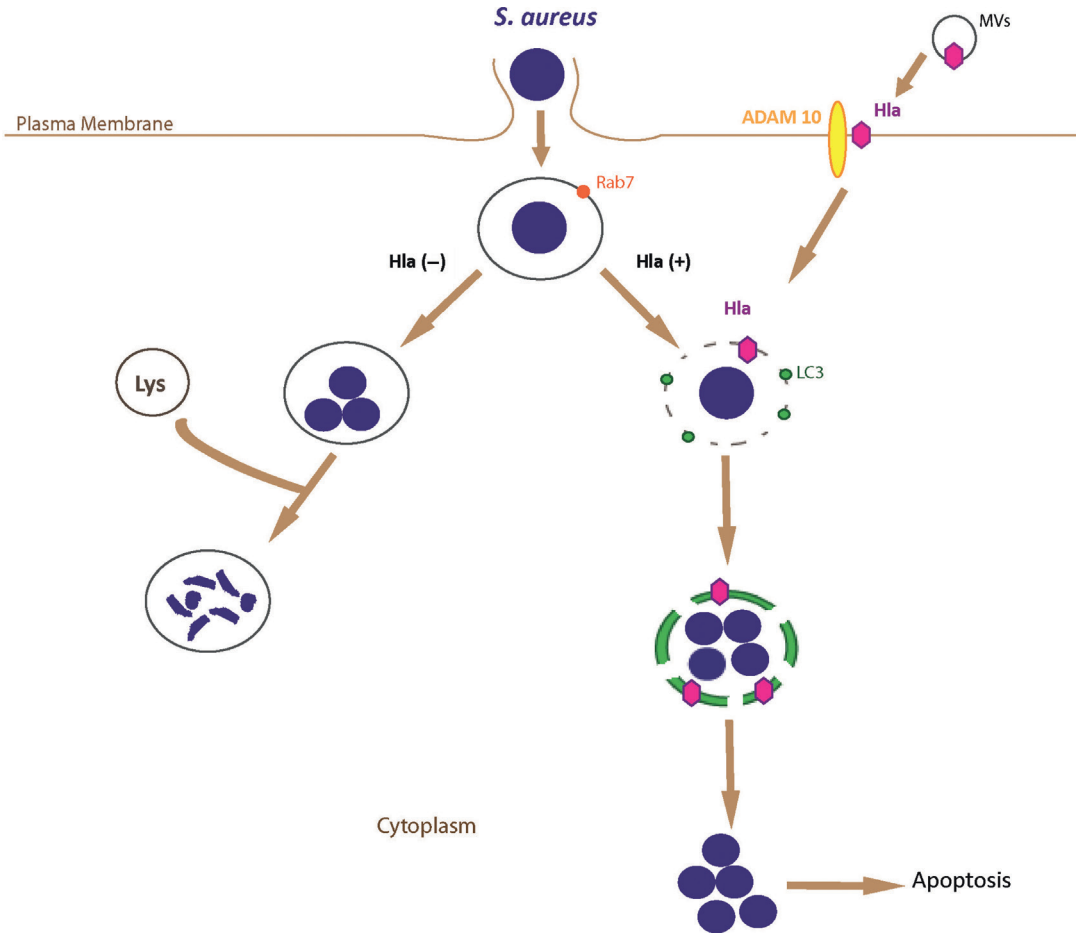

FIGURE 7.1 *S. aureus* enters the host cell via the endocytic pathway into a Rab7 decorated phago/endosome. *S. aureus* is able to secrete α-hemolysin (Hla), which induces pore formation and activates an autophagic response. The bacteria replicate in LC3-positive autophagosomes and prevent fusion with lysosomes. Eventually, *S. aureus* escapes toward the cytoplasm actively replicating and subsequently causing cell death. In contrast, Hla-deficient *S. aureus* are directed to the phagolysosomal pathway, where the bacteria are degraded. Alpha-hemolysin is also delivered to cells via membrane vesicles (MVs), and it is recognized by its receptor ADAM 10.

to take into account that Bcl-2 not only functions as an anti-apoptotic protein but also inhibits autophagy by interacting with Beclin-1. These data suggest that autophagy is necessary not only for *S. aureus* replication but also for killing of the host cell. To confirm that autophagy is essential for *S. aureus*-induced cell death, Schnaith *et al.* (2007) employed a genetic approach utilizing MEFs from atg5$^{-/-}$ mice. Wild-type MEFs infected with wt *S. aureus* strain showed marked signs of cell death 24h postinfection, while atg5$^{-/-}$ MEFs appear phenotypically unchanged. Staining with trypan blue dye revealed that *S. aureus*-induced cell death was

significantly reduced in atg5-deficient MEFs, supporting the idea that transit of *S. aureus* through the autophagosomal pathway is required for *S. aureus* cytotoxicity. Nevertheless, we cannot discard the possibility that specific autophagy related genes might be required for cell demise. Indeed, Atg5 has also been involved in cell apoptosis. Yousefi *et al.* (2006) has identified a truncated form of Atg5 that is cleaved by calpain 1 and 2. Interestingly, this truncated Atg5 translocates from the cytoplasm to mitochondria and causes cytochrome c release. This truncated form of Atg5 binds to Bcl-xl and may inactivate the Bcl-xl anti-apoptotic activity, promoting apoptotic cell death.

THE *S. AUREUS* α-HEMOLYSIN, A KEY SECRETED VIRULENCE FACTOR

Pore-Forming Toxin α-Hemolysin

As mentioned, *S. aureus* secretes a number of host-injurious toxins, among the most prominent of which is the small β-barrel pore-forming toxin α-hemolysin. Initially named based on its properties as a red blood cell lytic toxin, early studies suggested a far greater complexity of this α-hemolysin (Berube and Wardenburg, 2013). It was initially postulated that the toxin was secreted as a water-soluble monomer, capable of binding and oligomerization into a heptameric structure on the host cell membrane. However, more recent studies suggest an alternative secretion system (see the following). Nevertheless, this molecular transformation in host cells culminates in the extension of a membrane-perforating 1–3 nm pore through the eukaryotic lipid bilayer, allowing the flow of Ca^{2+} and K^{+}, ATP, and low molecular weight molecules through the barrel of the pore (Bhakdi and Tranum-Jensen, 1991).

Early evidence suggested that α-toxin monomers aggregated into an oligomeric structure on the host cell surface. Electron micrograph images led to the discovery of ring-like structures of 10 nm in diameter with 6–7 subunits and a central pore of approximately 2–3 nm (Füssle *et al.*, 1981). Song *et al.* (1996) postulate that the main structure is composed of three domains: (1) the cap domain on the extracellular face of the toxin, exposed to the aqueous environment, defining the entry of the pore; (2) the rim domain that is juxtaposed to the outer leaflet of the host plasma membrane; and (3) the stem domain that forms the membrane-perforating β-barrel pore. The accessory gene regulator (*agr*) locus codes for a quorum-sensing system that provides the primary control of Hla production via a regulatory RNA molecule, RNAIII, activated during late-log and stationary phases of growth (Novick *et al.*, 1993).

However, the secretion of these virulence factors from *S. aureus* and their delivery to host cells has not been fully characterized. Gurung *et al.* (2011) recently demonstrated that *S. aureus* produced membrane-derived vesicles (MVs) during *in vitro* culture, and many virulence-associated proteins were identified in these *S. aureus* MVs. Interestingly, proteomics analyses of *S. aureus* MV preparations identified a plethora of proteins and enzymes, including the α-toxin and the IgG-binding protein A. In addition, it has been evidenced by different techniques (immunoelectron microscopy, density gradient) that the *S. aureus* MVs are a vehicle to deliver biologically active Hla to human cells. In addition, a tight association of the toxin with the MVs has been shown. Apparently, the MVs interact with host cells via a

mechanism of MV fusion with the plasma membrane, and cholesterol is required for this interaction, as it can be abolished using the cholesterol-sequestering agent Filipin III.

S. aureus MVs are the first example of membrane-derived vesicles from a Gram-positive organism that can deliver virulence factors to host cells via membrane fusion, whereas this mode of delivery of effector proteins has been recognized for outer membrane vesicles (OMVs) from some Gram-negative bacteria.

ADAM 10, the Hla Receptor in Host Cells

The identification of A desintegrin and metalloprotease 10 (ADAM10) as an Hla-interacting protein required for initial toxin binding and its multiple cellular cytotoxic effects provides important insights into how Hla engages the host cell. *In vivo* interaction of Hla with ADAM10 revealed that this must occur in the context of an intact cell membrane, because solubilization of the membrane before toxin binding precludes the association of these proteins. A mechanistic view of the assembly of Hla suggests that its initial interaction with ADAM10 and the membrane directs the assembly of the Hla-ADAM10 complex in cholesterol/sphingolipid-rich caveolar rafts (Wilke and Bubeck Wardenburg, 2010).

ADAM10 functioning as the α-toxin receptor is supported by the following: (1) toxin binding to eukaryotic cells requires ADAM10 expression; (2) Hla physically interacts with ADAM10 *in vivo*; (3) the requirement for ADAM10 in Hla-mediated cytotoxicity is most apparent at low toxin concentrations wherein the need for a high-affinity cellular receptor was predicted to be most relevant (Berube and Wardenburg, 2013). Interestingly, it has been recently shown that mice harboring a conditional disruption of the ADAM10 gene in lung epithelium are resistant to lethal pneumonia. Investigation of the molecular mechanism of toxin-receptor function revealed that α-hemolysin upregulates ADAM10 metalloprotease activity in alveolar epithelial cells, resulting in cleavage of the E-cadherin protein (Inoshima *et al.*, 2012).

Hla is Capable of Inducing an Autophagic Response

Recent studies have demonstrated that α-hemolysin is the *S. aureus* secreted factor required for the activation of the autophagic pathway. This autophagic response is characterized by the formation of a large number of phagosomal structures containing the bacterium decorated by the protein LC3. *S. aureus* co-localization with LC3 reaches a maximum at 3h after infection to decline to basal levels within 5h (Schnaith *et al.*, 2007). During this period *S. aureus* autophagosomes were not acidified, as demonstrated by lysotracker staining. This indicates that the bacterium, in some way, inhibits autophagosomal maturation likely to prevent bacterial degradation before reaching the cytoplasm. As indicated previously, the Hla toxin induces cell damage by forming a lipid-bilayer penetrating pore at the containing vacuole membrane. This suggests that the generated pores would allow the diffusion of the protons across the membrane. As a consequence, the luminal pH of the bacteria-containing compartment would be neutralized. It is believed that modification of intravesicular pH may also cause alterations in the fusogenic capacity of the compartment (Jarry *et al.*, 2008). Thus, current knowledge indicates that the toxin Hla is likely one of the bacterial factors responsible for inhibiting the interaction between the bacterium-containing compartments and the lysosomes.

We have previously demonstrated (Mestre *et al.*, 2010) that *S. aureus* is able to induce an autophagic response in host cells and that α-hemolysin is the secreted factor required for the autophagy activation. To achieve this CHO GFP-LC3 cells were infected with different *S. aureus* strains: wt, a mutant deficient for α-hemolysin (Hla−) and the Hla(−) mutant complemented with an α-hemolysin plasmid. After infection, cells were washed and incubated for an additional period of time to allow bacteria replication. Results demonstrate that *S. aureus* wt as well as the complemented Hla(−) mutant induced an autophagic response upon infection evinced by the recruitment of LC3 to the phagosomes. Interestingly, these LC3-decorated vesicles were present in clusters containing bacteria inside. In contrast, the Hla(−) mutant was unable to activate this pathway and the number of bacteria did not seem to increase after the total incubation period, as clearly occurred with the wt strain or the Hla complemented mutant. These findings indicate that the autophagic response depends on the presence of Hla and that bacteria replication is reduced when *S. aureus* is deficient for Hla. In addition, we have shown that the purified Hla toxin alone is able to induce autophagy in the absence of bacterial infection. Interestingly, the toxin prevents the maturation of these autophagic vacuoles, leading to an increase in the number of autophagosomes.

More recently our group have also shown that the autophagic response induced by Hla does not occur by the canonical pathway of autophagy activation (Mestre and Colombo, 2012). Indeed, the autophagic response induced by the toxin is not suppressed by the classical autophagy inhibitors 3-mehtyladenine or wortmannin, suggesting that this process occurs independently of PI3Kinase activation. The toxin uses an alternative molecular mechanism to induce autophagy, which is independent of the PI3K/Beclin-1 complex but dependent on the autophagic protein Atg5.

Current knowledge indicates that cAMP plays a key role in the Hla-induced autophagic response. It is known that cAMP is able to stimulate the cAMP-activated guanine exchange factor (EPAC), which specifically turns on the monomeric G protein Rap. EPAC proteins are known to control a range of diverse effectors and to regulate several essential processes. We have shown that EPAC and its effector Rap2b participate in the regulation of Hla-induced autophagy. The direct activation of EPAC by cAMP or the overexpression of EPAC/Rap2b is sufficient to inhibit the autophagy response induced by the toxin.

When activated by cAMP, EPAC in turn activates Rap2b, which, through PLCε and an increase in the cytosolic levels of IP3, induces exit of calcium from the endoplasmic reticulum. Rise in intracytosolic Ca^{2+} activates the calcium-dependent, nonlysosomal cysteine–protease calpains. Inactivation of calpain 1, which in turn is able to cleave Atg5, leads to activation of autophagy by increasing the levels of the Atg5–Atg12 complex required for LC3 lipidation. It has been proved that the inhibition of calpains by the inhibitor calpeptin is sufficient to revert cAMP inhibition of the autophagy induced by Hla. All this evidence indicates that this signaling pathway participates in the regulation of the Hla-induced autophagic response and suggests that the toxin likely controls this pathway to allow autophagy activation, which is beneficial to the bacteria (Figure 7.2).

DISCUSSION

Several pathogens have the capacity to invade host cells to be sheltered from systemic immune defense mechanisms. However, once internalized they have to employ sophisticated

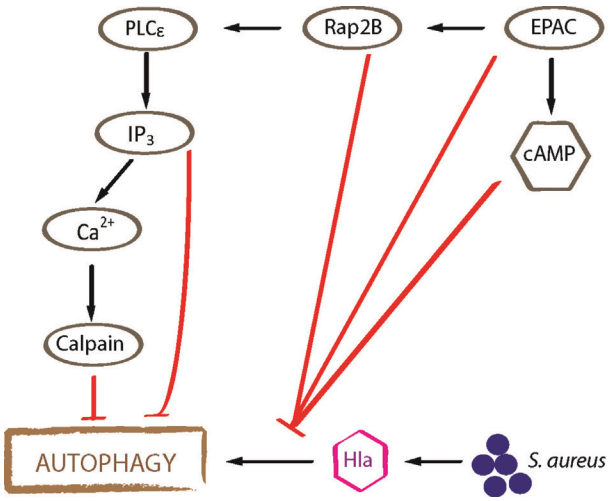

FIGURE 7.2 *S. aureus* secretes α-hemolysin (Hla) which induces a noncanonical autophagy pathway negatively modulated by cAMP. cAMP activates the exchange factor EPAC which, in turn, switches on the GTPase Rap2b. Afterwards, via PLCε, which increases cytosolic levels of IP3, calcium is released from the endoplasmic reticulum. The rise in intracellular Ca^{2+} activates calpain, which inhibits autophagy by cleaving the autophagic protein Atg5.

strategies to avoid being destroyed by the host cells. In the last few years numerous studies have documented that autophagy represents one of the most important devices of the host cell defense mechanisms that bacteria face upon intrusion in the cell. Nevertheless, several microorganisms subvert the autophagic pathway controlling this process as a strategy to establish a persistent infection. In this chapter we have summarized recent findings about the interaction of *S. aureus* with the autophagy pathway and how this pathway is manipulated, at the molecular level, allowing successful colonization and bacterial replication. In addition, evidence indicates that a functional autophagy pathway seems to be important for inducing cell death which, in turn, allows spreading of the pathogen.

Many key questions remain to be answered, mainly concerning the identity of the bacterial factors that govern individual pathways and specific transport steps of the target cell. The discovery of how these factors control key components of the autophagy machinery will constitute the foundation for novel therapeutic intervention against the persistent infection caused by intracellular microorganisms.

References

Berube, B., Wardenburg, J., 2013. *Staphylococcus aureus* α-toxin: nearly a century of intrigue. Toxins (Basel) 5, 1140–1166.
Bhakdi, S., Tranum-Jensen, J., 1991. Alpha-toxin of *Staphylococcus aureus*. Microbiol. Rev. 55, 733–751.
Burnet, F.M., 1930. The production of staphylococcal toxin. J. Pathol. Bacteriol. 33, 1–16.
Cassidy, P., Six, H.R., Harshman, S., 1974. Biological properties of staphylococcal alpha-toxin. Biochim. Biophys. 332, 413–423.
Craven, R., Gao, X., Allen, I.C., et al., 2009. *Staphylococcus aureus* alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells. PLoS ONE 4, e7446.
Fraunholz, M., Sinha, B., 2012. Intracellular *staphylococcus aureus*: live-in and let die. Front Cell Infect. Microbiol. 2, 43.

Füssle, R., Bhakdi, S., Sziegoleit, A., et al., 1981. On the mechanism of membrane damage by *Staphylococcus aureus* alpha-toxin. J. Cell Biol. 91, 83–94.
Gurung, M., Moon, D., Choi, C., et al., 2011. *Staphylococcus aureus* produces membrane-derived vesicles that induce host cell death. PLoS ONE 6, e27958.
Haslinger-Löffler, B., Kahl, B.C., Grundmeier, M., et al., 2005. Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells. Cell Microbiol. 7, 1087–1097.
Inoshima, I., Inoshima, N., Wilke, G., et al., 2012. A *Staphylococcus aureus* pore-forming toxin subverts the activity of ADAM10 to cause lethal infection. Nat. Med. 17, 1310–1314.
Jarry, T.M., Memmi, G., Cheung, A.L., 2008. The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalization in CFT-1 cells. Cell Microbiol. 10, 1801–1814.
Kirkegaard, K., Taylor, M.P., Jackson, W.T., 2004. Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat. Rev. Microbiol. 2, 301–314.
Mestre, M.B., Colombo, M.I., 2012. cAMP and EPAC are key players in the regulation of the signal transduction pathway involved in the α-hemolysin autophagic response. PLoS Pathog. 8, e1002664.
Mestre, M.B., Fader, C.M., Sola, C., et al., 2010. Alpha-hemolysin is required for the activation of the autophagic pathway in *Staphylococcus aureus*-infected cells. Autophagy 6, 110–125.
Novick, R.P., 2003. Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol. Microbiol. 48, 1429–1449.
Novick, R.P., Ross, H.F., Projan, S.J., et al., 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. 12, 3967–3975.
Ogston, A., 1881. Report upon micro-organisms in surgical diseases. Br. Med. J. 1 (1054), 369.b2–375.
Schnaith, A., Kashkar, H., Leggio, S., et al., 2007. *Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death. J. Biol. Chem. 282, 2695–2706.
Siegel, I., Cohen, S., 1964. Action of staphylococcal toxin on human platelets. J. Infect. Dis. 114, 488–502.
Song, L., Hobaugh, M.R., Shustak, C., et al., 1996. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. Sci. New York NY 274, 1859–1866.
Wesson, C.A., Liou, L.E., Todd, K.M., et al., 1998. *Staphylococcus aureus* Agr and Sar global regulators influence internalization and induction of apoptosis. Infect. Immun. 66, 5238–5243.
Wilke, G., Bubeck Wardenburg, J., 2010. Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. Proc. Natl Acad. Sci. USA 107, 13473–13478.
Yousefi, S., Perozzo, R., Schmid, I., et al., 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nat. Cell Biol. 8, 1124–1132.

CHAPTER

8

Modulation of Autophagy by Herpesvirus Proteins

Marion Lussignol and Audrey Esclatine

OUTLINE

Abstract

Autophagy is an evolutionarily conserved vacuolar mechanism for the autodigestion of recycling cellular components. It is involved in cellular homeostasis, and in the response to stressful environmental conditions. Autophagy also plays an important role during viral infections, when it may act either as part of the immune defense system, or as a proviral mechanism. Numerous viruses have developed mechanisms that modulate autophagy. In this chapter, we focus on the most recent data from the *Herpesviridae* family, and in particular the autophagy-regulating proteins that are encoded by these viruses. For several of these viruses, autophagy contributes to the host antiviral defenses, either by enhancing innate immunity or by helping antigen presentation. In order to counteract autophagy, herpesviruses have evolved various proteins that are able to inhibit this cellular mechanism. We highlight the four main steps in the autophagic process that are targeted by herpesvirus proteins. However, herpesviruses also encode proteins that activate autophagy

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at specific points in the virus life cycle, i.e., during lytic replication, latency, or reactivation. Interestingly, autophagy can be triggered not only by proteins, but also by the viral genome. Studies of autophagy regulation by herpesviruses help to give us a clearer understanding of the positive or negative impact of autophagy on viral infection, and on the long-term persistence of the virus in the host organism.

INTRODUCTION

Autophagy is essential for cell maintenance, for adaptation to stress, for the regulation of inflammation, and for the quality control of protein aggregates. However, autophagy is also an important intrinsic defense mechanism, which is involved in the control of infections and is linked to antigen presentation, and thus to the activation of T-cell responses. This process acts at several different levels in host/pathogen interactions:

i. Autophagy directly contributes to the degradation of intracellular pathogens, such as *M. tuberculosis* and herpes simplex virus type 1 (HSV-1), into autolysosomes in a process known as *xenophagy* (derived from the Greek *xenos* "stranger" and *phagein* "to eat") (Talloczy *et al.*, 2006). Xenophagy of HSV-1 depends on the cell type involved, and it has recently been reported that, in neurons, xenophagy limits HSV-1 infection both *in vivo* and *in vitro*, whereas in epithelial cells, it has no effect on viral replication (Alexander *et al.*, 2007; Yordy *et al.*, 2012). Autophagy has also been reported to target and degrade capsid proteins of the Sindbis virus in neurons, but without any impact on viral multiplication (Orvedahl *et al.*, 2010). Instead, autophagy contributes to protecting the host against Sindbis infection of the central nervous system (CNS) by limiting the accumulation of viral proteins that could lead to neuronal cell death.

ii. Autophagy also modulates innate immunity. Indeed, it contributes to the upstream activation of the innate immunity by delivering TLR (toll-like receptor) ligands to their cognate receptors within the endosomal compartments (Yordy *et al.*, 2013). In return, the activation of certain TLRs (such as TLR1, TLR3, TLR4, and TLR7) by microbial components conserved in pathogens triggers autophagy. Moreover, autophagy and innate immunity can both be stimulated by numerous cellular stresses induced by viruses, such as oxidative stress and ER stress.

iii. Autophagy is involved in the loading of peptides onto the major histocompatibility complex (MHC) class I or class II, and thus in initiating adaptive immune responses. Autophagy has subsequently been implicated in the thymic "education" of T cells and in autoimmunity, as well as in T-cell mediated antivirus and antitumor defenses. Autophagy facilitates the loading of at least two viral proteins, the Epstein-Barr virus (EBV)-encoded latency product EBNA-1, and glycoprotein gB of HSV-1, respectively, onto MHC class II and I (English *et al.*, 2009; Paludan *et al.*, 2005). An unconventional MHC class I antigen has recently been reported to contribute to the presentation of a human cytomegalovirus (HCMV) latency associated protein, pUL138 (which is independent of the transporters associated with antigen processing (TAPs) and independent of the proteasome) (Tey and Khanna, 2012). This processing of pUL138 is mediated by macroautophagy, and utilizes the vacuolar pathway. Therefore, autophagy supplements the conventional pathways, possibly to bypass the numerous viral immunity-evading mechanisms that target the MHC machinery.

In some situations, autophagy is also able to act as a proviral pathway that helps to boost viral replication. For example, stimulation of autophagy has been reported to increase the yields of poliovirus, hepatitis C virus (HCV), dengue virus, and coxsackie B virus. It has also been suggested that autophagy may help poliovirus particles to leave the cell during the late stages of infection. Dengue virus stimulates a selective form of autophagy, known as *lipophagy*, which degrades lipid droplets (Heaton and Randall, 2010). This regulation of the lipid metabolism results in the generation of free fatty acids and ATP, which promote viral replication by producing a metabolically favorable environment. It has been shown that measles virus induces sustained autophagy during viral infection, and that this is exploited by the virus to limit the cell death of infected cells, thus to improve viral production (Richetta *et al.*, 2013). A proviral effect of autophagy on the varicella zoster virus (VZV) has been reported very recently (Buckingham *et al.*, 2014). Induction of autophagy by VZV enhances infectivity, and improves the biosynthesis and the maturation of the major glycoprotein gE.

Highlighting the importance and complexity of these interactions, numerous viruses have developed a veritable arsenal of proteins that allow them to evade autophagy, whereas others hijack this machinery in order to replicate. Several recent reviews have addressed the interplay between viruses and autophagy (Richetta and Faure, 2013; Yordy *et al.*, 2013), and so in this chapter we have decided to focus on the modulation of autophagy by viral proteins encoded by members of the *Herpesviridae* family. The *Herpesviridae* family, which comprises large double-stranded DNA viruses, has been divided into three subfamilies, namely *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. Herpesviruses are widely disseminated, and more than 200 have already been identified. Few herpesviruses naturally infect more than one species, and only eight have humans as their primary host. These consist of *Herpes simplex* virus types 1 (HSV-1) and 2 (HSV-2) and VZV, which are alphaherpesviruses, whereas HCMV, human herpes virus 6 (HHV-6) and 7 (HHV-7) are betaherpesviruses. EBV and human herpes virus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), are gammaherpesviruses. We will see in this chapter that most of these viruses have the ability to modulate autophagy during infection. After the primary infection, all the herpesviruses enter latency, a dormant state, in the infected host and persist throughout the life of the host. However, the virus can be reactivated, leading to the production of new infectious viral particles.

We will first look at herpesvirus proteins with anti-autophagic properties, which act at different steps of the autophagic process. We will then see that in other situations, a few viral proteins have been identified that stimulate autophagy alongside viral nucleic acids.

INHIBITION OF AUTOPHAGY BY HERPESVIRUS PROTEINS

Inhibition of autophagy has been reported for several different herpesviruses, and can occur at different stages in the process: upstream by blocking the signaling pathways, or more directly by affecting the actual machinery by inhibiting the initiation of the autophagic process, the elongation of the phagophore or the maturation of autophagosomes into autolysosomes.

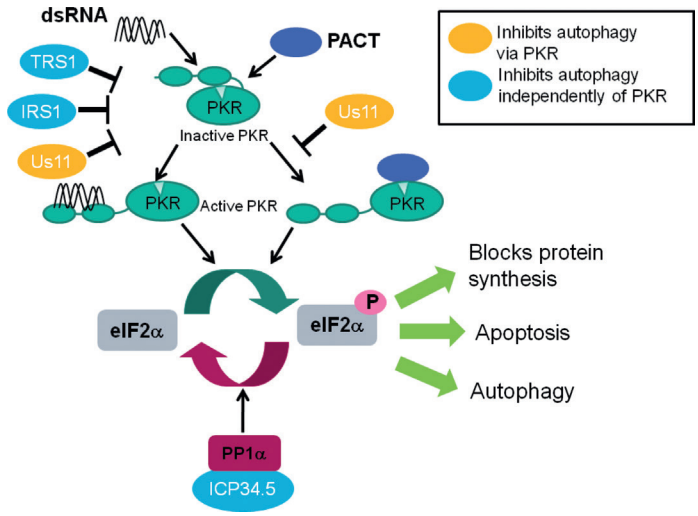

FIGURE 8.1 Activation of autophagy via the PKR-eIF2α signaling pathway. PKR associates with double-stranded RNA (dsRNA) or with PKR-associated activator (PACT), to induce a similar conformational change in PKR and leading to its activation. Phosphorylated eIF2α stimulates autophagy, blocks protein synthesis, and induces apoptosis. Several herpesvirus proteins block this signaling pathway by acting at different steps (e.g., TRS1 and IRS1 of HCMV; Us11 and ICP34.5 of HSV-1). All these proteins are able to inhibit autophagy, but most of them block autophagy independently of PKR. Us11 is the only one that has been reported to block autophagy via PKR.

Modulation of the Autophagy Signaling Pathways

Autophagy is regulated by several signal transduction pathways, including the mammalian TOR (mTOR) pathway; regulation of the two Beclin-1 complexes; regulation of the eIF2α phosphorylation pathway, which includes either PKR or the PKR-like endoplasmic reticulum kinase, PERK. Phosphorylation of eIF2α, a translation initiation factor, can activate autophagy in response to various stressors, such as HSV-1 infection, or nutrient deprivation (Talloczy *et al.*, 2002). Among eIF2α kinases, the PKR kinase acts as a sensor of viral infection, since PKR expression is induced by type-I interferon, and the kinase is activated by the double stranded RNA (dsRNA) produced during viral replication (Figure 8.1). PKR can also be activated by PACT (PKR-associated activator), which associates directly with PKR. Activated PKR phosphorylates eIF2α, leading to a shut-off of protein synthesis within the cell and the activation of autophagy. It has been reported that autophagy is stimulated during HSV-1 infection in fibroblasts, and that PKR is responsible for this activation (Talloczy *et al.*, 2002). Nevertheless, only infection with a deletion mutant virus was found to induce autophagy, because HSV-1 had developed anti-autophagic properties.

One of the inhibitory proteins to have been identified is the ICP34.5 viral protein. It has long been known that ICP34.5 is important to counteract the shutdown of the host protein during infection. Indeed, it interacts with the phosphatase 1α (PP1α), and redirects it to dephosphorylating eIF2α, via a domain known as GADD34, which is homologous to a cellular stress-induced protein. Infection of fibroblasts with a mutant virus lacking the ICP34.5

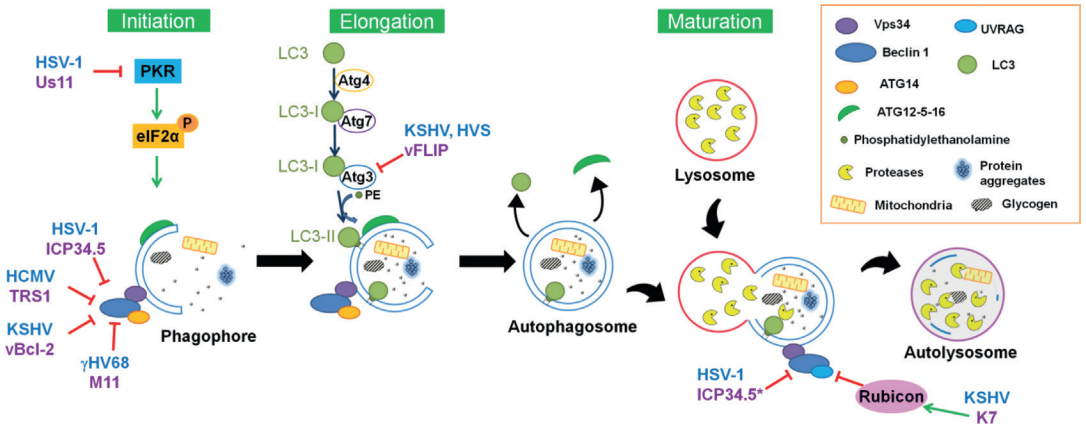

FIGURE 8.2 Anti-autophagic proteins encoded by herpesviruses. Numerous proteins encoded by herpesviruses can inhibit various different steps in the autophagy pathway. Herpes simplex virus 1 (HSV-1) possesses two proteins that inhibit the initiation of autophagosome formation: Us11, which blocks the PKR/eIF2α signaling pathway, and ICP34.5, which interacts with Beclin-1. Three other viral proteins block this initiation by interacting with Beclin-1: TRS1 from human cytomegalovirus (HCMV), and viral homologues of Bcl-2 from Kaposi's sarcoma-associated herpesvirus (KSHV) and γ-herpesvirus 68 (γHV68). KSHV and *Herpesvirus saimiri* (HVS) encode viral homologues of FLIP (vFLIP) that inhibit autophagosome elongation by interacting with Atg3, thus preventing LC3 conjugation with phosphatidylethanolamine (PE). ICP34.5 inhibits the maturation steps in dendritic cells. Finally, KSHV protein K7 binds to Rubicon, and consequently blocks the maturation complex Beclin-1/Vps34/UVRAG.

genes (ΔICP34.5) stimulates autophagy by activating the PKR/eIF2α signaling pathway. The most likely hypothesis is that ICP34.5 is able to block autophagy by inhibiting the PKR/eIF2α signaling pathway. However, although ICP34.5 is able to block autophagy, its mechanism of action is independent of the GADD34 domain, and therefore independent of eIF2α. In fact, as reported below, ICP34.5 can block autophagy by interacting with Beclin-1 (Figure 8.2), a major autophagic machinery protein, via another domain of ICP34.5 (Orvedahl *et al.*, 2007).

Viruses from different families have evolved numerous proteins that are able to block the PKR/eIF2α signaling pathway, because the general shut-off of protein synthesis hampers viral replication. HSV-1 encodes a protein, Us11, which can also inhibit this pathway by directly blocking PKR (Cassady and Gross, 2002). This late protein is produced after viral DNA replication, and is necessary to maintain protein expression late during the infection. Us11 has been described as capable of interacting with PKR, therefore preventing it from phosphorylating eIF2α (Figure 8.1). Us11 can block PKR activation via either dsRNA or PACT. We have demonstrated that Us11 is a second HSV-1 protein that is able to inhibit autophagy, in a PKR-dependent manner (Lussignol *et al.*, 2013). Indeed, ectopic expression of Us11 inhibits autophagy triggered by various stimuli, such as starvation or PKR activation by artificial dsRNA (Figure 8.3). We have demonstrated that Us11 was no longer able to inhibit autophagy when expressed in PKR-deficient cells. Moreover, by constructing various truncated forms of Us11, we confirmed that the binding of Us11 to PKR was essential for its anti-autophagic activity. Although the mechanisms of action of Us11 and ICP34.5 are

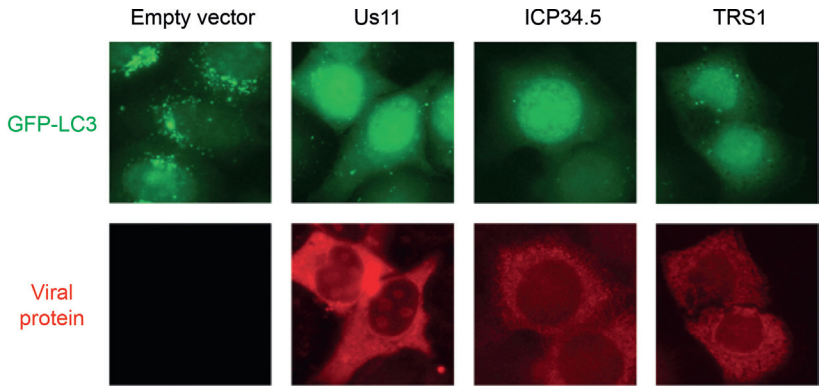

FIGURE 8.3 Various different proteins encoded by HCMV and HSV-1 block starvation-induced autophagy. When starved, HeLa cells expressing a hallmark of autophagy, GFP-LC3, reveal the presence of numerous autophagosomes in their cytoplasm (Empty vector). Expression of HSV-1 proteins, Us11, and ICP34.5 and that of HCMV TRS1 are able to block autophagosome formation; this is visualized by a diffuse staining of GFP-LC3 in the cytoplasm.

different, the early expression of Us11 in infected cells can compensate for a lack of ICP34.5, and inhibit autophagy during viral infection with a ΔICP34.5 mutant virus.

PKR is known to be activated by many viruses, and one attractive hypothesis is that viral proteins inhibiting this pathway are also able to block autophagy, as we observed in the case of Us11. ICP34.5 inhibits the PKR/eIF2α signaling pathway, but blocks autophagy via an interaction with Beclin-1. Other examples are TRS1 and IRS1, two anti-autophagic proteins encoded by HCMV and highly homologous, which have been described to be antagonists of the PKR/eIF2α signaling pathway. TRS1 binds to PKR and redirects it to the nucleus where it cannot phosphorylate its substrate, eIF2α. Although both TRS1 and IRS1 are also involved in the inhibition of autophagy by HCMV, their respective activities are not related (Chaumorcel *et al.*, 2012). We will see in the next paragraph that TRS1 and IRS1 inhibit autophagy by interacting with Beclin-1.

Inhibition of the Beclin-1 Initiation Complex

The Beclin-1/Vps34 complex is one of the two complexes, with the ULK1/Fip200 complex, regulating the early events in the formation of the autophagosome: initiation, nucleation, and assembly of the phagophore. Beclin-1 acts as a "platform," recruiting proteins that either activate or inhibit autophagy. For example, the anti-apoptotic protein Bcl-2 interacts with the BH3 (Bcl-2 homology-3) domain of Beclin-1, and possesses anti-autophagic activity (Pattingre *et al.*, 2005). When Bcl-2 is phosphorylated, it dissociates from Beclin-1, allowing autophagy to occur (Pattingre *et al.*, 2009). Other cellular proteins can activate the complex, including AMBRA1 (activating molecule in Beclin-1 regulated autophagy) and UVRAG (UV irradiation resistance associated protein).

Several viruses of the *Gammaherpesvirinae* subfamily encode viral homologues of Bcl-2 that inhibit autophagy by mimicking the interaction between cellular Bcl-2 and Beclin-1. These viral homologues have previously been reported to block apoptosis, and they are involved

in different steps in the life cycles and pathogenesis of viruses. The viral KSHV Bcl-2 homologue (vBcl-2) was the first viral protein reported to be able to inhibit autophagy by interacting with the BH3 domain of Beclin-1 in both yeast and mammalian cells (Pattingre *et al.*, 2005). Subsequently, the Bcl-2 homologue of another virus, the murine gammaherpesvirus 68 (γHV68), also referred to as M11, was shown to have the same anti-autophagic activity through its interaction with Beclin-1 (Sinha *et al.*, 2008). Whereas the interaction between cellular Bcl-2 and Beclin-1 is regulated by the phosphorylation of three different Bcl-2 sites when subjected to stress, such as starvation or ceramide treatment, KSHV vBcl2 and γHV68 M11 lack these phosphorylation sites. This results in a stable interaction between the viral homologue of Bcl-2 and Beclin-1, and leads to a constitutive repression of autophagy. As a result, these viral homologues exhibit a greater capacity for autophagy inhibition than cellular Bcl-2. The biological effects of vBcl-2, and in particular the vBcl-2-mediated antagonism of autophagy, have been explored in the lytic cycle and in latency. γHV68 M11 does not appear to be indispensable for lytic replication *in vitro* or for acute infection *in vivo*, but a Beclin-1-binding deficient vBcl-2 mutant virus has lost the capacity to maintain chronic infections in mice (E *et al.*, 2009). This finding seems to demonstrate that the viral evasion of autophagy plays an essential role in establishing latency. Another gammaherpesvirus, the EBV, encodes two viral homologues of Bcl-2, BHRF1 and BALF1, but their activity with regard to autophagy has not yet been tested.

Targeting Beclin-1 seems to be a conserved strategy for inhibiting autophagy among the viruses of the *Herpesviridae* family, as examples are to be found in all three subfamilies. Indeed, the viral proteins ICP34.5 of HSV-1 (an *Alphaherpesvirinae*), and TRS1 of HCMV (a *Betaherpesvirinae*) both possess a Beclin-1 binding domain that is necessary for their anti-autophagic activity (Chaumorcel *et al.*, 2012; Orvedahl *et al.*, 2007). ICP34.5, a neurovirulence factor, was the first viral anti-autophagic protein to be discovered: a ΔICP34.5 mutant virus activates autophagy, whereas the wild-type HSV-1 does not modulate the basal level of autophagy, and the ectopic expression of ICP34.5 is able to counteract autophagy by interacting directly with Beclin-1 in several cell types (Orvedahl *et al.*, 2007; Talloczy *et al.*, 2002). ICP34.5 possesses a Beclin-1-binding domain (BBD) located on a 20 amino acid region (amino acids 68–87), and this domain is required for the inhibition of autophagy. Interestingly, as shown by Orvedahl *et al.* (2007), this domain also seems to be important for the neurovirulence of ICP34.5. Indeed, HSV-1 is able to replicate in neuronal cells and to cause severe damage of the CNS, notably encephalitis. Using mouse infection models of HSV-1 encephalitis, ICP34.5 has been shown to be a neurovirulence factor, because a ΔICP34.5 mutant virus had lost its capacity to replicate and to damage the CNS of mice. Orvedahl *et al.* demonstrated that the Beclin-1-binding deficient ICP34.5 mutant virus is markedly neuroattenuated *in vivo*. They concluded from these results that inhibition of host autophagy is essential for HSV-1 neurovirulence.

HCMV induces autophagy during the early stages of infection in fibroblasts (Chaumorcel *et al.*, 2012; McFarlane *et al.*, 2011). Subsequently, autophagy is inhibited from 24 h p.i., and this inhibition depends on viral protein synthesis, as a UV-inactivated virus, which is able to enter cells but does not allow expression of viral protein synthesis, stimulates autophagy. We have identified a viral protein involved in this inhibition, TRS1, which possesses a Beclin-1-binding domain on its N-terminal part (Figure 8.3). TRS1 and its homologue IRS1 were previously described as being able to bind to dsRNA and PKR, thus preventing the activation of PKR (Marshall *et al.*, 2009). We have demonstrated that the Beclin-1-binding domain

is essential to repress autophagy, in contrast to the PKR-binding domain of TRS1. Moreover, TRS1 is still able to inhibit autophagy in murine fibroblasts lacking PKR. Importantly, TRS1 is involved in autophagy inhibition in the context of the infection. Indeed, a mutant virus lacking TRS1 is unable to block autophagy, but unlike a UV-inactivated virus, it does not stimulate autophagy. Thus, this finding led us to investigate whether HCMV encodes several anti-autophagic proteins, and whether even when TRS1 is lacking, other proteins can maintain autophagy at a basal level. We demonstrated that IRS1 also blocks autophagy, and we identified the Beclin-1-binding domain of IRS1 (unpublished data). It is likely that the expression of both TRS1 and IRS1 inhibits autophagy during HCMV infection.

Inhibition of the LC3 Conjugation Complex

The elongation of phagophores and the formation of autophagosomes require two ubiquitin-like conjugations: the former leads to the formation of the Atg12-Atg5 complex that associates with the external membrane of the autophagosome, and then dissociates during closure. The latter allows the autophagic protein LC3 to conjugate with phosphatidylethanolamine (PE) (Figure 8.2). After the cleavage of LC3 by Atg4, the conjugation reaction involves two enzymes: Atg7 and then Atg3. The conjugated form of LC3 remains on the autophagosomal membranes after their closure.

Two members of the *Herpesviridae* family, KSHV and herpesvirus saimiri (HVS), encode proteins that are able to block LC3 conjugation. These proteins are viral homologues of the cellular protein FLIP (FLICE-like inhibitor protein), which is a two death-effector domains-(DED1/2)-containing protein that regulates death receptor-mediated apoptosis. Cellular and viral FLIP proteins are able to block autophagy induced by starvation or by rapamycin (Lee *et al.*, 2009). The autophagy-inhibition mechanism relies on the interaction between FLIP and Atg3. FLIP proteins bind to Atg3 at the same sites as LC3 does. Therefore, interaction between FLIP and Atg3 prevents LC3 binding to Atg3, and consequently LC3 conjugation to PE. This results in the inhibition of both phagophore elongation and autophagosome formation. Specific inhibition of vFLIP interaction with Atg3 induces autophagy and an increase of cell death associated with autophagy in KSHV-infected cells *in vitro*. Similarly, *in vivo*, vFLIP inhibition induces tumor regression. These findings lead to the conclusion that vFLIP might prevent autophagic cell death, and this protein could be an interesting therapeutic target in order to prevent KSHV-associated tumor formation.

The important role of autophagy during KSHV pathogenesis has been confirmed recently, since, during KSHV-latent infection, autophagy triggers oncogene-induced senescence (OIS) (Leidal *et al.*, 2012). This mechanism is induced by oncogenic stress and leads to a blockade of the cell cycle. The expression of vFLIP limits autophagy and subsequently OIS, allowing infected cell proliferation to occur. OIS has been described as an important mechanism that suppresses tumors and limits the effect of oncogenic viruses.

Inhibition of the Maturation Complex

Beclin-1 can also be involved in the maturation of the autophagosome, i.e., the fusion with the lysosome and maturation to form the autolysosome, in a complex containing

Vps34 and UVRAG. Inhibition of these late stages of autophagy results in the accumulation of autophagosomes in the cytosol and blockade of autophagic degradation.

Gobeil and Leib (2012) observed that during HSV-1 infection of dendritic cells (DCs), the maturation of autophagosomes is blocked whereas, in fibroblasts, HSV-1 blocks the biogenesis of autophagosomes. In fact, the modulation of autophagy by HSV-1 depends on the cell type, since in murine fibroblasts, in HeLa cells, in MCF7 cells (a human mammary cell line), and in primary neurons, we and others have observed inhibition of the early steps of autophagosome biogenesis (Lussignol *et al.*, 2013; Orvedahl *et al.*, 2007; Talloczy *et al.*, 2002). The accumulation of autophagosomes observed in DCs infected by HSV-1 depends on ICP34.5 expression, and stable expression of ICP34.5 in DCs leads to the same effect. The Beclin-1-binding domain of ICP34.5 is required for the inhibition of autophagosome maturation to occur (Figure 8.2). It is unclear why ICP34.5 inhibits the initiation of autophagosome formation in some cell lines, but inhibits autophagosome maturation in others. Two hypotheses have been advanced: the first is that ICP34.5 can interact with Beclin-1 within both the initiation complex and the maturation complex, depending on the cell type. The second hypothesis involves TBK1 (TANK-binding kinase 1), a cellular protein known both to be involved in autophagy regulation and also to be a component of the TLR-signaling pathway. ICP34.5 interacts with TBK1 and blocks this latter pathway by inhibiting the phosphorylation of interferon regulatory factor 3/7 (IRF3/7). TBK1 also plays an important role in the capture of autophagic cargos and in the maturation of autophagosomes into autolysosomes, which notably allows the degradation of intracellular microorganisms to occur (Pilli *et al.*, 2012). Thus, it is possible that the mechanism by which ICP34.5 blocks the late stages of autophagy involves a direct interaction with TBK1; this is all the more likely because the TBK1-binding domain (aa 72-106) and the Beclin-1-binding domain (aa 68-87) of ICP34.5 partially overlap.

Recently, a new anti-autophagic protein of KSHV has been identified (Liang *et al.*, 2013). This protein, called K7, blocks autophagosome maturation by inhibiting the Beclin-1/Vps34/UVRAG complex. This complex can be regulated by Rubicon, a cellular protein that interacts with the complex, blocks Vps34 lipid kinase activity, and so inhibits the fusion between autophagosome and lysosome. The viral K7 interacts with Rubicon, promotes its interaction with the Beclin-1/Vps34/UVRAG complex, and enhances the suppression of Vps34 kinase activity. Based on what we have described here, we can see that KSHV encodes three anti-autophagic proteins that inhibit different stages of the autophagy pathway: vBcl-2 blocks the initiation of formation, vFLIP the elongation, and K7 the maturation of autophagosomes.

AUTOPHAGY ACTIVATION BY HERPESVIRUSES

In some cases, autophagy is stimulated during viral infection, either because the virus is unable to block it, or because it is involved in the viral life cycle. Expression of viral proteins can trigger autophagy in infected cells, but autophagy can also be stimulated by detecting viral components, such as its genome.

Herpesviridae Proteins that Activate Autophagy

The expression of gH, an HSV-1 glycoprotein, triggers a specific type of autophagy known as NEDA (nuclear envelope-derived autophagy), which differs from conventional macroautophagy (English *et al.*, 2009). NEDA involves four-layered membrane autophagosomes that are connected to the nucleus. This kind of autophagy does not occur in cells infected with a ΔICP34.5 defective mutant virus, because gH, which is a true late protein of HSV-1, is therefore not expressed. First observed in murine macrophages, NEDA seems to be a conserved mechanism induced by HSV-1 in several celltypes, including epithelial cells and fibroblasts (Radtke *et al.*, 2013). Although the mechanism of NEDA activation by gH has not yet been revealed, it has been shown that NEDA is involved in the presentation of viral antigens on MHC class I in macrophages (English *et al.*, 2009). During HSV-1 infection, the processing and presentation of the viral antigen gB on MHC class I follows two successive phases: the first phase depends on the classical pathway of MHC class I presentation, but the second depends on autophagy. Both macroautophagy and NEDA are able to contribute to the processing of gB, but since macroautophagy is blocked during HSV-1 infection, NEDA can allow the host to bypass autophagy inhibition.

Whereas autophagy is highly regulated by HSV-1 during the lytic cycle of the virus, nothing is yet known about the activation of autophagy by this virus during latency or early reactivation. However, all herpesviruses have the ability to maintain their genome in the host organism in a latent state, and expression of latent membrane protein (LMP1) of EBV has been reported to modulate autophagy (Lee and Sugden, 2008). During latency, the viral genome persists in cells, and the transcription of viral genes is limited to latency-specific genes. After infection of the oral epithelium, EBV establishes its latency in B cells and can induce B-cell proliferation. LMP1 is required for this proliferation to occur, and is able to induce autophagy. LMP1 expression is highly variable in EBV-infected B cells, and the level of autophagy depends on the quantity of LMP1. Whereas cells that express low levels of LMP1 preferentially accumulate early autophagosomes, high levels of LMP1 expression induce an accumulation of autolysosomes, corresponding to later stages of autophagy. Furthermore, in cells expressing high levels of LMP1, the authors observed a degradation of LMP1, which appeared to depend on autophagy, since they observed an accumulation of LMP1 in autophagy-deficient cells. Triggering autophagy can be beneficial in EBV. Indeed, the autophagy-dependent degradation of LMP1 seems to be necessary for the proliferation of infected B cells. The mechanism of autophagy activation by LMP1 is not known, but one possibility is that it could be related to the property of LMP1 to phosphorylate the eIF2α kinase PERK. However, it remains to be confirmed whether LMP1 is able to stimulate autophagy by PERK activation.

Regulating autophagy can give the virus a way of maintaining latency, as autophagy is involved in the regulation of cell death. For example, autophagy induction has a positive effect during the latency of rhesus monkey rhadinovirus (RRV), a virus close to KSHV (Ritthipichai *et al.*, 2012). During latency, this virus encodes a homologue of cellular FLIP, vFLIP, which activates autophagy. Under pro-apoptotic treatment, ectopic expression of vFLIP enhances autophagosome formation, and also inhibits apoptosis. Autophagy can be a pro-survival mechanism, and during RRV latent infection, vFLIP protects cells against apoptosis. Therefore, the hypothesis is that vFLIP stimulates autophagy during latent infection

in order to prevent cell death. Surprisingly, KSHV also encodes a vFLIP homologue (see previous section "Inhibition of the LC3 Conjugation Complex"), but in contrast KSHV-vFLIP inhibits autophagy by interacting with Atg3. This may be related to the fact that the stimulation of autophagy during latent infection has a deleterious effect on KSHV (Leidal *et al.*, 2012). As reported previously, autophagy triggers OIS during latent KSHV infection, a mechanism that induces cell growth arrest. This senescence is activated by another viral latency protein known as v-cyclin, which has been found to be capable of activating autophagy. V-cyclin induces the transcription of several autophagy genes (ULK1, ATG7, LC3), and leads to an inhibition of mTOR, which allows the activation of autophagy. The senescence induced by v-cyclin activation of autophagy is counteracted by vFLIP.

Autophagy can be also modulated during the initiation of KSHV reactivation, a process by which a latent virus switches to a lytic phase of replication. RTA (replication and transcription activator) is a viral transcriptional factor necessary for the lytic reactivation, and it is also able to activate autophagy (Wen *et al.*, 2010). Wen *et al*. observed that autophagy is stimulated during KSHV reactivation, and that RTA alone induces autophagosome formation in both 293T and B cells. Furthermore, autophagy inhibition affects KSHV lytic reactivation, suggesting that autophagy probably plays an important role during this step in the virus life cycle. One possible hypothesis for the mechanism of autophagy activation by RTA might be an upregulation of autophagic gene expression, as an increase of Beclin-1 expression has been observed during KSHV reactivation.

Activation of Autophagy by Viral Nucleic Acids

HCMV and HSV-1 stimulate autophagy during the early steps of infection of fibroblasts, but this occurs independently of *de novo* viral protein expression (Chaumorcel *et al.*, 2012; McFarlane *et al.*, 2011). Similarly, infection with an HSV-1 defective mutant that does not express either early or late proteins stimulates LC3 conversion, a hallmark of autophagy (McFarlane *et al.*, 2011). These findings suggest that viral components of HCMV can directly trigger autophagy; however, it is interesting to note that noninfectious viral particles of HSV-1 and HCMV (L particles and dense bodies, respectively) do not stimulate autophagy. Whereas these particles can bind to and enter the cells and release tegument proteins, they lack viral genome and capsid proteins. This implies that nucleic acids could be responsible for triggering autophagy. In fact, the HCMV genome alone seems able to stimulate autophagy in human fibroblasts, and more generally, cytoplasmic DNA from various different origins can also activate autophagy.

The activation of autophagy by HSV-1 DNA has been also reported in murine myeloid cells, which are nonpermissive cells (Rasmussen *et al.*, 2011). In these cells, HSV-1 stimulates autophagy in a PKR/eIF2α-independent manner during the first hours of infection. Viral DNA triggers autophagy via STING (stimulator of IFN genes), a transmembrane protein, that mediates a type-I IFN antiviral response. This study has provided some insights into the role of early autophagy induction during viral infection; indeed, autophagy inhibition decreased IFN-β production following HSV-1 infection. Similar results have been observed following infection with PrV (pseudorabies virus), another *Alphaherpesvirinae* virus.

CONCLUSION

The *Herpesviridae* are highly adapted to their host, and after infection they can persist throughout the lifetime of the organism. Thus, it is not surprising to find that they possess proteins that are able to modulate an essential cellular process, such as autophagy, which can both contribute to antiviral immunity and be involved in the viral life cycle. Nevertheless, it is interesting to note that they have developed various different strategies for repressing autophagy, targeting virtually every step in the process. Whereas xenophagy seemed to be the most obvious antiviral defense, no recent data have confirmed the degradation of viral particles in the autophagic vacuoles. In contrast, autophagy clearly contributes to several aspects: first, viral antigen presentation on MHC class I and II; second, to the regulation of cell death; and third, to the control of tumor progression. Most of the various mechanisms by which herpesvirus proteins inhibit autophagy have been identified, whereas less is known about how autophagy is activated by these viruses. Autophagy may be stimulated as a consequence of the detection of viral components, or can be triggered by viral protein expression, but the way viral proteins act on the autophagic pathway remains to be elucidated. At the physiopathological level, little is known about why a herpesvirus activates autophagy. Although recent data demonstrate that autophagy is involved in the maturation of envelope glycoproteins in the case of VZV, and has a prosurvival effect in cells infected with a monkey rhadinovirus. In conclusion, it is now certain that autophagy plays a complex role during herpesvirus infections, and this can be exemplified by the KSHV life cycle. During latency, autophagy has a harmful effect on the virus, since it triggers senescence and cell death, and consequently threatens the ability of the virus to persist inside these cells. Subsequently, autophagy is stimulated during KSHV reactivation, but this time, this activation promotes the onset of lytic replication. Afterwards, autophagy is controlled by K7, a protein expressed during the lytic cycle of KSHV, but the role of autophagy after viral reactivation and the requirement for the virus to control it remain to be elucidated. Further investigations on the impact of autophagy on herpesvirus infections are required, but autophagy is a potential new target for the development of antiviral therapeutics.

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References

Alexander, D.E., Ward, S.L., Mizushima, N., et al., 2007. Analysis of the role of autophagy in replication of herpes simplex virus in cell culture. J. Virol. 81, 12128–12134.

Buckingham, E.M., Carpenter, J.E., Jackson, W., et al., 2014. Autophagy and the effects of its inhibition on varicella-zoster virus glycoprotein biosynthesis and infectivity. J. Virol. 88, 890–902.

Cassady, K.A., Gross, M., 2002. The herpes simplex virus type 1 U(S)11 protein interacts with protein kinase R in infected cells and requires a 30-amino-acid sequence adjacent to a kinase substrate domain. J. Virol. 76, 2029–2035.

Chaumorcel, M., Lussignol, M., Mouna, L., et al., 2012. The human cytomegalovirus protein TRS1 inhibits autophagy via its interaction with Beclin-1. J. Virol. 86, 2571–2584.
English, L., Chemali, M., Duron, J., et al., 2009. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. Nat. Immunol. 10, 480–487.
E, X., Hwang, S., Oh, S., et al., 2009. Viral Bcl-2-mediated evasion of autophagy aids chronic infection of gammaherpesvirus 68. PLoS Pathog. 5, e1000609.
Gobeil, P.A., Leib, D.A., 2012. *Herpes simplex* virus gamma34.5 interferes with autophagosome maturation and antigen presentation in dendritic cells. MBio 3, e00267–00212.
Heaton, N.S., Randall, G., 2010. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 8, 422–432.
Lee, D.Y., Sugden, B., 2008. The latent membrane protein 1 oncogene modifies B-cell physiology by regulating autophagy. Oncogene 27, 2833–2842.
Lee, J.S., Li, Q., Lee, J.Y., et al., 2009. FLIP-mediated autophagy regulation in cell death control. Nat. Cell Biol. 11, 1355–1362.
Leidal, A.M., Cyr, D.P., Hill, R.J., et al., 2012. Subversion of autophagy by Kaposi's sarcoma-associated herpesvirus impairs oncogene-induced senescence. Cell Host Microbe 11, 167–180.
Liang, Q., Chang, B., Brulois, K.F., et al., 2013. Kaposi's sarcoma-associated herpesvirus K7 modulates Rubicon-mediated inhibition of autophagosome maturation. J. Virol. 87, 12499–12503.
Lussignol, M., Queval, C., Bernet-Camard, M.F., et al., 2013. The herpes simplex virus 1 Us11 protein inhibits autophagy through its interaction with the protein kinase PKR. J. Virol. 87, 859–871.
Marshall, E.E., Bierle, C.J., Brune, W., et al., 2009. Essential role for either TRS1 or IRS1 in human cytomegalovirus replication. J. Virol. 83, 4112–4120.
McFarlane, S., Aitken, J., Sutherland, J.S., et al., 2011. Early induction of autophagy in human fibroblasts after infection with human cytomegalovirus or herpes simplex virus 1. J. Virol. 85, 4212–4221.
Orvedahl, A., Alexander, D., Talloczy, Z., et al., 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin-1 autophagy protein. Cell Host Microbe 1, 23–35.
Orvedahl, A., MacPherson, S., Sumpter Jr., R., et al., 2010. Autophagy protects against Sindbis virus infection of the central nervous system. Cell Host Microbe 7, 115–127.
Paludan, C., Schmid, D., Landthaler, M., et al., 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307, 593–596.
Pattingre, S., Bauvy, C., Carpentier, S., et al., 2009. Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. J. Biol. Chem. 284, 2719–2728.
Pattingre, S., Tassa, A., Qu, X., et al., 2005. Bcl-2 antiapoptotic proteins inhibit Beclin-1-dependent autophagy. Cell 122, 927–939.
Pilli, M., Arko-Mensah, J., Ponpuak, M., et al., 2012. TBK-1 promotes autophagy-mediated antimicrobial defense by controlling autophagosome maturation. Immunity 37, 223–234.
Radtke, K., English, L., Rondeau, C., et al., 2013. Inhibition of the host translation shutoff response by herpes simplex virus 1 triggers nuclear envelope-derived autophagy. J. Virol. 87, 3990–3997.
Rasmussen, S.B., Horan, K.A., Holm, C.K., et al., 2011. Activation of autophagy by alpha-herpesviruses in myeloid cells is mediated by cytoplasmic viral DNA through a mechanism dependent on stimulator of IFN genes. J. Immunol. 187, 5268–5276.
Richetta, C., Faure, M., 2013. Autophagy in antiviral innate immunity. Cell Microbiol. 15, 368–376.
Richetta, C., Gregoire, I.P., Verlhac, P., et al., 2013. Sustained autophagy contributes to measles virus infectivity. PLoS Pathog. 9, e1003599.
Ritthipichai, K., Nan, Y., Bossis, I., et al., 2012. Viral FLICE inhibitory protein of rhesus monkey rhadinovirus inhibits apoptosis by enhancing autophagosome formation. PLoS ONE 7, e39438.
Sinha, S., Colbert, C.L., Becker, N., et al., 2008. Molecular basis of the regulation of Beclin-1-dependent autophagy by the gamma-herpesvirus 68 Bcl-2 homolog M11. Autophagy 4, 989–997.
Talloczy, Z., Jiang, W., Virgin, H.W.T., et al., 2002. Regulation of starvation- and virus-induced autophagy by the eIF2alpha kinase signaling pathway. Proc. Natl Acad. Sci. USA 99, 190–195.
Talloczy, Z., Virgin, H.W.T., Levine, B., 2006. PKR-dependent autophagic degradation of herpes simplex virus type 1. Autophagy 2, 24–29.

Tey, S.K., Khanna, R., 2012. Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway. Blood 120, 994–1004.
Wen, H.J., Yang, Z., Zhou, Y., et al., 2010. Enhancement of autophagy during lytic replication by the Kaposi's sarcoma-associated herpesvirus replication and transcription activator. J. Virol. 84, 7448–7458.
Yordy, B., Iijima, N., Huttner, A., et al., 2012. A neuron-specific role for autophagy in antiviral defense against herpes simplex virus. Cell Host Microbe 12, 334–345.
Yordy, B., Tal, M.C., Hayashi, K., et al., 2013. Autophagy and selective deployment of Atg proteins in antiviral defense. Int. Immunol. 25, 1–10.

CHAPTER

9

Autophagy Induced by Varicella-Zoster Virus and the Maintenance of Cellular Homeostasis

Charles Grose

OUTLINE

Abstract

Varicella-zoster virus (VZV) induces abundant autophagy in human cells. This human herpesvirus causes the childhood exanthematous disease known as varicella or chickenpox. Thereafter, VZV establishes a latent infection in the dorsal root ganglia; VZV subsequently reactivates and causes the dermatomal exanthem known as herpes zoster. Examination of VZV infected cells collected from these exanthems revealed the

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presence of numerous autophagosomes, after immunolabeling with anti-LC3 antibody and a fluoroprobe. Subsequent reconstruction of these Z-stacks of confocal microscopic images into a 3D animation by Imaris software facilitated enumeration of >100 autophagosomes per infected cell. Further visualization of infected cells documented a markedly enlarged endoplasmic reticulum (ER) as an indicator of ER stress. Evidence of the unfolded protein response (UPR) consisted of detecting by immunoblotting both the spliced variant of the X-box binding protein-1 (XBP1s) as well as the CCAAT/enhancer-binding protein homologous protein (CHOP). Thus, VZV infection leads to ER stress that is a precursor to autophagy through the induction of at least two of the three signal transduction UPR pathways: the IRE1 (inositol requiring enzyme-1) pathway and the PERK (PRK-like eukaryotic translation initiation factor 2a kinase) pathway.

INTRODUCTION

Autophagy is a subject of considerable research interest in virology (Deretic and Levine, 2009; Grose, 2010). The sentinel organelle of autophagy is the autophagosome (Dunn, 1990a,b). Autophagosomes are easily detected by confocal microscopy after immunolabeling with specific antibody probes. During most virus infections of humans, however, autophagosomes are induced in internal organs, sites not easily amenable to sampling and microscopic examination (Buckingham *et al.*, 2014; Carpenter and Grose, 2014). One notable exception is the disease varicella, also called chickenpox. Varicella is a common childhood illness throughout the world. The characteristic feature of varicella is a vesicular exanthem (Weller, 1983). This chapter will describe the detection and enumeration of autophagosomes within the exanthem. The overlying hypothesis is that autophagy acts to relieve cell stress and thereby maintain cellular homeostasis following infection with varicella-zoster virus (VZV), a response that also allows sufficient time for viral replication and assembly to occur before cell death.

VARICELLA-ZOSTER VIRUS

VZV is one of nine human herpesviruses. VZV is also called human herpesvirus 3. The herpesviruses most closely related to VZV include herpes simplex virus (HSV) type 1 and type 2. As common to all herpesviruses, VZV structure includes a capsid surrounded first by a tegument and then by an envelope. The diameter of the complete enveloped virion is around 200 nm. However, when grown in cultured cells, many viral particles have an aberrant appearance (Carpenter *et al.*, 2009). The double stranded DNA genome is housed within the capsid. The genome codes for approximately 70 open reading frames (Davison and Scott, 1986).

Complete sequencing of over 40 different VZV genomes has facilitated detection of single nucleotide polymorphisms (SNPs). In turn, extensive bioinformatics analysis has revealed that the SNPs separate into five clusters, which segregate roughly based on geography (Chow *et al.*, 2012). This clustering has led to the designation of five VZV clades, enumerated 1 through 5. Further clade analysis points to an out-of-Africa model as an explanation for the origin of the clades, based on co-evolution of VZV with humankind over past millennia.

With regard to autophagy, the VZV genome contains no known inhibitors of autophagy. In sharp contrast, the HSV-1 genome contains the inhibitory ICP34.5 protein (Chou *et al.*, 1990; Orvedahl *et al.*, 2007). The HSV ICP 34.5 protein is composed of 263 amino acids; these include a 159-residue N-terminal domain, ten repeats of the tripeptide AlaThrPro, followed by a 74-residue C-terminal domain. The C-terminus binds to protein phosphatase 1 alpha; in turn, this complex dephosphorylates eukaryotic translation factor 2 alpha. Of note, the C-terminus is closely homologous to the GADD34, the growth arrest and DNA damage-inducible protein. GADD34 functions as a sensor of endoplasmic reticulum stress, after which it interacts with a phosphatase to dephosphorylate the transcription factor. The final result is a feedback loop to recover protein syntheses within the cells.

THE DISEASE VARICELLA

The primary infection is called varicella or chickenpox (Grose, 1981). The virus is spread via aerosol. After an initial infection within an epithelial site on the face, the virus replicates locally, probably in the tonsillar tissues (Ross, 1962). The duration of viral replication within the tonsillar tissues is 4–6 days after which the viremia occurs. In an experimental model, both CD4+ and CD8+ T cells from tonsils were infected. The percentages of VZV infection were 20% for CD4+ cells and 13% for CD8+ cells. When examined by scanning electron microscopy (SEM), viral particles were easily detected on the surface of the lymphocytes. The virions were spherical and ranged in diameter from 150–200 nm.

Cell surface phenotypes of the T lymphocytes were investigated in order to determine relative VZV susceptibility (Ku *et al.*, 2002). Many tonsillar CD4+ lymphocytes were CD69+, including naïve (CD45RA+) as well as memory (CD45RA−) subpopulations. When the frequency of VZV infection was assessed between the latter two subsets, memory T cells were significantly more likely to be infected than naïve T cells. To further investigate the VZV susceptibility of the T cell subsets, memory T lymphocytes were analyzed based on expression of the transferrin receptor (CD71). CD71+ was detected in 10–15% of the memory CD4+ and CD69+ tonsillar cells. The percentage of VZV infection was higher in the CD4+ and CD71+ memory T cells. Since the final stage of VZV replication occurs on the skin, the expression on skin homing marker CLA (cutaneous leucocyte associated antigen) and CCR4 (chemokine receptor type 4) of an infected CD4+ cell was also ascertained. VZV-infected CD4+ T lymphocytes were more likely to have CLA and CCR4 markers. In one experiment, 21% of CCR4+ and CD4+ T lymphocytes were infected as compared with 9% in CD4+ lymphocytes lacking a CCR4 marker. These data clearly demonstrated that CD4+ cells with skin homing phenotypes were preferentially infected with VZV during the viremia phase of the infectious cycle.

CHARACTERISTIC EXANTHEMS OF VARICELLA AND HERPES ZOSTER

Varicella Exanthem

The viremia described above occurs during the first 2 weeks after transfer of infection from a child with acute varicella to a naïve host. Thereafter, the typical vesicular exanthem

of varicella is evident. The appearance of the exanthem marks the end of the incubation period of varicella. VZV has entered the epidermis via the CD4+ lymphocytes, which have extravasated from the capillaries within the skin. Viral inclusions are detectable in the endothelial cells lining the capillaries. The first changes in the epidermal cells consist of swelling of both the nuclei and cytoplasm. The swelling progresses to ballooning degeneration, a term describing greatly enlarged multinucleated skin cells. The characteristic VZV vesicles are formed by the exudation of clear fluid at discrete sites of degenerated epithelium. The chamber of each vesicle lies in the middle layer of the epidermis; the floor of each vesicle consists of numerous polykaryons induced by the fusogenic effects of VZV infection.

The exanthem typically is first seen on the face, often along the hair line. Thereafter, the rash emerges in successive crops over a 3–6 day period (Ross, 1962). The rash spreads from the face to the trunk and arms. Occasionally, the legs are spared. The average course of the exanthem ranges from 200–300 vesicles, although more than 500 vesicles are seen in severe cases of varicella.

Herpes Zoster Dermatomal Exanthem

After the primary disease varicella abates, VZV enters the sensory nerves in the skin and travels retrograde to the dorsal root ganglia. The virus establishes latency within the ganglia. The virus remains quiescent through several decades of adulthood. In late adulthood, however, the virus frequently reactivates and travels anterograde along the same nerve to the skin. The velocity of the viral particle within the nerve is 12 cm per day (Tannous and Grose, 2011). The dermatomal exanthem caused by replication of the reactivated VZV on the skin is known as herpes zoster or shingles. The basic pathological features of the exanthem after herpes zoster are similar to those after varicella. Typically, herpes zoster occurs only once in the lifetime of an otherwise healthy older adult.

AUTOPHAGY AND ITS VISUALIZATION BY CONFOCAL MICROSCOPY

Autophagy is induced through inhibition of the mammalian target of rapamycin (mTOR) under various conditions, e.g., starvation or infection (Kuma *et al.*, 2004). After induction of autophagy, the mTOR substrate complex (ULK1, ULK2, ATG13, FIP200 and ATG101) translocates from the cytosol to the ER, where the complex associates with vacuole membrane protein 1 (VMP1). This interaction activates the ER localized class III phosphatidylinositol-3-kinase PI(3)K, to generate phosphatidylinositol 3-phosphate PI3P (Klionsky *et al.*, 2007). This PI(3)K is a positive regulator of autophagy; the kinase complex includes VPS34 (PIK3C3 gene product), VPS15 (PIK3R4 gene product and p150), Beclin-1 and ATG14. The kinase complex recruits PI3P binding proteins including WIPIs on the ER membrane. WIPIs are WD-repeat proteins interacting with phosphoinositides, members of a larger PROPPIN family that bind to both PI(3)P and the vacuolar lipid PI(3,5)P2 (Baskaran *et al.*, 2012).

These described events initiate the nucleation and the remodeling of the phagophore (preautophagosomal) membranes. At the same time, the C-terminus of the microtubule-associated protein 1 light chain 3, abbreviated LC3, is cleaved by a protease Atg4 to produce

LC3-I, an 18 kDa protein that is distributed throughout the cytoplasm. During the subsequent stage of autophagosome formation, the double-membraned vacuolar structure elongates and closes, a step requiring the ATG12-ATG5 conjugate together with its partner ATG16LI. The latter complex also effects the conjugation of LC3-I with phosphatidylethanolamine (PE) to create the LC3-II isoform, which migrates slightly faster by SDS-PAGE (16 kd). Lipidated LC3-II is attached to both the inner and outer membranes of the maturing autophagosome. Thereafter, autophagosomes are easily identified by immunostaining of the LC3-II proteins with fluorescent probes; the distinctive autophagosomes are usually described as puncta. Subsequently, autophagosomes fuse with lysosomes to form autolysosomes. The cargo within the autophagosome is degraded by the lysosomally derived hydrolases. LC3-II isoforms located on the inner membrane are degraded while LC3-II isoforms on the outer membrane are recycled after delipidation by ATG4. At this point, in the absence of LC3-II, the autolysosome becomes a lysosome and is no longer detectable with LC3 antibody probes.

High-resolution imaging of autophagosomes is performed with an upright Zeiss LSM710 Spectral confocal microscope using 40× and 63× high numerical-aperture oil immersion objective lenses (Jackson *et al.*, 2013). Image size is set to either 512 × 512 or 1024 × 1024 pixels. Multitrack sequential acquisition settings are used to avoid interchannel crosstalk. Excitation occurs via a 561 nm diode-pumped solid-state laser and the 488 nm line of an argon ion laser. Optimized emission detection bandwidths are configured by Zeiss Zen control software. The confocal pinhole is set to 1 Airy unit. Z-stack acquisition intervals are selected to satisfy Nyquist sampling criteria.

In order to detect autophagosomes by confocal microscopy, a high avidity antibody probe is required. To this end, we evaluated anti-LC3 antibodies purchased from five companies (Figure 9.1A). We also examined the ability of each of the five antibodies to precipitate the LC3 complex. As mentioned above, the cytosolic LC3-I form (18 kd) is lipidated when it is inserted into the double membraned wall of the autophagosome, as the LC3-II form (16 kd) (Kabeya *et al.*, 2000). As shown in Figure 9.1B, all five antibodies precipitated the radiolabeled LC3 complex to varying degrees. In general, most antibodies precipitated more of the cytosolic 18 kd LC3-I form. We subsequently discovered that some commercial antibodies were better probes during confocal microscopy than other antibodies. Generally, the anti-LC3 antibodies that failed under conditions of confocal microscopy did detect the LC3 forms, according to technical data provided by the respective commercial bioreagent companies. Since detection can be greatly enhanced by peroxidase-labeled secondary antibodies during immunoblotting, the simplest explanation is that the anti-LC3 titer of some commercial antibodies is too low for optimal sensitivity under conditions of confocal microscopy but is adequate for immunoblotting.

AUTOPHAGOSOMES IN THE EXANTHEMS OF VARICELLA AND HERPES ZOSTER

In our experiments to detect autophagosomes in human tissues, we have selected both mouse and rabbit anti-LC3 monoclonal antibody (MAb) reagents as well as a rabbit polyclonal antibody (Carpenter *et al.*, 2011; Takahashi *et al.*, 2009). We have avoided reagents that

A. List of antibody reagents

Lane	Catalog	Company	Animal	Type
1	L7543	Sigma	Rabbit	Poly
2	sc-28266	Santa cruz	Rabbit	Poly
3	2057-1	Epitomics	Rabbit	Mono
4	38685	Cell signaling	Rabbit	Mono
5	M186-3	MBL	Mouse	Mono

B. Immunoprecipitations

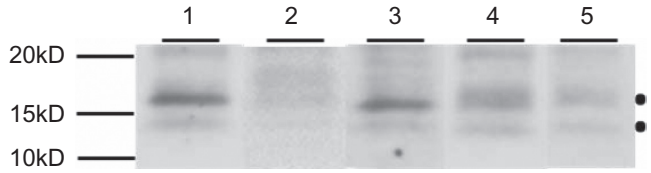

FIGURE 9.1 Comparative analysis of antibody reagents against the human LC3 protein. A. List of the antibody reagents. Column labeled type indicates whether the antibody is polyclonal or monoclonal. B. Immunoprecipitation of the LC3 protein. Antigen for immunoprecipitation was prepared from a VZV infected monolayer radiolabeled with 35[S] methionine/cysteine (1175 Curies/mmol). Immunoprecipitation and SDS-PAGE were carried out by procedures described by this laboratory (Montalvo and Grose, 1986). Bands representing the higher molecular weight LC3-I form and the lower molecular weight LC3-II form are indicated by closed circles in the right margin.

would lead to nonspecific detection of the human blood group A antigen (Zerboni *et al.*, 2012). When the vesicle cells were inspected by confocal microscopy after immunolabeling with anti-LC3 MAb, not all cells contained autophagosomes. Nevertheless, the positive cells contained large numbers of puncta. We found no major differences after examination of multiple sets of archived samples. The tally of autophagosomes within 20 vesicular cells from a representative 2D confocal micrograph showed a mean of 52 ± 9, with a range of 38 to 72.

Because of the potential of newer sophisticated 3D software programs for visualization of subcellular components, we postulated that we could extend the quantification data obtained from a 2D image of one slice of a cell to include a tally of all autophagosomes within an entire cell. To this end, we acquired a considerably larger number of confocal Z-stacks of our vesicle samples labeled with LC3 antibody. In turn, these data sets were loaded into Imaris software version 7.6 for a true 3D reconstruction of a single cell and subsequent analysis using the Imaris MeasurementPro module (Figure 9.2). We selected parameters based on both pixel intensity threshold and target object size, in order to identify putative superimposed autophagosomes within the maximum intensity projection images of the raw confocal data sets. Subsequently, 50 individual cells containing fluorescent structures were cropped from the data sets and then visualized within a boundary frame. Under these conditions of 3D enumeration, the average number of autophagosomes was 118 per

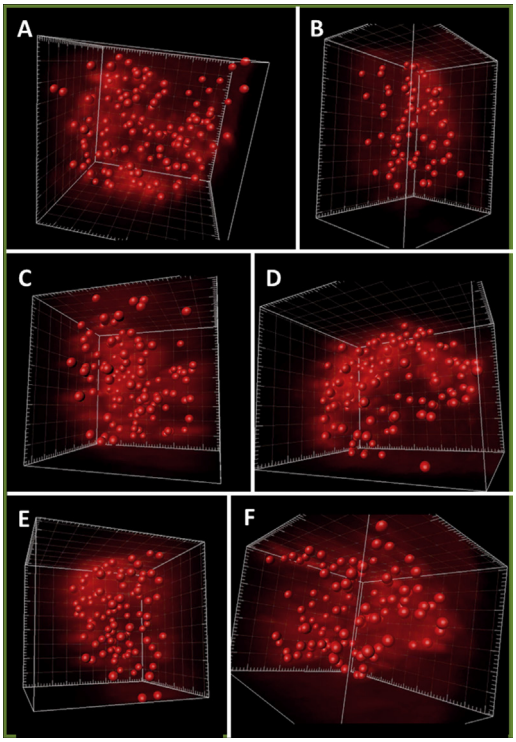

FIGURE 9.2 Reconstructed 3D images of autophagosomes in a VZV-infected cell. Vesicle cells from a varicella patient were immunolabeled with anti-LC3 antibody and a fluoroprobe as described in the text. A large Z-stack series was collected with a Zeiss LSM710 laser scanning microscope and 3D animations were assembled using Imaris software. Six individual 2D examples of the 3D animation are portrayed in panels A–F. Each red sphere represents an individual autophagosome within a single cell. To view the 3D animation, see supplemental video in the article by Jackson *et al.* (2013).

cell, although a few cells had over 200 (Jackson *et al.*, 2013). The features of autophagy in VZV-infected cells are very similar to those seen in human cells removed from an exanthem. In all likelihood, therefore, VZV studies in cultured cells accurately reflect events during natural infection of the human host. In short, autophagy within VZV-infected cultured cells is not an artifact of *in vitro* culture conditions.

EVIDENCE FOR ER STRESS AND UNFOLDED PROTEIN RESPONSE

In order to document ER stress in VZV-infected cells, we quantitated the increase in ER size during VZV infection (Carpenter *et al.*, 2011). For this analysis, we labeled the ER membranes with DiOC6, a dye that preferentially localizes to polar membranes. After taking a series of confocal images of the ER found within both uninfected and infected cells, we calculated that the ER size in VZV-infected cells was up to 10× larger than the ER size in

uninfected cultured cells. When visualized by electron microscopy, the enlarged ER structures in infected cells closely resembled enlarged ER structures previously associated with ER stress under other noninfectious conditions (Dorner *et al.*, 1989). This result complemented an earlier observation that viral glycoprotein biosynthesis is extremely abundant within 24 hpi (Grose, 1980). We also investigated ER stress and autophagic flux by measurement of the level of polyubiquitin-binding protein called p62, the product of the sequestosome 1 gene (SQSTM1) found on the long arm of human chromosome 5 (Takahashi *et al.*, 2009). The p62 protein may act as a receptor for cargo including ubiquitinated aggregates, to sort them to the autophagosome. Later, p62 and its cargo are specifically degraded by the autophagosome/lysosome pathway. When compared with uninfected cells, p62 levels were lower in VZV-infected cells when measured by immunoblotting, a result that suggested degradation of p62 as part of autophagic flux during VZV infection.

Any condition that leads to ER stress creates an imbalance that is relieved by one or more of three signal transduction UPR pathways (Schroder and Kaufman, 2005). When the IRE1 (inositol-requiring enzyme-1) pathway is activated, the endoribonuclease IRE1 effects cleavage of an intron from the X-box binding protein-1 (XBP1) mRNA to produce a spliced transcript that codes for a transcriptional activator of the UPR called the XBP1s protein. The alternative splicing of the XBP1 mRNA results in a translational frameshift in the C-terminus of the translated protein that exposes a basic leucine zipper (bZIP) transcription factor; the spliced XBP1s variant contains 115 more amino acids than the unspliced 261-residue protein. We observed that the bands for the spliced form, XBP1s, first became detectable at 48 hpi and were readily visible at both 72 and 96 hpi (Carpenter *et al.*, 2011). These results demonstrated unequivocally that the IRE1 arm of the UPR was upregulated in VZV-infected cells. We have also examined a second signal transduction pathway, PERK (PKR-like eukaryotic translation initiation factor 2a kinase). A transcription factor closely associated with the PERK UPR pathway under conditions of severe ER stress is CHOP or GADD153 (growth arrest and DNA damage 153 protein). Under experimental conditions similar to those required for detecting the XBP1s protein, we immunodetected CHOP in VZV infected cells (Carpenter *et al.*, 2011).

Our final important observations about the UPR during VZV infection were the fortuitous result of a completely independent set of experiments. The major VZV glycoprotein called gE (ORF68) had been purified by affinity chromatography, and the purified product was analyzed by Orbitrap mass spectrometry in an attempt to identify any viral binding partners. Instead of finding unexpected viral partners, we detected four cellular proteins co-purifying with gE that are associated with the UPR, including three heat shock proteins – HSPA5, HSPA8, and HSPD1 (Carpenter *et al.*, 2010).

Acknowledgments

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References

Baskaran, S., Ragusa, M.J., Hurley, J.H., 2012. How Atg18 and the WIPIs sense phosphatidylinositol 3-phosphate. Autophagy 8, 1851–1852.

Buckingham, E.M., Carpenter, J.E., Jackson, W., et al., 2014. Autophagy and the effects of its inhibition on varicella-zoster virus glycoprotein biosynthesis and infectivity. J. Virol. 88, 890–902.

Carpenter, J.E., Grose, C., 2014. Varicella-zoster virus glycoprotein expression differentially induces the unfolded protein response in infected cells. Frontiers Microbiol 5, 322.
Carpenter, J.E., Henderson, E.P., Grose, C., 2009. Enumeration of an extremely high particle to Pfu ratio for varicella zoster virus. J. Virol. 83, 6917–6921.
Carpenter, J.E., Jackson, W., Benetti, L., et al., 2011. Autophagosome formation during varicella-zoster virus infection following endoplasmic reticulum stress and the unfolded protein response. J. Virol. 85, 9414–9424.
Carpenter, J.E., Jackson, W., de Souza, G.A., et al., 2010. Insulin-degrading enzyme binds to the nonglycosylated precursor of varicella-zoster virus gE protein found in the endoplasmic reticulum. J. Virol. 84, 847–855.
Chou, J., Kern, E.R., Whitley, R.J., et al., 1990. Mapping of herpes simplex virus-1 neurovirulence to gamma 1 934.5, a gene nonessential for growth in culture. Science 250, 1262–1266.
Chow, V.T., Tipples, G.A., Grose, C., 2013. Bioinformatics of varicella-zoster virus: single nucleotide polymorphisms define clades and attenuated vaccine genotypes. Infect. Genet. Evol. 18, 351–356.
Davison, A.J., Scott, J.E., 1986. The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. 67, 1759–1816.
Deretic, V., Levine, B., 2009. Autophagy, immunity, and microbial adaptations. Cell Host Microbe 5, 527–549.
Dorner, A.J., Wasley, L.C., Kaufman, R.J., 1989. Increased synthesis of secreted proteins induces expression of glucose-regulated proteins in butyrate-treated Chinese hamster ovary cells. J. Biol. Chem. 264, 20602–20607.
Dunn Jr., W.A., 1990a. Studies on the mechanisms of autophagy: formation of the autophagic vacuole. J. Cell Biol. 110, 1923–1933.
Dunn Jr., W.A., 1990b. Studies on the mechanisms of autophagy: maturation of the autophagic vacuole. J. Cell Biol. 110, 1935–1945.
Grose, C., 1980. The synthesis of glycoproteins in human melanoma cells infected with varicella-zoster virus. Virology 101, 1–9.
Grose, C., 1981. Variation on a theme by Fenner: the pathogenesis of chickenpox. Pediatrics 68, 735–737.
Grose, C., 2010. Autophagy during common bacterial and viral infections of children. Pediatr. Infect. Dis. J. 29, 1040–1042.
Jackson, W., Yamada, M., Moninger, T., et al., 2013. Visualization and quantitation of abundant macroautophagy in virus-infected cells by confocal three-dimensional fluorescence imaging. J. Virol. Methods 193, 244–250.
Kabeya, Y., Mizushima, N., Ueno, T., et al., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19, 5720–5728.
Klionsky, D.J., Cuervo, A.M., Seglen, P.O., 2007. Methods for monitoring autophagy from yeast to human. Autophagy 3, 181–206.
Ku, C., Padilla, J., Grose, C., et al., 2002. Tropism of varicella-zoster virus for tonsillar CD4(+) lymphocytes that express activation, memory and skin homing markers. J. Virol. 76, 11425–11433.
Kuma, A., Hatano, M., Matsui, M., et al., 2004. The role of autophagy during the early neonatal starvation period. Nature 432, 1032–1036.
Montalvo, E.A., Grose, C., 1986. Varicella zoster virus glycoprotein gpI is selectively phosphorylated by a virus-induced protein kinase. Proc. Natl Acad. Sci. USA 83, 8967–8971.
Orvedahl, A., Alexander, D., Talloczy, Z., et al., 2007. HSV-1 ICP34.5 confers neurovirulence by targeting the Beclin 1 autophagy protein. Cell Host Microbe 1, 23–35.
Ross, A.H., 1962. Modification of chicken pox in family contacts by administration of gamma globulin. N. Engl. J. Med. 267, 369–376.
Schroder, M., Kaufman, R.J., 2005. The mammalian unfolded protein response. Annu. Rev. Biochem. 74, 739–789.
Takahashi, M.N., Jackson, W., Laird, D.T., et al., 2009. Varicella-zoster virus infection induces autophagy in both cultured cells and human skin vesicles. J. Virol. 83, 5466–5476.
Tannous, R., Grose, C., 2011. Calculation of the anterograde velocity of varicella-zoster virions in a human sciatic nerve during shingles. J. Infect. Dis. 203, 324–326.
Weller, T.H., 1983. Varicella and herpes zoster. Changing concepts of the natural history, control, and importance of a not-so-benign virus. N. Engl. J. Med. 309, 1434–1440.
Zerboni, L., Sobel, R.A., Lai, M., et al., 2012. Apparent expression of varicella-zoster virus proteins in latency resulting from reactivity of murine and rabbit antibodies with human blood group a determinants in sensory neurons. J. Virol. 86, 578–583.

CHAPTER

10

Autophagy and Hepatitis B Virus

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OUTLINE

Abstract

Autophagy is a catabolic process by which long-lived proteins and damaged organelles are removed from cells. It is important for maintaining cellular homeostasis and also can be used by cells to remove intracellular microbial pathogens. Although autophagy suppresses the replication of some viruses, it enhances the replication of others including hepatitis B virus (HBV). HBV is a hepatotropic virus that can cause hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). Recent studies indicated that HBV could activate the autophagic pathway without inducing the autophagic protein degradation. This activation of the autophagic pathway by HBV may be due to the activities of its X protein (HBx) and/or the induction of endoplasmic reticulum (ER) stress and plays a positive role in HBV replication. In this chapter, we discuss the molecular pathways of HBV-induced autophagy and how autophagy impacts HBV replication. In addition, we also discuss the possible effect of autophagy on HBV-induced hepatocarcinogenesis.

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INTRODUCTION

Macroautophagy, hereafter referred to as autophagy, is an evolutionarily conserved catabolic process found in all eukaryotic cells. During autophagy, double-membrane vesicles, known as autophagosomes, are generated to sequester part of the cytoplasm that may contain damaged organelles, long-lived proteins and other protein aggregates (Sir and Ou, 2010). These autophagosomes will subsequently fuse with lysosomes to form autolysosomes, in which the cargos of autophagosomes are digested by lysosomal enzymes and recycled (Levine and Kroemer, 2008; Mizushima, 2007). Autophagy may be induced by a variety of factors including nutrient starvation and microbial infections (He *et al.*, 2009). Many cellular factors that regulate autophagy have been identified. The class III phosphatidylinositol-3-kinase (PI3KC3), which phosphorylates phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3P), is important for the initiation of autophagy. PI3KC3 consists of a catalytic subunit Vps34 and a regulatory subunit Vps15. Additional factors that participate in the autophagic process also include the two ubiquitin-like conjugation systems that mediate the conjugation of Atg12 to Atg5 and the conjugation of phosphatidylenthanolamine to LC3, a microtubule-associated protein. Autophagy can also be negatively regulated, such as by the mammalian target of rapamycin (mTOR) and the B-cell chronic lymphocytic leukemia/lymphoma 2 (Bcl-2) protein (Kroemer *et al.*, 2010).

Autophagy plays an important role in maintaining cellular homeostasis, as it prevents the accumulation of protein aggregates and damaged organelles in the cell (Mizushima, 2007). A defect in the autophagic pathway can cause a variety of diseases including cancers, neurodegenerative diseases, cardiovascular diseases, metabolic diseases, pulmonary diseases, and aging (Choi *et al.*, 2013; He *et al.*, 2009).

Autophagy can also serve as innate immunity to remove intracellular microbial pathogens. This process is called xenophagy. For that reason, a defect in autophagy can increase the susceptibility of the host to microbial infections. However, autophagy is not always antimicrobial, as many viruses have evolved mechanisms to use this pathway to benefit their own replications (Dong and Levine, 2013; Sir and Ou, 2010). In this review, we will focus on the relationship between hepatitis B virus (HBV) and autophagy and discuss how HBV induces autophagy and uses it to benefit its own replication and how autophagy may affect HBV-induced hepatocarcinogenesis.

THE HBV LIFE CYCLE

HBV is an important human pathogen that can cause severe liver diseases including acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. There are two billion people in the world that have been exposed to this virus and among them, 350 million people failed to clear the viral infection and are chronic HBV carriers. HBV belongs to the hepadnavirus family. It is an enveloped virus with a circular and partially double-stranded DNA genome of about 3.2 Kb (Figure 10.1). The HBV genome contains four genes named C, S, P and X. The C gene codes for the core protein and a related protein termed the precore protein (Figure 10.1). The core protein forms the viral core particle and the precore protein is the precursor of the e antigen found in the serum of HBV patients. The S gene codes for

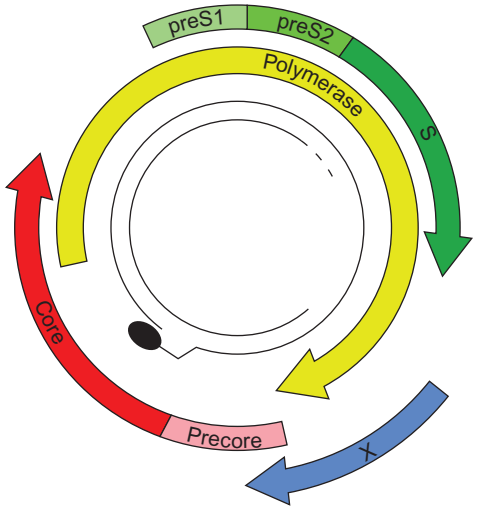

FIGURE 10.1 Genomic organization of hepatitis B virus. The S gene codes for the three co-carboxy-terminal surface (envelope) proteins. The C gene codes for the precore protein and the core protein. The P gene codes for the viral DNA polymerase. This protein is covalently linked to the 5′-end of the long strand of the HBV genomic DNA (denoted by a solid circle). The X gene codes for the regulatory protein HBx.

the three co-carboxy-terminal envelope proteins termed large (LHBs), middle (MHBs) and small surface antigens (SHBs) or pre-S1, pre-S2 and major S proteins. The P gene codes for the viral DNA polymerase, which is also a reverse transcriptase. This protein is covalently linked to the 5′-end of the long strand of the HBV DNA (Figure 10.1). The X gene codes for a 17-kDa regulatory protein. The expression of the HBV genes is controlled by four different promoters and two enhancer elements.

The HBV infection of hepatocytes is initiated by the binding of HBV to the cell surface heparan sulfate proteoglycans (Schulze *et al.*, 2007). This is followed by the binding of the virus to high-affinity receptors. A recent study identified the sodium taurocholate cotransporting polypeptide (NTCP) as a receptor for HBV (Yan *et al.*, 2012). After binding to its receptor, HBV is internalized into hepatocytes through a mechanism that is still poorly understood. This internalization process results in the release of the viral core particle, which then transports HBV DNA into the nucleus where it is converted to the fully double-stranded and covalently closed circular DNA (cccDNA) (Figure 10.2). This cccDNA then serves as the template to direct the transcription of viral mRNAs. The core protein mRNA is larger than the genome size and is also called the pregenomic RNA (pgRNA). It codes for the core protein as well as the viral DNA polymerase. After the synthesis of the core protein, it packages the pgRNA to form the core particle, in which the pgRNA is converted to the partially double-stranded viral genome by the viral DNA polymerase that is also packaged. The mature core particle, which contains the viral genomic DNA, may transport the viral genome back to the nucleus to amplify the cccDNA pool or interact with viral envelope proteins on intracellular membranes to form progeny virions for release from infected cells (Beck and Nassal, 2007; Sir *et al.*, 2010a).

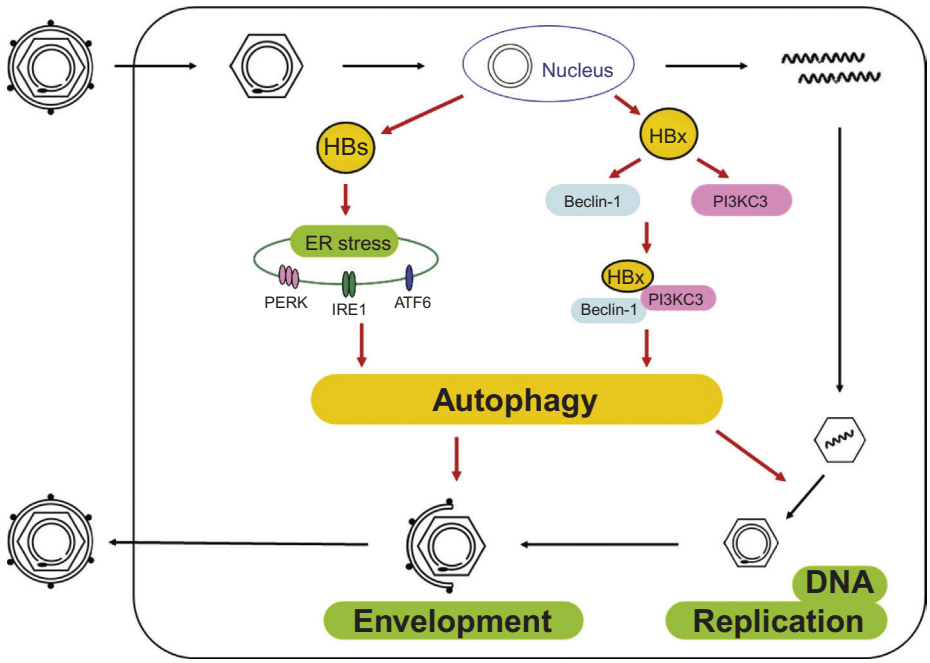

FIGURE 10.2 Effects of HBV and autophagy on each other. After the infection of hepatocytes, HBV genomic DNA is delivered to the nucleus where it is repaired to mediate the transcription of HBV mRNAs. HBV may induce autophagy via its HBx protein to induce the expression of Beclin-1 or directly bind to and activate PI3KC3. It may also induce autophagy via ER stress, which may be induced by its HBs proteins. Autophagy may then enhance HBV DNA replication and/or viral envelopment to facilitate the release of progeny viral particles.

MECHANISM OF HBV-INDUCED AUTOPHAGY

HBV has been shown to induce autophagy in cell cultures, in the liver of transgenic mice that carry the entire HBV genome, and during natural infection (Li *et al.*, 2011; Sir *et al.*, 2010b; Wang *et al.*, 2010). However, different mechanisms regarding how HBV may induce autophagy have been proposed. It has been reported that the HBV X protein (HBx), a regulatory protein, could activate the Beclin-1 promoter to induce the expression of Beclin-1 (Tang *et al.*, 2009), which can activate the PI3KC3 complex. This induction of Beclin-1 by HBx enhanced autophagy when cells were nutrient starved (Tang *et al.*, 2009). In a separate study, Sir *et al.* did not observe the induction of Beclin-1 by HBV or the HBx protein (Sir *et al.*, 2010b). However, they found that HBx, whether by itself or when it was expressed from the HBV DNA genome, could bind to and activate PI3KC3. This activation of PI3KC3 led to the production of a high level of phosphatidylinositol-3-phosphate (PtdIns(3)P) in cells and an increased amount of autophagic vacuoles including autophagosomes and autolysosomes. Interestingly, this increase of autophagic vacuoles by HBx did not lead to an increase of the autophagic protein degradation rate, suggesting that the increased amount of autophagic vacuoles induced by HBx did not sequester more cellular proteins or organelles for degradation (Sir *et al.*, 2010b). The induction of autophagic vacuoles was not only observed in cells that expressed

HBx, but also in cells productively replicating HBV and in the liver of transgenic mice that carried the complete HBV genome (Sir *et al.*, 2010b). However, it was not observed in mice carrying the HBV genomic mutant that was not capable of expressing HBx (Sir *et al.*, 2010b).

The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) can lead to ER stress, which can activate IRE1, PERK and ATF6 to result in the activation of a cascade of downstream signaling events collectively called the unfolded protein response (UPR). HBV has also been shown to use this pathway to induce autophagy without promoting the autophagic protein degradation (Li *et al.*, 2011) (Figure 10.2). It was found that this induction of ER stress and UPR was mediated by SHBs, and the effect of SHBs on autophagy was abolished if the expression of any of the three sensors of the ER stress (i.e., IRE1, PERK and ATR6) was suppressed with siRNAs (Li *et al.*, 2011). The HBV genomic mutant incapable of expressing SHBs could not activate UPR and induce autophagy. Although the activation of UPR can induce autophagy (Hoyer-Hansen and Jaattela, 2007; Kouroku *et al.*, 2007; Ogata *et al.*, 2006; Yorimitsu *et al.*, 2006), and hepatitis C virus has been shown to induce autophagy via this mechanism (Ke and Chen, 2011; Sir *et al.*, 2008), the finding that HBV could use SHBs to induce ER stress and autophagy was rather surprising. This is because previous studies on HBV indicated that HBx, LHBs and the LHBs mutant with a deletion in the pre-S2 region could induce the ER stress (Hsieh *et al.*, 2004; Li *et al.*, 2007; Xu *et al.*, 1997). In contrast, SHBs was not known to induce ER stress and, instead, a reduction of its expression could actually cause the accumulation of LHBs in the ER lumen (Ou and Rutter, 1987; Persing *et al.*, 1986), resulting in the induction of ER stress and hepatocellular injury (Chisari *et al.*, 1989; Xu and Yen, 1996). LC3 is a cytosolic protein. It is covalently linked to phosphatidylethanolamine during autophagy and this lipidation process has been frequently used as a marker for autophagy. A recent report, which did not detect any effect of SHBs on the lipidation of LC3 (Lazar *et al.*, 2012), did not support the role of SHBs in the induction of autophagy. Further studies will be required to understand this unusual finding of Li *et al.* and the possible role of HBV surface proteins in the induction of autophagy.

Besides HBV gene products, HBV genotypes may also affect the ability of HBV to induce autophagy. It was found that HBV genotype C virus was more potent than genotype B virus in the induction of autophagy (Wang *et al.*, 2010). HBV genotype C is associated with a more severe liver disease outcome than genotype B (Lin and Kao, 2008). It is unclear whether the increased virulence of genotype C HBV is related to its increased ability to induce autophagy.

AUTOPHAGY ON HBV REPLICATION

Autophagy plays a positive role in the replication of HBV, as the inhibition of autophagy led to the reduction of HBV replication in cells (Li *et al.*, 2011; Sir *et al.*, 2010a,b). Vps34 is the catalytic component of PI3KC3 and Atg7 is critical for the lipidation of LC3 (Tanida *et al.*, 2012). The suppression of the expression of either one of these two proteins with the siRNA resulted in the inhibition of HBV DNA replication with only a slight effect on HBV RNA transcription, protein synthesis or the packaging of the pgRNA into the core particle (Sir *et al.*, 2010b). Similar results were obtained if the activity of PI3KC3 was inhibited using its inhibitor 3-methyladenine (Sir *et al.*, 2010b). This finding was supported by the studies conducted using HBV transgenic mice, which carried the entire HBV genome and actively replicated HBV in the liver (Xu *et al.*, 2002). The liver-specific knockout of Atg5, a gene essential

for the formation of autophagosomes, in these transgenic mice abolished autophagy in the liver and significantly reduced the HBV titer in the mouse blood (Tian *et al.*, 2011). Further analysis revealed that this Atg5 knockout abolished the replication of HBV DNA in the mouse liver with only a slight effect on HBV RNA transcription and protein synthesis (Tian *et al.*, 2011). How autophagy is involved in HBV DNA replication is unclear. However, it does have a profound effect on the subcellular localization of the HBV core protein, as the HBV core protein was localized primarily to the nuclei of the hepatocytes in wild-type mice but it was diffusely localized to the cytoplasm in the hepatocytes of mice with liver-specific knockout of Atg5 (Tian *et al.*, 2011). Curiously, in the studies conducted by Li *et al.*, it was shown that the inhibition of autophagy with 3-methyladenine or with siRNA knockdown of either Beclin-1 or Atg5 had little effect on HBV RNA and DNA syntheses, but it inhibited the release of enveloped viral particles. Based on the observation that SHBs colocalized with the autophagosomes and co-immunoprecipitated with LC3, they suggested that HBV might use autophagosomes as the scaffold for viral envelopment (Li *et al.*, 2011). The possibility that HBV may use autophagosomes for viral envelopment certainly requires further investigation, as the report by Sir *et al.* also indicated the colocalization of both HBV core/precore protein and surface proteins with autophagic vacuoles (Sir *et al.*, 2010b). It is important to note, however, that this localization of HBV core and surface proteins to autophagic vacuoles might not be due to the need of the virus to use these membrane vesicles as the sites for viral envelopment, but rather, it might be due to the involvement of autophagy in the removal of HBV surface proteins. It has recently been demonstrated that HBV could activate the ER-associated degradation (ERAD) pathway and induce the expression of ER degradation-enhancing mannosidase-like proteins (EDEMs), which then mediate the degradation of HBV surface proteins apparently via the autophagic pathway (Lazar *et al.*, 2012).

It is unclear why autophagy was found not to affect HBV DNA replication in the report by Li *et al.* (2011), as it was found to affect HBV DNA replication both in cell cultures and in HBV transgenic mice with liver-specific knockout of Atg5, which abolished autophagy in hepatocytes (Sir *et al.*, 2010b; Tian *et al.*, 2011). One possible explanation to this discrepancy is the use of different HBV strains by different laboratories. Most of the studies conducted by Li *et al.* used Huh7 hepatoma cells. HBx is dispensable for the replication of HBV in this cell line and the suppression of autophagy had no effect on viral DNA replication of the HBx-negative HBV mutant in this cell line (Sir *et al.*, 2010b). It will be interesting to test whether the HBx expressed by the HBV strain used by Li *et al.* has altered biological activities. If it does, it will provide an explanation for why the HBx expressed from their HBV strain did not have a significant effect on autophagy. Nevertheless, the lack of effect of autophagy on the replication of the HBx-negative HBV mutant in Huh7 cells suggested that HBV was also able to replicate in an autophagy-independent manner under certain conditions.

AUTOPHAGY AND HBV-INDUCED HEPATOCARCINOGENESIS

Autophagy plays an important role in maintaining cellular homeostasis and its dysfunction has been linked to a variety of diseases including cancer. The chronic HBV infection can lead to liver cirrhosis and hepatocellular carcinoma. It is unclear whether the ability of HBV to activate the autophagic pathway without increasing autophagic protein degradation contributed

to HBV pathogenesis. However, a recent report indicated that autophagy was downregulated in HBV-associated HCC and there was an inverse correlation between the autophagic activity and the level of microRNA-224 (miR-224) in these tumor tissues (Lan *et al.*, 2013). Interestingly, such downregulation of autophagy and the inverse correlation between autophagy and miR-224 were not observed in HCV-associated HCC (Lan *et al.*, 2013). Further studies by the authors indicated that miR-224 could be sequestered in autophagosomes and subsequently degraded in the autophagic pathway. The increase of miR-224 led to the suppression of expression of its target gene Smad4 (Lan *et al.*, 2013), a transcription factor of the TGF-β signaling pathway. Since the suppression of Smad4 expression could convert TGF-β from a tumor suppressor to a tumor promoter (Zhang *et al.*, 2010), and miR-224 could enhance the tumorigenicity of hepatoma cells in NOD/SCID mice via the silencing of Smad6 (Lan *et al.*, 2013), it was concluded that miR-224 promoted HBV carcinogenesis through the silencing of Smad4 (Lan *et al.*, 2013). The inhibition of autophagy in HBV-associated HCC was apparently due to the reduction of the expression of Atg5 and Beclin-1, which led to the increase of p62, a protein that is removed by autophagy. How HBV participated in the suppression of Atg5 and Beclin-1 during tumorigenesis is not clear, although based on the observation that the liver tumors developed in transgenic mice that carried the HBV X gene also had reduced expression levels of Atg5 and increased levels of p62 and miR-224, it is conceivable that HBx alone was sufficient to reduce the expression of Atg5 to inhibit autophagy during tumorigenesis. As the protein levels of Atg5 and Beclin-1 were abundant in nontumor liver tissues of HBV patients or HBx-transgenic mice, and HBV induced rather than suppressed autophagy in cultured hepatoma cells, the effect of HBV or HBx on Atg5 and Beclin-1 was limited to tumor tissues *in vivo*. For that reason, it is likely that the reduction of Atg5 and Beclin-1 expression by HBV during HBV tumorigenesis in patients involves specific factors in the tumor microenvironment.

CONCLUSION

Studies up to now have indicated that HBV could induce autophagy without increasing the autophagic protein degradation. However, different mechanisms regarding how HBV may induce autophagy have been proposed. These mechanisms, which may be through the activities of the HBx protein or through the induction of ER stress, are illustrated in Figure 10.2. Similarly to a number of RNA viruses such as HCV and poliovirus, HBV induces autophagy to enhance its own replication. Although autophagy enhances HBV replication, it may also suppress HBV-induced hepatocarcinogenesis, as the HBV-associated HCC had reduced autophagic activity and increased tumorigenicity. These findings indicate that there are interesting interplays between autophagy and HBV and between autophagy and HBV carcinogenesis.

References

Beck, J., Nassal, M., 2007. Hepatitis B virus replication. World J. Gastroenterol. 13, 48–64.

Chisari, F.V., Klopchin, K., Moriyama, T., et al., 1989. Molecular pathogenesis of hepatocellular carcinoma in hepatitis B virus transgenic mice. Cell 59, 1145–1156.

Choi, A.M., Ryter, S.W., Levine, B., 2013. Autophagy in human health and disease. N. Engl. J. Med. 368, 651–662.

Dong, X., Levine, B., 2013. Autophagy and viruses: adversaries or allies? J. Innate Immun. 5, 480–493.

He, C., Bassik, M.C., Moresi, V., et al., 2009. Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature 481, 511–515.

Hoyer-Hansen, M., Jaattela, M., 2007. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell Death Differ. 14, 1576–1582.
Hsieh, Y.H., Su, I.J., Wang, H.C., et al., 2004. Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage. Carcinogenesis 25, 2023–2032.
Ke, P.Y., Chen, S.S., 2011. Activation of the unfolded protein response and autophagy after hepatitis C virus infection suppresses innate antiviral immunity in vitro. J. Clin. Invest. 121, 37–56.
Kouroku, Y., Fujita, E., Tanida, I., et al., 2007. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 14, 230–239.
Kroemer, G., Marino, G., Levine, B., 2010. Autophagy and the integrated stress response. Mol. Cell 40, 280–293.
Lan, S.H., Wu, S.Y., Zuchini, R., et al., 2013. Autophagy suppresses tumorigenesis of hepatitis B virus-associated hepatocellular carcinoma through degradation of miR-224. Hepatology 59 (2), 505–517.
Lazar, C., Macovei, A., Petrescu, S., et al., 2012. Activation of ERAD pathway by human hepatitis B virus modulates viral and subviral particle production. PLoS ONE 7, e34169.
Levine, B., Kroemer, G., 2008. Autophagy in the pathogenesis of disease. Cell 132, 27–42.
Li, B., Gao, B., Ye, L., et al., 2007. Hepatitis B virus X protein (HBx) activates ATF6 and IRE1-XBP1 pathways of unfolded protein response. Virus Res. 124, 44–49.
Li, J., Liu, Y., Wang, Z., et al., 2011. Subversion of cellular autophagy machinery by hepatitis B virus for viral envelopment. J. Virol. 85, 6319–6333.
Lin, C.L., Kao, J.H., 2008. Hepatitis B viral factors and clinical outcomes of chronic hepatitis B. J. Biomed. Sci. 15, 137–145.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.
Ogata, M., Hino, S., Saito, A., et al., 2006. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol. Cell Biol. 26, 9220–9231.
Ou, J.H., Rutter, W.J., 1987. Regulation of secretion of the hepatitis B virus major surface antigen by the preS-1 protein. J. Virol. 61, 782–786.
Persing, D.H., Varmus, H.E., Ganem, D., 1986. Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science 234, 1388–1391.
Schulze, A., Gripon, P., Urban, S., 2007. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology 46, 1759–1768.
Sir, D., Ou, J.H., 2010. Autophagy in viral replication and pathogenesis. Mol. Cells 29, 1–7.
Sir, D., Chen, W.L., Choi, J., et al., 2008. Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. Hepatology 48, 1054–1061.
Sir, D., Ann, D.K., Ou, J.H., 2010a. Autophagy by hepatitis B virus and for hepatitis B virus. Autophagy 6, 548–549.
Sir, D., Tian, Y., Chen, W.L., et al., 2010b. The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc. Natl. Acad. Sci. USA 107, 4383–4388.
Tang, H., Da, L., Mao, Y., et al., 2009. Hepatitis B virus X protein sensitizes cells to starvation-induced autophagy via up-regulation of beclin 1 expression. Hepatology 49, 60–71.
Tanida, I., Yamasaki, M., Komatsu, M., et al., 2012. The FAP motif within human ATG7, an autophagy-related E1-like enzyme, is essential for the E2-substrate reaction of LC3 lipidation. Autophagy 8, 88–97.
Tian, Y., Sir, D., Kuo, C.F., et al., 2011. Autophagy required for hepatitis B virus replication in transgenic mice. J. Virol. 85, 13453–13456.
Wang, J., Shi, Y., Yang, H., 2010. Infection with hepatitis B virus enhances basal autophagy. Wei Sheng Wu Xue Bao (Chinese) 50, 1651–1656.
Xu, Z., Jensen, G., Yen, T.S., 1997. Activation of hepatitis B virus S promoter by the viral large surface protein via induction of stress in the endoplasmic reticulum. J. Virol. 71, 7387–7392.
Xu, Z., Yen, T.S., 1996. Intracellular retention of surface protein by a hepatitis B virus mutant that releases virion particles. J. Virol. 70, 133–140.
Xu, Z., Yen, T.S., Wu, L., et al., 2002. Enhancement of hepatitis B virus replication by its X protein in transgenic mice. J. Virol. 76, 2579–2584.
Yan, H., Zhong, G., Xu, G., et al., 2012. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife 1, e00049.
Yorimitsu, T., Nair, U., Yang, Z., et al., 2006. Endoplasmic reticulum stress triggers autophagy. J. Biol. Chem. 281, 30299–30304.
Zhang, B., Halder, S.K., Kashikar, N.D., et al., 2010. Antimetastatic role of Smad4 signaling in colorectal cancer. Gastroenterology 138 (969–80), e1–e3.

CHAPTER

11

Toll-Like Receptors Serve as Activators for Autophagy in Macrophages Helping to Facilitate Innate Immunity

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OUTLINE

Abstract

Toll-like receptors (TLRs) play critical roles in host defense by recognizing specific molecular patterns from a wide variety of pathogens. Using a small set of adaptor proteins TLR engagement leads to the activation of nuclear factor-κB (NF-κB) and interferon regulatory factors (IRFs). This results in the upregulation of downstream target genes including an array of pro-inflammatory cytokines, chemokines, and interferon-responsive genes. In macrophages TLR signaling also induces autophagy. Autophagy is a cellular response to starvation that helps remove damaged organelles and long-lived proteins from the cytosol. It also has a cytoprotective function helping to limit the replication of intracellular pathogens. Many pathogens target the autophagy pathway to thwart its effectiveness. This review focuses on how TLRs can activate autophagosome formation in macrophages and briefly reviews the role of autophagy in innate immunity.

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INTRODUCTION

The innate immune system provides a first line of defense against the invasion of pathogens (Kumar *et al.*, 2011). Unlike the adaptive immune system, the cells of the innate system recognize and respond to pathogens via generic receptors, which are encoded in the host's germline. This contrasts with the adaptive immune system where pathogen-specific receptors are acquired during the lifetime of the organism by processes that depend upon genomic rearrangements. As an initial defense system, innate immune cells and their components exhibit a fast, constitutive, antigen nonspecific response with minimal memory of past exposures. Adaptive immune reactions develop slower, exhibit specificity for pathogens and pathogenic antigens, and possess memory of previous exposures. While the innate immune system constitutes an evolutionarily older defense strategy considerable cross-talk and mutual communication between these two arms of the immune system lead to an integrated immune response.

The innate immune system consists of three main components: anatomical barriers, humoral barriers, and cellular barriers. For example, the skin acts as a physical barrier to prevent invading microorganisms from getting access to the interior of the body. The movement of cilia in the gastrointestinal and respiratory tracts is another anatomic defensive barrier. Humoral barriers include chemicals such as lysozyme and phospholipase found in tears, saliva and mucus that inhibit the growth of bacteria. Once an invader passes these barriers or a tissue injury occurs, acute inflammatory responses are initiated. These include activation of the complement system, coagulation system, and the production of certain cytokines and proteins possessing antimicrobial properties. Numerous cells are recruited to the site of inflammation and/or infection for the defense of the host. These cells form the third barrier and they include macrophages, monocytes, neutrophils, natural killer cells, and eosinophils.

Macrophages are a front-line component of host defense. They participate in innate immunity, but also help mobilize adaptive immune responses. Macrophages as other cells of the innate immune system recognize pathogens via a large repertoire of extracellular and intracellular receptors/sensors (Kawai and Akira, 2010). These germline-encoded receptors termed pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) displayed by bacteria, viruses, parasites and fungi. Among the PAMPs are microorganism-derived lipids, nucleic acids, proteins, lipoproteins, and glycans. The PRRs link to downstream signaling pathways that trigger innate immune responses. Prominent among these are the induction of NF-κB target genes and interferon-responsive genes. Induced genes include those that encode chemokines and cytokines. These serve as alarms to recruit and activate nearby leukocytes to promote and control the inflammatory response (Newton and Dixit, 2012). The engagement of some PRRs can activate inflammasomes, which trigger IL-1β and IL-18 secretion, also helping to mobilize innate immune defenses. Macrophages detect pathogens not only via PRR receptors, but also by receptors that recognize opsonized pathogens such as Fc and complement receptors. These receptors can trigger phagocytosis, whereby macrophages and other phagocytic cells internalize extracellular material including microorganisms within membrane bound vesicles termed phagosomes. The phagocytized material can be degraded by the fusion of the phagosomes with late endosomes and/or lysosomes. If microorganisms breech the plasma membrane invading the cytosol, or if they escape from phagosomes into the cytosol, they can be targeted by autophagy.

A primordial form of eukaryotic innate immunity, autophagy allows the capture and disposal of intracellular pathogens. Autophagy sequesters not only invading pathogens, but also damaged intracellular organelles and misfolded proteins. These targets are enclosed within a double-membrane-bound compartment termed an autophagosome, which delivers its contents to lysosomes for degradation (Yang and Klionsky, 2010). Besides eliminating pathogens both phagocytosis and autophagy deliver foreign material for antigen presentation, an initial step in the elicitation of adaptive immunity. Exposure of macrophages to PRRs can serve as a trigger for autophagosome formation. This provides a link between macrophage pathogen recognition and the activation of an intracellular host defense mechanism.

TOLL-LIKE RECEPTORS

Toll-like receptors (TLRs) are PRRs localized either on the cell surface or on the lumen of intracellular vesicles. TLRs are type I transmembrane proteins consisting of three parts. The extracellular portion of TLRs containing leucine-rich repeats, which are responsible for the recognition of PAMPs; the transmembrane domain; and the intracellular Toll/interleukin-1 (IL-1) receptor (TIR) domains, which recruit downstream adaptors and effectors that mediate downstream signaling (Kawai and Akira, 2010). Thirteen TLRs have been identified in mice and ten in humans. The cellular localizations of specific TLRs are as follows: TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are localized on the plasma membrane and recognize lipids, lipoproteins, and proteins. TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles such as endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes and largely detect microbial nucleic acids. Each particular TLR recognizes specific conserved structures in pathogens, triggering specific innate immune responses and helping to prime antigen-specific adaptive immunity (Pasare and Medzhitov, 2005).

A broad array of PAMPs such as lipopeptides, peptidoglycan, lipoteichoic acid, zymosan derived from bacteria, fungi, parasites and viruses are recognized by TLR2, which forms heterodimers with other TLRs (e.g., TLR1, TLR6). The heterodimerization confers specificity for PAMP recognition. Lipopolysaccharide (LPS), a major bacterial signature molecule found on the outer membrane of Gram-negative bacteria, is recognized by TLR4. TLR5 recognizes flagellin, a globular protein that is the principal substituent of bacterial flagella and is found in most flagellated bacteria. TLR3 detects viral double-stranded RNA (dsRNA) and polyinosinic-polycytidylic acid, poly(I:C), a synthetic analogue commonly used experimentally. TLR7 and human TLR8 detect viral single-stranded RNAs (ssRNAs), imidazoquinoline derivatives such as imiquimod and resiquimod (R-848), and various guanine analogues. TLR9 recognizes unmethylated *CpG* motifs prevalent in microbial, but not in vertebrate genomic DNA.

When PAMPs are sensed by TLRs, their engagement leads to the recruitment of adaptor proteins and the orchestration of different signal transduction pathways leading to distinct biological responses (West *et al.*, 2006). The diversity of the response is related to the adaptor proteins, which contain a TIR domain and are recruited to distinct TLRs as downstream effectors. The adaptor proteins include myeloid differentiation factor 88 (MyD88), TIR-domain-containing adaptor-inducing interferon-β (TRIF), MyD88 adaptor-like (Mal), also termed TIRAP, TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo motif-containing protein (SARM). MyD88 is recruited by all TLRs except TLR3 and activates

transcription factor NF-κB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines. TRIF is recruited by TLR3 and TLR4 and results in activation of IRF3 and NF-κB and the consequent induction of type I interferon and inflammatory cytokines.

Among the TLR adaptors MyD88 is particularly critical. It is a death domain (DD)-containing cytosolic protein, which is recruited to activated TLRs and takes a hexameric form leading to further recruitment of DD-containing kinases, IL-1 receptor (IL-1R)-associated kinase 1 (IRAK1) and IRAK4 (Into *et al.*, 2012). Activation of IRAKs through phosphorylation increases the association with an E3 ubiquitin ligase and scaffolding protein named tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). TRAF6 catalyzes K63-linked polyubiquitination of IRAK-1 and TRAF6 auto-ubiquitination. TRAF6 then binds through these ubiquitin proteins to transforming growth factor-β (TGF-β)-activated protein kinase 1 (TAK1) and TAK1-binding protein (TAB1) leading to the phosphorylation of the inhibitor of nuclear factor (NF)-κB IκB kinase (IKK) complex. As a result, IκB is degraded freeing NF-κB to translocate to the nucleus to induce transcription of inflammatory related genes. Besides its classical role in NF-κB activation, MyD88 signaling also participates in TLR-induced autophagy in macrophages.

AUTOPHAGY

Macroautophagy (hereafter referred to as autophagy) is a catabolic process enabling cellular homeostasis by exerting a cytoprotective role. As mentioned previously, the process of autophagy involves sequestration of regions of the cytosol within autophagosomes and the delivery of those contents to lysosomes for degradation (Yang and Klionsky, 2010). Autophagy consists of three main steps that are termed initiation, elongation, and fusion. During the initiation step an isolation membrane or phagophore is formed around a portion of cytoplasm. The developing phagophores are derived from and connected to phosphatidylinositol-3-phosphate positive regions of the endoplasmic reticulum (ER) termed omegasomes. Other membrane compartments besides the ER can also contribute to phagophore formation. Following elongation and fusion, a double membrane autophagosome encompasses the selected cargo. The formation of autophagosomes and cargo recruitment are dependent upon evolutionarily conserved protein kinases, a lipid kinase, and ubiquitin-like protein conjugation networks. Following their closure autophagosomes fuse with lysosomes, which results in the degradation of the contents. At that time the inner membrane of the two outer membranes is also degraded. We refer the reader to more detailed reviews that cover the complex and dynamic molecular mechanisms that underlie autophagosome biogenesis (Levine *et al.*, 2011; Yang and Klionsky, 2010). However, a few key protein components of the autophagic pathway are relevant to this review. One of them is the mammalian target of rapamycin (mTOR), which inhibits autophagy by binding and inactivating the UNC-51-like kinase (ULK)1/2 kinase. Signaling via the ULK1/2 kinase is important to recruit autophagy-related gene (ATG) proteins to the site of autophagosomal biogenesis. Another important protein is Atg8 (microtubule-associated light chain 3, LC3 in mammals), which is commonly used as a marker for the induction of autophagy. LC3 is conjugated to phosphatidylethanolamine on autophagosomal membranes following the activation of autophagy. By determining the amount of conjugated LC3 on autophagic

membranes, the degree of autophagic response can be assessed. Other important proteins include VPS34, the catalytic subunit of a type III phosphatidylinositol 3-kinase, and Beclin-1 (Atg6), which together with other regulatory proteins helps promote autophagosomal membrane expansion and fusion (Mintern and Villadangos, 2012).

Autophagy is induced in a number of different situations including following nutrient deprivation; the presence of excessive numbers or damaged organelles; the accumulation of misfolded proteins; as a consequence of ER or oxidative stress; and following the exposure to certain chemicals, radiation, and hypoxia (Mihalache and Simon, 2012). Depending on the nature of the trigger, autophagy may either proceed as a nonselective bulk degradation process or by a more selective targeting of labeled substrates (Shaid *et al.*, 2013). Autophagy is involved in a wide array of cellular and organismal responses playing a significant role in physiologic and pathologic pathways needed for cell survival, cell differentiation and development. Defects in autophagic machinery can lead to a variety of diseases including cancer, neurodegenerative syndromes, myopathies, and accelerated aging (Levine *et al.*, 2011).

In the context of host defense, autophagy can amplify immune and inflammatory responses, but in other instances it suppresses such responses (Levine *et al.*, 2011; Xu and Eissa, 2010). Studies of autophagy in innate immunity have shown that autophagy acts as a direct effector to help eliminate invading pathogens, that it helps regulate pathogen recognition, and it participates in the control of inflammatory signals (Oh and Lee, 2012). In adaptive immunity, autophagy contributes to antigen presentation via major histocompatibility complex (MHC) class II molecules, impacts B- and T-cell development, and plasma cell function (Mintern and Villadangos, 2012). Given the significance of autophagy during immune response regulation, the defects in autophagic machinery have been linked to the pathogenesis of some infectious diseases and inflammatory syndromes (Deretic, 2012).

Initial Reports that Linked Autophagy to the Clearance of Intracellular Pathogens

The investigation of the intracellular fate of *Streptococcus pyogens* (Group A Streptococcus) in nonphagocytic cells following bacterial invasion revealed a potential role for autophagy in bacterial clearance (Nakagawa *et al.*, 2004). Bacterial invasion of the cells led to an increase of LC3-II in cell lysates. The cytosolic LC3-I protein has a molecular weight of 18 kDa; however, following autophagic induction it becomes conjugated to phosphotidylethanolamine on the autophagic vesicle membranes as the 16 kDa LC3-II form. The two forms can be distinguished by LC3 immunoblotting. The intracellular bacteria also colocalized with LC3 and LAMP-1 positive vesicles, markers of autophagosomes and autolysosomes, respectively. Indicating that autophagy enhanced the clearance of intracellular bacteria, infected autophagy-deficient cells (from *Atg5*$^{-/-}$ mice) lacked autophagosomes and intracellular bacterial viability increased as a consequence (Nakagawa *et al.*, 2004). Shortly after this study, autophagy was found to limit *Mycobacterium tuberculosis* replication in the murine macrophage cell line RAW 264.7 and in murine bone marrow-derived macrophages (BMDM) (Gutierrez *et al.*, 2004). *Mycobacterium tuberculosis* is an intracellular pathogen that inhibits the phagosome maturation pathway by reducing phagolysosome formation. To understand the effects of autophagic induction on *Mycobacterium tuberculosis* infection of macrophages, infected RAW 264.7 cells were starved or treated with rapamycin, a

pharmacological inducer of autophagy. This resulted in the co-localization of *Mycobacterium tuberculosis* containing vesicles with the autophagic markers LC3 and Beclin-1. Moreover, autophagy induction inhibited *Mycobacterial tuberculosis* survival in the infected macrophages. These results indicated that autophagic induction resulted in the entrapment of *Mycobacterium tuberculosis* containing phagosomes by autophagic vesicles, which later fused with lysosomes overcoming the previously alluded to intracellular trafficking block. Apart from these early studies, there are many additional reports in literature depicting the recognition and modes of autophagic attack on other bacteria (i.e., *Shigella flexneri*, *Listeria monocytogenes*, *Toxoplasma gondii*, etc.) and bacterial evasion of autophagy (Gong *et al.*, 2012). The term xenophagy has been coined to describe the removal or elimination of intracellular pathogens (bacteria, viruses, fungi, parasites) by autophagic degradation. We recommend the reader to refer to other excellent reviews covering this topic (Gong *et al.*, 2012; Into *et al.*, 2012; Levine *et al.*, 2011).

TLR-INDUCED AUTOPHAGY

The recognition that autophagy contributes to the control of intracellular bacteria led to efforts to elucidate the molecular mechanisms and signaling pathways that trigger autophagy in macrophages and other cells involved in innate immunity. Our laboratory screened a panel of TLR ligands to assess their impact on autophagy using the mouse macrophage cell line RAW 264.7, which stably expressed green fluorescent protein (GFP) fused to LC3, GFP-LC3 (Shi and Kehrl, 2008). The induction of autophagy in this cell line could be quantitated by counting the number of GFP-LC3 dots in the cytosol. In this particular study we stimulated the cell line with 100 ng/mL of Pam_3CSK_4.3HCl (TLR1), 20 μg/mL of Poly(I:C), (TLR3), 100 ng/mL of LPS (TLR4), 50 ng/mL of flagellin (TLR5), 50 ng/mL of macrophage-activating lipopeptide-2 (TLR6), 10 μg/mL of ssRNA40 (TLR7), or 5 μg/mL of CpG oligodeoxynucleotides (TLR9). Seven hours later we assessed the number and size of GFP-LC3 cytoplasmic dots and we collected cell lysates to immunoblot LC3-I and LC3-II levels. We concluded from these experiments that all the TLR ligands with the exception of the TLR9 ligand were capable of triggering autophagosome formation. Among the different ligands tested, LPS provided the strongest induction. To determine which of the TLR adaptor proteins transduced signals leading to the induction of autophagy, we overexpressed various adaptor proteins along with GFP-LC3. This led to the conclusion that both MyD88 and TRIF triggered downstream signaling pathways that enhanced autophagosome formation. Consistent with this result, MyD88 or TRIF dominant negative constructs, and shRNAs targeting MyD88 or TRIF, all decreased TLR4-induced autophagy while targeting TRIF reduced TLR3-induced autophagy. To link TLR signaling to the autophagy pathway, we immunoprecipitated a TLR4 signaling complex using an agonistic TLR4-specific antibody and assessed the co-immunoprecipitating proteins. This revealed the presence of Beclin-1, MyD88, TRIF, and IRAK4. The death domain of MyD88 proved critical for Beclin-1 recruitment. Since Beclin-1 binds the anti-apoptotic protein BCL-2, and that interaction inhibits autophagy, we tested whether TLR-signaling altered the BCL-2/Beclin-1 interaction and found a strong decrease in their association. These studies led to the conclusion that one of the targets of TLR-signaling pathways was the interaction between Beclin-1 and BCL-2.

A close examination of the Beclin-1 immunoblot of the TLR4 immunoprecipitates revealed that a portion of Beclin-1 migrated with a slower mobility on the gel (Shi and Kehrl, 2008). This observation provided the basis for another study where we explored the nature of this Beclin-1 modification (Shi and Kehrl, 2010). We found that, following LPS stimulation of RAW264.7 cells, Beclin-1 underwent lysine 63- (K63-) linked ubiquitination. K63-linked ubiquitination is involved in the regulating molecules involved in signal transduction pathways, particularly those elicited by infectious agents; in certain cellular stress responses; and in the intracellular trafficking of membrane proteins. The known role of the E3 ligase TRAF6 in TNF-receptor signaling led us to test TRAF6's involvement in Beclin-1 ubiquitination. We found that TRAF6 overexpression led to a marked increase in the K63-linked ubiquitination of Beclin-1, while a TRAF6 knockdown impaired TLR-induced Beclin-1 ubiquitination. Further studies revealed that TRAF6 bound Beclin-1 and that the addition of TRAF6 to an *in vitro* ubiquitination reaction resulted in Beclin-1 ubiquitination. The involvement of TRAF6 suggested a possible regulatory role for A20, a protein known to deubiquitinate TRAF6, and the reduction of A20 expression resulted in enhanced Beclin-1 ubiquitination. The overexpression of an A20 point mutant unable to deubiquitinate TRAF6 led to enhanced Beclin-1 ubiquitination consistent with an inhibitory role for A20. Furthermore, our group identified Beclin-1 as a ubiquitin binding protein that had a preference for K63-linked ubiquitin and found a key lysine residue (K117) that serves as a ubiquitination site for Beclin-1. Moreover, the ubiquitination at this site promoted the oligomerization of Beclin-1 and influenced the autophagic state by modulating the lipid kinase activity of VPS34 (Shi and Kehrl, 2010).

Next, we investigated the functional role of Beclin-1 ubiquitination in LPS-induced autophagy. Using GFP-LC3 expressing RAW 264.7 cells, LPS-induced autophagosome formation was quantified via GFP-LC3 dots in TRAF6 and A20 silenced cells. The TRAF6 knockdown decreased the number of autophagic vesicles, whereas the A20 knockdown increased them. To verify the importance of K63-linked ubiquitination of Beclin-1 in LPS-induced autophagy, wild-type ubiquitin and mutants with impaired K48- (K48R) or K63-linked (K63R) ubiquitination were transfected into GFP-LC3 expressing RAW 264.7 cells. The autophagy levels were similar in WT-Ub and Ub-K48R expressing cells, whereas it was reduced in the Ub-K63R expressing cells consistent with a role for K63-linked ubiquitination in the regulation of TLR-induced autophagy. Interestingly, exposure of the cells to IL-1 or IFN-γ, and amino acid starvation all resulted in Beclin-1 ubiquitination leading to the induction of autophagy (Shi and Kehrl, 2010). Taken together, these data indicated that Beclin-1 ubiquitination has a regulatory role in the induction of autophagosomes in response to a variety of different stimuli. Furthermore, the TRAF6/A20 axis regulates Beclin-1 in a manner similar to its defined role in controlling NF-κB activation. Figure 11.1 illustrates our current understanding of the mechanisms by which TLR signaling enhances autophagy in macrophages.

Other studies have examined how LPS stimulation causes the formation of ubiquitin-positive aggresome-like induced structures in the cytosols of macrophages (Canadien *et al.*, 2005; Szeto *et al.*, 2006). These structures incorporate LC3, but reducing ATG5 or ATG7 expression did not affect their numbers. This suggested that LPS-induced their formation via a mechanism independent of the classical autophagic machinery. However, their clearance from the cytosol did depend upon classical autophagy. A role for the adaptor protein

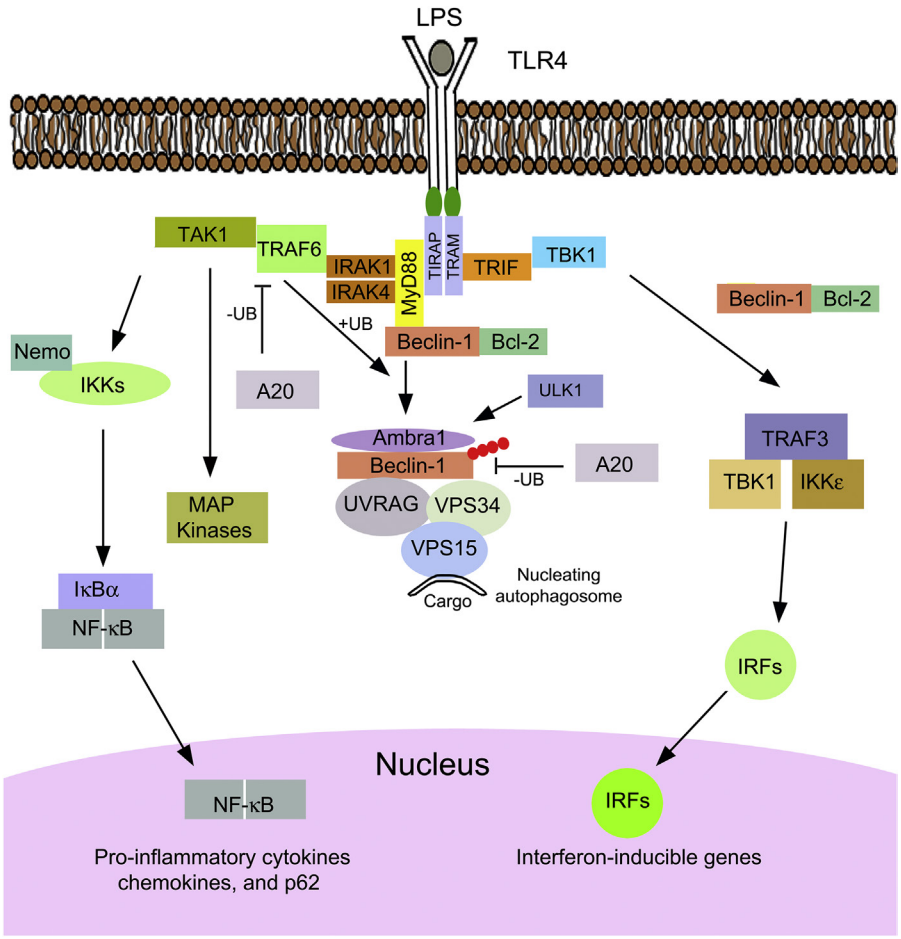

FIGURE 11.1 Model of TLR4-induced autophagosome biogenesis in macrophages. Illustrated is the TLR4 signaling pathway interfaced with its role in inducing autophagy. Ubiquitin chains are indicated by red dots.

p62 (SQSTM1) in the formation of aggresome-like induced structures in macrophages was found. p62 mediates LC3 binding to ubiquitinated targets and it helped recruit LC3 and ubiquitin to the aggresome-like induced structures as a p62 knockdown impaired their recruitment. The reduced expression of adaptor proteins such as MyD88, TRAF6, TRIF, and IRAK4 led to lower p62 levels suggesting that MyD88-dependent TLR4 signaling was essential for p62 induction and the formation of aggresome-like induced structures. The reactive oxygen species-p38 axis was found to activate Nrf2, which upregulated p62 in TLR4-stimulated macrophages at the transcriptional level (Fujita *et al.*, 2011). Thus, TLR signaling can lead not only to classical autophagy but also to the clearance of aggresome-like induced structures.

DISCUSSION

The induction of autophagy through PAMP-activated TLR signaling was demonstrated nearly simultaneously by three groups (Delgado *et al.*, 2008; Shi and Kehrl, 2008; Xu *et al.*, 2007). The first report showed that LPS-induced autophagy in macrophages was TRIF dependent, but MyD88 independent. Receptor-interacting protein (RIP1) and p38 mitogen-activated protein kinase functioned as downstream components in the pathway. Class III PI3K inhibition reduced LPS-induced autophagy. Moreover, the induction with LPS reduced the phagosomal block caused by *M. tuberculosis* and led to the mycobacterial phagosomes co-localizing with autophagosomes enhancing degradation (Xu *et al.*, 2007). Shortly thereafter, two other groups also reported that TLR signaling triggered autophagy in macrophages, although both found a role for MyD88 signaling (Delgado *et al.*, 2008; Shi and Kehrl, 2008). One group focused on engagement of TLR7 by ssRNAs and the induction of autophagy. The induction of autophagy depended upon the presence of Beclin-1, MyD88, and TLR7. The measurement of the half-life of long-lived protein following either TLR7 engagement or starvation revealed increased protein degradation, a result consistent with the induction of autophagy. Furthermore, the survival of Bacille Calmette-Guerin (BCG) in macrophages was decreased by either exposure of the cells to a TLR7 ligand or by starvation (Delgado *et al.*, 2008). As indicated above our study focused on how TLR4 signaling engaged the autophagosome pathway finding roles for MyD88, Beclin-1, IRAK4, and BCL-2 in regulating autophagosome formation. Some of the similarities and differences between the three studies have been discussed (Shi and Kehrl, 2008).

Shortly after the initial TLR-autophagy publication another group reported that the engagement of TLR receptors during phagocytosis resulted in the utilization of components of the autophagy pathway in the phagocytic pathway, blurring the line between autophagy and phagocytosis (Sanjuan *et al.*, 2007). Macrophage phagocytosis induced using TLR agonist-coated beads resulted in the recruitment of LC3 and Beclin-1 to the phagosomes. This process was termed "LC3-associated phagocytosis (LAP)." ATG5 and ATG7 were needed for the LC3 recruitment. Beclin-1 was also recruited before the association with LC3 and acidification of the phagosome occurred. However, the phagosome was not associated with an observable double-membrane structure normally seen during conventional autophagy. The TLR-induced association of autophagy elements to the phagosome enhanced phagosome maturation by promoting its fusion with lysosomes (Sanjuan *et al.*, 2007).

Our follow-up study revealed a role for Beclin-1 ubiquitination in the control of autophagy and showed how TRAF6 and A20 modulate the level of autophagy triggered by TLR signaling (Shi and Kehrl, 2010). TRAF6 has also been shown to support the K63-linked ubiquitination of ULK1, an upstream serine/threonine kinase in the autophagy pathway (Nazio *et al.*, 2013). TLR signaling was also shown to induce oxidative stress, which activated Nrf2, a transcription factor that upregulated p62 expression and promoted the assembly of aggresome-like induced structures. These structures were then degraded by autophagy (Fujita *et al.*, 2011). Hence, TLR signaling can induce selective autophagy targeting ubiquitin proteins in aggresome-like induced structures via a nonclassical autophagic pathway (Fujita and Srinivasula, 2011).

The involvement of adaptor proteins such as p62, nuclear domain 10 protein 52 (NDP52) and optineurin (OPTN) in recognition of ubiquitin has revealed a cross-talk between the ubiquitin-dependent proteolytic system and autophagy (Shaid *et al.*, 2013). Recent

studies have shown that xenophagy is not just a nonspecific induction of bulk autophagy. Ubiquitinated cytosolic bacteria can be recognized by specific adaptor proteins that deliver their ubiquitinated cargos to autophagosomes for degradation (Shaid *et al.*, 2013; Sriram *et al.*, 2011; Thurston *et al.*, 2009). Implicating TLR signaling in cargo selection the autophagy receptor OPTN is a downstream effector of TLR4 signaling. LPS stimulation led to TANK-binding kinase 1 (TBK-1), a TRIF effector, mediated phosphorylation of OPTN. This increased the affinity of OPTN for LC3, which promoted the capture and clearance of ubiquitin-coated cytosolic *Salmonella* (Wild *et al.*, 2011).

Autophagy in macrophages functions as a cytoprotective response against intracellular pathogens. However, autophagy can also negatively regulate innate immune response particularly during stressful conditions. For example, TLR stimulation of macrophages deficient in autophagy component-deficient macrophages – i.e., missing LC3, Beclin-1, Atg16, or Atg7 – leads to elevated production of IL-1β and IL-18, suggesting that autophagy can limit extensive inflammation (Nakahira *et al.*, 2011; Saitoh *et al.*, 2008). Moreover, the activation of autophagy decreases IL-1β production by inflammasome activators by selectively targeting ubiquitinated inflammasomes for degradation (Shi *et al.*, 2012).

In conclusion, considerable evidence now supports important interactions between autophagy and immunity. Triggers that engage the innate immune system also serve to upregulate autophagy in macrophages and likely in other cell types that participate in both the innate and adaptive immune responses. That pathogens have developed strategies to disrupt autophagy underscores its importance in pathogen clearance. Further studies of the role of autophagy in innate and adaptive immunity may provide some useful targets for the development of drugs that either limit or augment autophagy in inflammatory and infectious diseases.

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References

Canadien, V., Tan, T., Zilber, R., et al., 2005. Cutting edge: microbial products elicit formation of dendritic cell aggresome-like induced structures in macrophages. J. Immunol. 174, 2471–2475.

Delgado, M.A., Elmaoued, R.A., Davis, A.S., et al., 2008. Toll-like receptors control autophagy. EMBO J. 27, 1110–1121.

Deretic, V., 2012. Autophagy: an emerging immunological paradigm. J. Immunol. 189, 15–20.

Fujita, K., Maeda, D., Xiao, Q., et al., 2011. Nrf2-mediated induction of p62 controls toll-like receptor-4-driven aggresome-like induced structure formation and autophagic degradation. Proc. Natl Acad. Sci. USA 108, 1427–1432.

Fujita, K., Srinivasula, S.M., 2011. Tlr4-mediated autophagy in macrophages is a p62-dependent type of selective autophagy of aggresome-like induced structures (alis). Autophagy 7, 552–554.

Gong, L., Devenish, R.J., Prescott, M., 2012. Autophagy as a macrophage response to bacterial infection. IUBMB Life 64, 740–747.

Gutierrez, M.G., Master, S.S., Singh, S.B., et al., 2004. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. Cell 119, 753–766.

Into, T., Inomata, M., Takayama, E., et al., 2012. Autophagy in regulation of toll-like receptor signaling. Cell Signal. 24, 1150–1162.

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat. Immunol. 11, 373–384.
Kumar, H., Kawai, T., Akira, S., 2011. Pathogen recognition by the innate immune system. Int. Rev. Immunol. 30, 16–34.
Levine, B., Mizushima, N., Virgin, H.W., 2011. Autophagy in immunity and inflammation. Nature 469, 323–335.
Mihalache, C.C., Simon, H.U., 2012. Autophagy regulation in macrophages and neutrophils. Exp. Cell Res. 318, 1187–1192.
Mintern, J.D., Villadangos, J.A., 2012. Autophagy and mechanisms of effective immunity. Front Immunol. 3, 60.
Nakagawa, I., Amano, A., Mizushima, N., et al., 2004. Autophagy defends cells against invading group A streptococcus. Science 306, 1037–1040.
Nakahira, K., Haspel, J.A., Rathinam, V.A., et al., 2011. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the nalp3 inflammasome. Nat. Immunol. 12, 222–230.
Nazio, F., Strappazzon, F., Antonioli, M., et al., 2013. MTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. Nat. Cell Biol. 15, 406–416.
Newton, K., Dixit, V.M., 2012. Signaling in innate immunity and inflammation. Cold Spring Harb. Perspect. Biol. 4.
Oh, J.E., Lee, H.K., 2012. Modulation of pathogen recognition by autophagy. Front Immunol. 3, 44.
Pasare, C., Medzhitov, R., 2005. Toll-like receptors: linking innate and adaptive immunity. Adv. Exp. Med. Biol. 560, 11–18.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Sanjuan, M.A., Dillon, C.P., Tait, S.W.G., et al., 2007. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature 450, 1253–1257.
Shaid, S., Brandts, C.H., Serve, H., et al., 2013. Ubiquitination and selective autophagy. Cell Death Differ. 20, 21–30.
Shi, C.S., Kehrl, J.H., 2008. MyD88 and Trif target beclin 1 to trigger autophagy in macrophages. J. Biol. Chem. 283, 33175–33182.
Shi, C.S., Kehrl, J.H., 2010. Traf6 and A20 regulate lysine 63-linked ubiquitination of beclin-1 to control tlr4-induced autophagy. Sci. Signal. 3, ra42.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Sriram, S.M., Han, D.H., Kim, S.T., 2011. Partners in crime: ubiquitin-mediated degradation and autophagy. Sci. Signal. 4, jc4.
Szeto, J., Kaniuk, N.A., Canadien, V., et al., 2006. ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy. Autophagy 2, 189–199.
Thurston, T.L., Ryzhakov, G., Bloor, S., et al., 2009. The TBK1 adaptor and autophagy receptor ndp52 restricts the proliferation of ubiquitin-coated bacteria. Nat. Immunol. 10, 1215–1221.
West, A.P., Koblansky, A.A., Ghosh, S., 2006. Recognition and signaling by toll-like receptors. Annu. Rev. Cell Dev. Biol. 22, 409–437.
Wild, P., Farhan, H., McEwan, D.G., et al., 2011. Phosphorylation of the autophagy receptor optineurin restricts salmonella growth. Science 333, 228–233.
Xu, Y., Eissa, N.T., 2010. Autophagy in innate and adaptive immunity. Proc. Am. Thorac. Soc. 7, 22–28.
Xu, Y., Jagannath, C., Liu, X.D., et al., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144.
Yang, Z., Klionsky, D.J., 2010. Mammalian autophagy: core molecular machinery and signaling regulation. Curr. Opin. Cell Biol. 22, 124–131.

CHAPTER

12

Autophagy in Antigen Processing for MHC Presentation to T Cells

Christian Münz

OUTLINE

Abstract

T cells recognize infected tissues via the display of small protein fragments that are bound to major histocompatibility complex (MHC) molecules. These peptides are generated by the cellular proteolytic machineries, primarily proteasomes and lysosomes. Proteasomal products are primarily presented on MHC class I molecules to cytotoxic $CD8^+$ T cells and lysosomal products primarily on MHC class II molecules to helper $CD4^+$ T cells. Autophagy encompasses at least three pathways, by which cytoplasmic material is transported to lysosomes for degradation. These pathways, therefore, allow cytoplasmic antigens to be presented on MHC class II molecules. However, in recent years additional functions of the molecular machinery for one of these pathways, macroautophagy, have been shown to regulate other membrane transport mechanisms, influencing antigen phagocytosis and exocytosis. In addition, vesicular loading of MHC class I molecules, which usually bind proteasomal products in the endoplasmic reticulum, might benefit from macroautophagy. We will discuss these different pathways and their possible contribution to autoimmunity, some of which are genetically linked with macroautophagy. Modulation of these vesicular transport functions in order to regulate cell intrinsic, beneficial effects of autophagy, for example in cancer and neurodegeneration, should consider the discussed effects on immune regulation.

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INTRODUCTION

The adaptive immune system consists of T and B cells. While B cells recognize their cognate antigens, such as viral proteins, as whole proteins and sometimes even structural epitopes (Victora and Nussenzweig, 2012), T cells detect processed antigens, mostly in the form of peptides, that are displayed on the cell surface via major histocompatibility complex (MHC) molecules (Rammensee *et al*., 1999). Cytotoxic $CD8^+$ T cells recognize octa- or nonamers on MHC class I molecules, while helper $CD4^+$ T cells detect nonameric core sequences, which are mostly extended on the C- and N-terminus for better binding to MHC class II molecules. These two classes of MHC molecules patrol the output of different proteolytic machineries in the cells and present their peptide products on the cell surface. MHC class I and II molecules are primarily loaded with proteasome- and lysosome-generated peptides, respectively (Trombetta and Mellman, 2005). The proteasomal products are generated in cytosol and nucleus for this purpose, then imported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) and finally loaded onto MHC class I molecules in the MHC class I loading complex, which includes chaperones and oxidoreductases. Afterwards they reach the cell surface via the Golgi apparatus. MHC class II molecules are also co-translationally inserted into the ER, but associate there with the chaperone invariant chain (Ii), which blocks peptide loading in this organelle and diverts MHC class II molecules to late endosomes, so-called MHC class II-containing compartments (MIICs). These fuse with lysosomes and their hydrolases degrade antigens, which have reached these vesicles, and destroy the invariant chain. Generated antigenic peptides are finally loaded onto MHC class II molecules with the help of the chaperone HLA-DM in humans or H2-M in mice, and then transported to the cell surface. Due to the location of the proteolytic machineries for MHC class I and II ligand generation the classical paradigm of antigen processing suggests that cytosolic and nuclear antigens can only be presented on MHC class I molecules, and extracellular and membrane antigens are presented on MHC class II molecules after endocytosis. However, studies in recent years have demonstrated that extracellular material can be presented on MHC class I via so-called cross-presentation (Joffre *et al*., 2012), while cytosolic and nuclear antigens can also be presented on MHC class II molecules, at least in part via autophagy (Münz, 2009). This later pathway will be discussed in this chapter.

Autophagy encompasses at least three pathways that transport cytosolic material into lysosomes. These are macro-, micro- and chaperone-mediated autophagy (Mizushima *et al*., 2010). While micro- and chaperone-mediated autophagy engulf or transport cytosolic substrates directly at late endosomal or lysosomal membranes, macroautophagy generates dedicated vesicles, so-called autophagosomes, which incorporate large portions of the cytoplasm for transport into lysosomes. Thirty-five gene products (autophagy-related proteins or atgs) are involved in the generation of these vesicles and various membrane sources can be used for this purpose (Mizushima *et al*., 2010). Therefore, macroautophagy can degrade damaged organelles, cytosolic protein aggregates and pathogens. These are incorporated into these autophagosomes by one of at least three mechanisms (Randow and Münz, 2012). Firstly, for some organelles, like mitochondria, dedicated organelle proteins exist that bind to Atg8/LC3B, which is directly coupled to the autophagosomal membrane by its ligase complex of Atg5, Atg12 and Atg16L1. Secondly, loss of endosomal membrane integrity and

exposure of luminal glycosylation leads to recruitment of galectins, some of which bind NDP52, which in turn associates with Atg8/LC3B. Finally, at least four anchor proteins exist in higher eukaryotic cells (sequestosome/p62, NBR-1, NDP52 and optineurin) that bridge ubiquitination with Atg8/LC3B. These recognition mechanisms mediate the transport of cytoplasmic constituents to lysosomes and late endosomes. We will discuss in the following paragraphs how these pathways affect antigen presentation on MHC molecules and provide alternative routes for T cell stimulation.

CYTOSOLIC ANTIGEN PRESENTATION ON MHC CLASS II MOLECULES

Already in the first studies of peptide ligand elution from MHC molecules, it was noted that peptides from cytosolic and nuclear antigens were not restricted to MHC class I molecules, but also constituted 20–30% of MHC class II ligands (Dengjel *et al.*, 2005). Furthermore, presentation of cytosolic and nuclear peptides can be enhanced by stimulating macroautophagy via starvation (Dengjel *et al.*, 2005). Among these, peptides of two mammalian Atg8 orthologues have been found (two peptides of LC3B presented on HLA-DR, and one peptide of GABARAP presented on H2-A^{g7}) (Dengjel *et al.*, 2005; Suri *et al.*, 2008). These characterizations of the ligand repertoire of MHC class II molecules suggested that macroautophagy provides cytosolic substrates for MHC class II loading. Indeed, autophagosomes could be found to fuse with MIICs (Kasai *et al.*, 2009; Schmid *et al.*, 2007). In these studies fusion proteins of LC3B with fluorescent proteins were used and their colocalization with HLA-DR and -DM analyzed. Late endosomal multivesicular bodies (MVBs) were identified as the vesicles, in which the MHC class II loading machinery and autophagosomes converge (Figure 12.1). The fusion of autophagosomes with MVBs was suggested to be mediated by the GTPase Rab11 and the SNARE VAMP3 (Fader *et al.*, 2008). These studies suggest that autophagosomes deliver substrates for MHC class II antigen presentation by fusion with MVBs that contain the MHC class II loading machinery.

This hypothesis was tested for several viral and bacterial antigens. For example, the nuclear antigen 1 of the Epstein Barr virus (EBNA1) was found to gain access to MHC class II presentation by an intracellular pathway (Münz *et al.*, 2000). Upon lysosomal inhibition, EBNA1 accumulates in autophagosomes and inhibition of macroautophagy by atg-specific RNA silencing compromises MHC class II restricted antigen presentation to $CD4^+$ T cell clones (Paludan *et al.*, 2005). Furthermore, this antigen processing can be enhanced if EBNA1's nuclear import sequence is mutated and the protein is no longer protected from macroautophagy by nuclear localization (Leung *et al.*, 2010). EBNA1 was the first antigen which was found to gain access to MHC class II presentation via macroautophagy at physiological expression levels. Overexpression of the bacterial antigen neomycin phosphotransferase II (NeoR) leads to intracellular antigen processing for MHC class II presentation (Nimmerjahn *et al.*, 2003). Pharmacological inhibition of macroautophagy or lysosomal function compromises this presentation. Interestingly, and seemingly in contrast to findings with EBNA1, a fusion protein of NeoR with a nuclear localization sequence did not impair its presentation on MHC class II molecules (Riedel *et al.*, 2008). Therefore, nuclear localization by itself does not necessarily prevent antigen processing for MHC class II

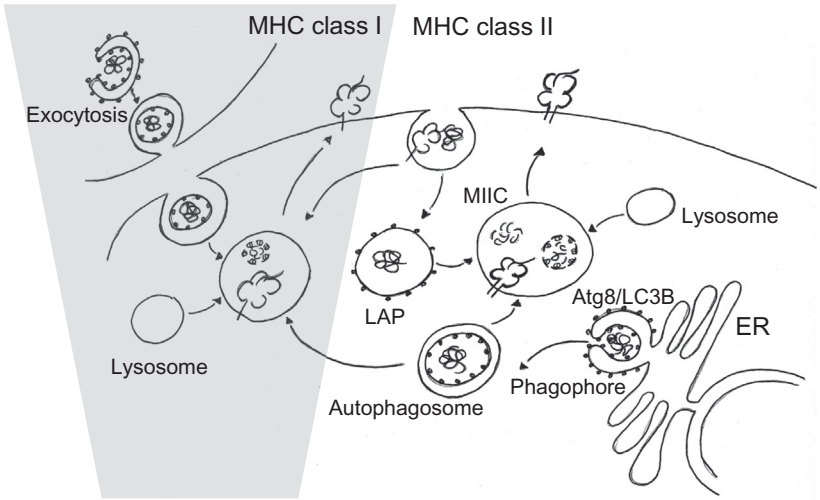

FIGURE 12.1 Regulation of antigen processing for MHC class I and II presentation by macroautophagy. Autophagosomes are formed from phagophores on membrane sources like the endoplasmic reticulum (ER). They transport cytoplasmic constituents to late endosomes, some of which are equipped with the MHC class II loading machinery (MIICs). Upon fusion with lysosomes their content is degraded, loaded onto MHC class II and transported to the cell surface for $CD4^+$ T cell stimulation. MIICs also receive input from phagosomes, some of which are modified by the molecular machinery of macroautophagy in a process termed LC3-associated phagocytosis (LAP). This regulation seems to enhance antigen presentation on MHC class II molecules. In contrast to its contribution to MHC class II antigen presentation, the role of macroautophagy in antigen presentation on MHC class I molecules is much less understood (gray shaded area). Exocytosis, which can be supported by macroautophagy, facilitates antigen cross-presentation on MHC class I molecules of neighboring antigen presenting cells. Moreover, intracellular antigen processing, especially under conditions that inhibit the classical MHC class I antigen processing pathway, such as TAP inhibition by viral infection, can be supported by macroautophagy. However, it remains unclear if this then leads to vesicular loading of MHC class I molecules, before these get transported to the cell surface for $CD8^+$ T cell stimulation.

presentation via macroautophagy. Moreover, targeting matrix protein 1 (MP1) of influenza A virus to autophagosomes via fusion to LC3B enhanced MHC class II antigen presentation in epithelial, B and dendritic cells (Schmid *et al.*, 2007). This increased presentation did not occur after mutating the C-terminal glycine residue of LC3B, which is used for the conjugation of this Atg8 orthologue to the autophagosomal membrane. Along the same lines, a hemagglutinin derived $CD4^+$ T cell epitope of influenza A virus was coupled to LC3B or NeoR (Comber *et al.*, 2011). Both fusion constructs demonstrated augmented MHC class II presentation to $CD4^+$ T cells and this presentation was macroautophagy dependent. In addition to these examples of viral and bacterial antigens that are presented to $CD4^+$ T cells after macroautophagy, self-protein presentation by this pathway seems to influence thymic T cell selection. Indeed, atg5 deficient thymic tissue was shown to inefficiently select certain T cell receptors positively (Nedjic *et al.*, 2008). Moreover, when the model antigen pigeon cytochrome C (PCC) was transgenically expressed, either in mitochondria or membrane

bound, T cells with the respective antigen-specific T cell receptor (TCR) were negatively selected, but only the mitochondrial expression required macroautophagy for this thymic deletion (Aichinger *et al*., 2013). Furthermore, when another $CD4^+$ T cell epitope of the human C-reactive protein (CRP) was fused to LC3B and this fusion protein was transgenically expressed in mice, it again mediated negative thymic selection of the respective TCR transgenic T cells. This negative selection was macroautophagy dependent and did not occur when the C-terminal amino acid of LC3B, which is used to conjugate this protein to the autophagosomal membrane during macroautophagy, is mutated (Aichinger *et al*., 2013). These findings suggest that macroautophagy substrates can be presented to $CD4^+$ T cells.

AUTOPHAGY REGULATION OF PHAGOCYTOSIS

Consistent with these contributions of macroautophagy for antigen presentation on MHC class II molecules *in vitro* and *in vivo*, $CD4^+$ T cell responses to herpes simplex virus (HSV) infection were found to be diminished in mice with atg5 deficiency in dendritic cells (Lee *et al*., 2010). However, in the same study it was noted that extracellular antigen presentation, using the model antigen ovalbumin, was also compromised in macroautophagy deficient dendritic cells. Especially, ovalbumin loaded and LPS coated apoptotic splenocytes were much better presented by wild-type dendritic cells. Furthermore, the authors observed that phagocytosis of toll-like receptor (TLR) ligand coated cargo led to accelerated recruitment of lysosomal proteases to the respective endosomes (Lee *et al*., 2010). These findings are reminiscent of LC3-associated phagocytosis (LAP), a process that was originally described for TLR2 ligand coated beads in mouse macrophage cell lines (Sanjuan *et al*., 2007). In this and successive studies, TLR ligand coated, antibody opsonized or apoptotic material was shown to recruit LC3B to the phagosomal membrane, which seemed to enhance fusion with lysosomes and degradation of the endocytosed antigens or pathogens (Henault *et al*., 2012). In one study it was found that this LAP process facilitates extracellular antigen processing for MHC class II presentation (Ma *et al*., 2012). These findings suggest that, in addition to building autophagosomes and delivering cytoplasmic constituents for lysosomal degradation, the autophagic core machinery, which modifies membranes by coupling Atg8 orthologues to them, can regulate the fate of phagosomes by modulating their maturation and fusion with lysosomes (Figure 12.1).

A second pathway by which macroautophagy can influence processing of extracellular antigens consists of the transport of lysosomal hydrolases to endosomal compartments. It was noted that starvation-induced macroautophagy diminished the levels of lysosomal proteases, cathepsins, in antigen presenting cells (Dengjel *et al*., 2005). Furthermore, citrullinated peptides are recognized as autoantigens by B and T cells in rheumatoid arthritis. The arginine to citrullin conversion is performed by peptidylargenine deiminases (PADs) in endosomal compartments. It was recently shown that PAD2 and 4 reach these compartments via macroautophagy (Ireland and Unanue, 2011). Citrullinated epitopes of the model antigen hen egg lysozyme were only presented at decreased levels to specific T cells by macroautophagy deficient antigen presenting cells. Therefore, macroautophagy influences the composition of the endosomal antigen processing machinery and can thereby regulate extracellular antigen presentation on MHC class II molecules.

ANTIGEN PACKAGING FOR CROSS-PRESENTATION VIA MACROAUTOPHAGY

In addition to the alternate usage of the macroautophagy machinery for the regulation of phagocytosis, it seems to also regulate exocytosis. The signal peptide independent secretion of acyl coenzyme A-binding protein (ACBP) was described to depend on macroautophagy in yeast (Duran *et al*., 2010; Manjithaya *et al*., 2010). This exocytosis required a SNARE involved in vesicle fusion with the plasma membrane, but not peroxisome turnover or autophagosome fusion with the lysosomal vacuole. Such exocytosis with the help of the macroautophagy machinery might be conserved in higher eukaryotes, because signal peptide-independent export of the cytokine IL-1β was described to rely on macroautophagy (Dupont *et al*., 2011). Moreover, lysosomal content secretion by bone resorbing macrophages, osteoclasts, was found to depend on the macroautophagy protein Atg5 and the GTPase Rab7 (DeSelm *et al*., 2011). Similarly it was noted that antigen cross-presentation on MHC class I molecules benefited from macroautophagy in the antigen donor cell (Li *et al*., 2008; Uhl *et al*., 2009). Both influenza A virus infected and tumor cells apparently packaged their antigens via macroautophagy for more efficient cross-presentation, and enriched autophagosomes of tumor cells constituted a superior antigen formulation for cross-presentation (Li *et al*., 2008). Therefore, it is tempting to speculate that antigen exocytosis using the macroautophagy machinery allows more efficient antigen presentation on neighboring cells (Figure 12.1).

REGULATION OF MHC CLASS I ANTIGEN PROCESSING BY MACROAUTOPHAGY

The initial studies on contributions of macroautophagy for antigen processing and MHC presentation did not observe any effects on MHC class I presentation. Targeting of antigens to autophagosomes by LC3B fusion constructs did not seem to affect recognition by $CD8^+$ T cell clones (Schmid *et al*., 2007), positive thymic selection of $CD8^+$ T cells did not appear deficient in Atg5 deficient thymi (Nedjic *et al*., 2008) and cross-presentation of extracellular ovalbumin for $CD8^+$ T cell stimulation did not occur at decreased levels in Atg5 deficient dendritic cells (Lee *et al*., 2010). However, several recent studies might suggest that under conditions, in which the canonical MHC class I antigen processing pathway is compromised, for example after viral inhibition of TAP, macroautophagy could be involved in antigen presentation on MHC class I molecules. HSV is such a pathogen. Indeed, late after HSV infection in cell culture, MHC class I presentation of the viral gB antigen was found to depend on macroautophagy (English *et al*., 2009). At this time point, HSV particles were getting enveloped in vesicles with multiple surrounding membranes, which originated from the outer nuclear membrane. Despite its dependency on macroautophagy HSV gB antigen processing for MHC class I presentation remained proteasome dependent. In contrast, pUL138 antigen processing for $CD8^+$ T cell stimulation after human cytomegalovirus (HCMV) infection was also found to be dependent on macroautophagy, but did not require proteasomal processing or TAP transport (Tey and Khanna, 2012). Similarly, cross-presentation of the F protein of respiratory syncytial virus (RSV) depends on macroautophagy and lysosomal processing, but is TAP independent (Johnstone *et al*., 2012). Finally, chlamydial antigen processing for

MHC class I presentation was also observed to be macroautophagy dependent, but required proteasomal processing and TAP transport in addition (Fiegl *et al.*, 2013). All these studies argue for a vesicular pathway of MHC class I antigen loading (Figure 12.1), which might become especially prominent when the TAP and proteasome-dependent classical antigen processing is compromised, for example during viral infections. However, whether macroautophagy facilitates access of MHC class I molecules to this compartment or rather actively degrades antigens for MHC class I presentation remains to be established.

AUTOPHAGY AND AUTOIMMUNITY

These mechanisms of antigen processing for MHC presentation by macroautophagy might, however, not only affect immune responses to pathogen challenge, but also contribute to autoimmunity. Along these lines, single nucleotide polymorphisms (SNPs) in autophagy associated genes have been found to be associated with Crohn's disease and systemic lupus erythematosus (SLE). In familial Crohn's disease, which is characterized by intestinal inflammation due to an uncontrolled immune reaction against gut commensals and possibly also autoantigens, associations with SNPs in atg16L1 (Hampe *et al.*, 2007), a component of the Atg8/LC3B ligase enzyme complex, and IRGM (Parkes *et al.*, 2007), a GTPase that assists autophagic intracellular pathogen clearance, have been described. In SLE, a systemic autoimmune disease, however, SNPs in atg5 (Harley *et al.*, 2008), another component of the Atg8/LC3B ligase complex, are associated with the disease. It is still unclear how these SNPs influence the function of the affected proteins, and if they impair their function in macroautophagy. Furthermore, restriction of bacterial burden, innate immune activation, such as IL-1 production, and/or clearance of apoptotic bodies rather than antigen processing might be affected (Saitoh *et al.*, 2008). Nevertheless, compromised autophagy during negative thymic selection due to Atg5 deficiency resulted in severe colitis, which could be transferred by the insufficiently tolerized T cells of these mice (Nedjic *et al.*, 2008). Moreover, citrullinated peptides, which constitute autoantigens in rheumatoid arthritis, were generated with the assistance of macroautophagy in mice (Ireland and Unanue, 2011). And finally, the autoantigens glutamate decarboxylase 65 (GAD65) and mutant immunoglobulin κ light chain SMA of diabetes mellitus and autoimmune hepatitis, respectively, have been reported to be processed by chaperone mediated autophagy for MHC class II presentation to $CD4^+$ T cells (Zhou *et al.*, 2005). Therefore, antigen processing by autophagy might be involved in autoimmunity. However, its tolerizing versus disease aggravating functions needs to be assessed for these diseases individually and the function of the associated gene variants rather than the complete knockout of the respective genes should be analyzed in the future.

DISCUSSION

The evidence, summarized in this chapter, suggests that the immune system has learned to utilize autophagy during immune responses. It not only monitors the products of macroautophagic degradation by MHC presentation in order to detect pathogen presence, but also harnesses the vesicular fusion processes of this pathway to enhance exo- and

endocytosis. This complexity makes it difficult to predict the outcome of macroautophagy regulation in human diseases. Therefore, experimental models for these diseases have to be carefully designed to investigate the role of macroautophagy inhibition or stimulation in the accompanying adaptive immune responses, before introducing such treatments to the clinic.

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References

Aichinger, M., Wu, C., Nedjic, J., et al., 2013. Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. J. Exp. Med. 210, 287–300.

Comber, J.D., Robinson, T.M., Siciliano, N.A., et al., 2011. Functional macroautophagy induction by influenza A virus without a contribution to MHC-Class II restricted presentation. J. Virol. 85, 6453–6463.

Dengjel, J., Schoor, O., Fischer, R., et al., 2005. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc. Natl Acad. Sci. USA 102, 7922–7927.

DeSelm, C.J., Miller, B.C., Zou, W., et al., 2011. Autophagy proteins regulate the secretory component of osteoclastic bone resorption. Dev. Cell. 21, 966–974.

Dupont, N., Jiang, S., Pilli, M., et al., 2011. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J. 30, 4701–4711.

Duran, J.M., Anjard, C., Stefan, C., et al., 2010. Unconventional secretion of Acb1 is mediated by autophagosomes. J. Cell. Biol. 188, 527–536.

English, L., Chemali, M., Duron, J., et al., 2009. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. Nat. Immunol. 10, 480–487.

Fader, C.M., Sanchez, D., Furlan, M., et al., 2008. Induction of autophagy promotes fusion of multivesicular bodies with autophagic vacuoles in K562 cells. Traffic 9, 230–250.

Fiegl, D., Kagebein, D., Liebler-Tenorio, E.M., et al., 2013. Amphisomal route of MHC class I cross-presentation in bacteria-infected dendritic cells. J. Immunol. 190, 2791–2806.

Hampe, J., Franke, A., Rosenstiel, P., et al., 2007. A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat. Genet. 39, 207–211.

Harley, J.B., Alarcon-Riquelme, M.E., Criswell, L.A., et al., 2008. Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat. Genet. 40, 204–210.

Henault, J., Martinez, J., Riggs, J.M., et al., 2012. Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes. Immunity 37, 986–997.

Ireland, J.M., Unanue, E.R., 2011. Autophagy in antigen-presenting cells results in presentation of citrullinated peptides to CD4 T cells. J. Exp. Med. 208, 2625–2632.

Joffre, O.P., Segura, E., Savina, A., et al., 2012. Cross-presentation by dendritic cells. Nat. Rev. Immunol. 12, 557–569.

Johnstone, C., Ramos, M., Garcia-Barreno, B., et al., 2012. Exogenous, TAP-independent lysosomal presentation of a respiratory syncytial virus CTL epitope. Immunol. Cell. Biol. 90, 978–982.

Kasai, M., Tanida, I., Ueno, T., et al., 2009. Autophagic compartments gain access to the MHC class II compartments in thymic epithelium. J. Immunol. 183, 7278–7285.

Lee, H.K., Mattei, L.M., Steinberg, B.E., et al., 2010. In vivo requirement for Atg5 in antigen presentation by dendritic cells. Immunity 32, 227–239.

Leung, C.S., Haigh, T.A., Mackay, L.K., et al., 2010. Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. Proc. Natl Acad. Sci. USA 107, 2165–2170.

Li, Y., Wang, L.X., Yang, G., et al., 2008. Efficient cross-presentation depends on autophagy in tumor cells. Cancer Res. 68, 6889–6895.
Ma, J., Becker, C., Lowell, C.A., et al., 2012. Dectin-1-triggered recruitment of light chain 3 protein to phagosomes facilitates major histocompatibility complex class II presentation of fungal-derived antigens. J. Biol. Chem. 287, 34149–34156.
Manjithaya, R., Anjard, C., Loomis, W.F., et al., 2010. Unconventional secretion of Pichia pastoris Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. J. Cell. Biol. 188, 537–546.
Mizushima, N., Yoshimori, T., Ohsumi, Y., 2010. The role of atg proteins in autophagosome formation. Annu. Rev. Cell. Dev. Biol. 27, 107–132.
Münz, C., 2009. Enhancing immunity through autophagy. Annu. Rev. Immunol. 27, 423–429.
Münz, C., Bickham, K.L., Subklewe, M., et al., 2000. Human $CD4^+$ T lymphocytes consistently respond to the latent Epstein-Barr virus nuclear antigen EBNA1. J. Exp. Med. 191, 1649–1660.
Nedjic, J., Aichinger, M., Emmerich, J., et al., 2008. Autophagy in thymic epithelium shapes the T-cell repertoire and is essential for tolerance. Nature 455, 396–400.
Nimmerjahn, F., Milosevic, S., Behrends, U., et al., 2003. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur. J. Immunol. 33, 1250–1259.
Paludan, C., Schmid, D., Landthaler, M., et al., 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. Science 307, 593–596.
Parkes, M., Barrett, J.C., Prescott, N.J., et al., 2007. Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat. Genet. 39, 830–832.
Rammensee, H., Bachmann, J., Emmerich, N.P., et al., 1999. SYFPEITHI: database for MHC ligands and peptide motifs. Immunogenetics 50, 213–219.
Randow, F., Münz, C., 2012. Autophagy in the regulation of pathogen replication and adaptive immunity. Trends Immunol. 33, 475–487.
Riedel, A., Nimmerjahn, F., Burdach, S., et al., 2008. Endogenous presentation of a nuclear antigen on MHC class II by autophagy in the absence of CRM1-mediated nuclear export. Eur. J. Immunol. 38, 2090–2095.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Sanjuan, M.A., Dillon, C.P., Tait, S.W., et al., 2007. Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis. Nature 450, 1253–1257.
Schmid, D., Pypaert, M., Münz, C., 2007. MHC class II antigen loading compartments continuously receive input from autophagosomes. Immunity 26, 79–92.
Suri, A., Walters, J.J., Rohrs, H.W., et al., 2008. First signature of Islet beta-cell-derived naturally processed peptides selected by diabetogenic class II MHC molecules. J. Immunol. 180, 3849–3856.
Tey, S.K., Khanna, R., 2012. Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway. Blood 120, 994–1004.
Trombetta, E.S., Mellman, I., 2005. Cell biology of antigen processing *in vitro* and *in vivo*. Annu. Rev. Immunol. 23, 975–1028.
Uhl, M., Kepp, O., Jusforgues-Saklani, H., et al., 2009. Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific $CD8^+$ T cells. Cell. Death. Differ. 16, 991–1005.
Victora, G.D., Nussenzweig, M.C., 2012. Germinal centers. Annu. Rev. Immunol. 30, 429–457.
Zhou, D., Li, P., Lott, J.M., et al., 2005. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity 22, 571–581.

CHAPTER

13

Autophagy Controls the Production and Secretion of IL-1β: Underlying Mechanisms

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OUTLINE

Abstract

Although essential for the eradication of pathogens and tissue repair, inflammatory responses can be detrimental for the host when uncontrolled. Thus, the production of inflammatory mediators, including the proinflammatory cytokine IL-1β, must be tightly regulated to prevent excessive inflammation and collateral damage. Autophagy can regulate the production and secretion of the IL-1 cytokine family members IL-1α, IL-1β and IL-18, suggesting a pivotal role for autophagy in the regulation of inflammatory responses. This chapter focuses on the molecular mechanisms by which autophagy controls IL-1β secretion and addresses the relevance of this regulation *in vitro* and *in vivo*.

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INTRODUCTION

Inflammation is induced by infectious agents and danger signals that are recognized by pattern recognition receptors on innate immune cells. This recognition triggers the production of inflammatory cytokines that recruit neutrophils and macrophages to the site of infection or injury, where they engulf and contain the insult and recruit additional cells by the production of cytokines and chemokines. Once the insult has been contained, inflammatory responses must be resolved to restore tissue homeostasis and prevent host damage.

The interleukin-1 (IL-1) family of cytokines, which includes IL-1β, IL-1α, IL-1Ra, IL-18, IL-33, IL-36, IL-37 and IL-38, play a central role in the regulation of inflammatory responses to both infections and sterile inflammation (Dinarello, 2009). In particular, IL-1β exerts multiple proinflammatory effects and, consequently, its production and secretion is closely regulated within the cells. Moreover, dysregulation of IL-1β activity has been described to influence the development of some autoimmune and autoinflammatory diseases (Sims and Smith, 2010).

Several studies have demonstrated that autophagy can modulate the secretion of a number of cytokines, including the IL-1 family members IL-1α, IL-1β and IL-18 (Harris *et al.*, 2011; Nakahira *et al.*, 2011; Saitoh *et al.*, 2008; Zhou *et al.*, 2011a), and IL-23 (Peral de Castro *et al.*, 2012). This role of autophagy confirms this process as a major regulator of cytokine secretion and an attractive therapeutic target for the treatment of inflammatory conditions.

INTERLEUKIN-1β: BIOLOGICAL FUNCTIONS AND REGULATION

IL-1β is largely produced by macrophages and monocytes, although it can also be secreted by other cell types, such as dendritic cells, mast cells, neutrophils, B and T cells, endothelial and epithelial cells and it is commonly released by dying cells (Sims and Smith, 2010). IL-1β mediates diverse inflammatory responses, such as fever, vasodilatation and hypotension, mainly by the induction of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). IL-1β is also able to increase the expression of chemokines and adhesion molecules, which promotes the infiltration of inflammatory and immunocompetent cells from the circulation into the tissues (Dinarello, 2009).

Due to its potent and extensive functions, the production and activity of IL-1β are tightly regulated. IL-1β is first produced as an inactive pro-form that is cleaved by caspase-1 to form the bioactive, mature cytokine and this is then secreted. Activation of caspase-1 is mediated by an inflammasome. Inflammasomes are formed from at least one member of the cytosolic innate immune sensor family, the NOD-like receptors (NLRs), which include NLRP1, NLRP3 and NLRC4, coupled with the adaptor apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC or PYCARD). Inflammasomes can be activated by microbial and nonmicrobial signals. The NLRP1 inflammasome is activated by anthrax lethal toxin; the NLRP3 inflammasome is activated by a broad range of toxic stimuli including particulates, like uric acid crystals and protein aggregates; the NLRC4 inflammasome is triggered by bacterial flagellin and the AIM2 inflammasome is activated by double-stranded DNA (Schroder and Tschopp, 2010). To date, the NLRP3 inflammasome is the best

characterized. Mice lacking NLRP3, ASC or caspase-1 show a considerable defect in the production of mature IL-1β after challenge with LPS and ATP and are resistant to LPS-induced lethality (Li *et al.*, 1995). However, caspase-1-independent processing of IL-1β has also been described, involving serine kinases controlled by cathepsin C (Kono *et al.*, 2012).

ROLE OF AUTOPHAGY IN INTERLEUKIN-1β SECRETION

Recent studies have shown that autophagy regulates IL-1β secretion through at least two separate mechanisms, which are discussed below (Figure 13.1).

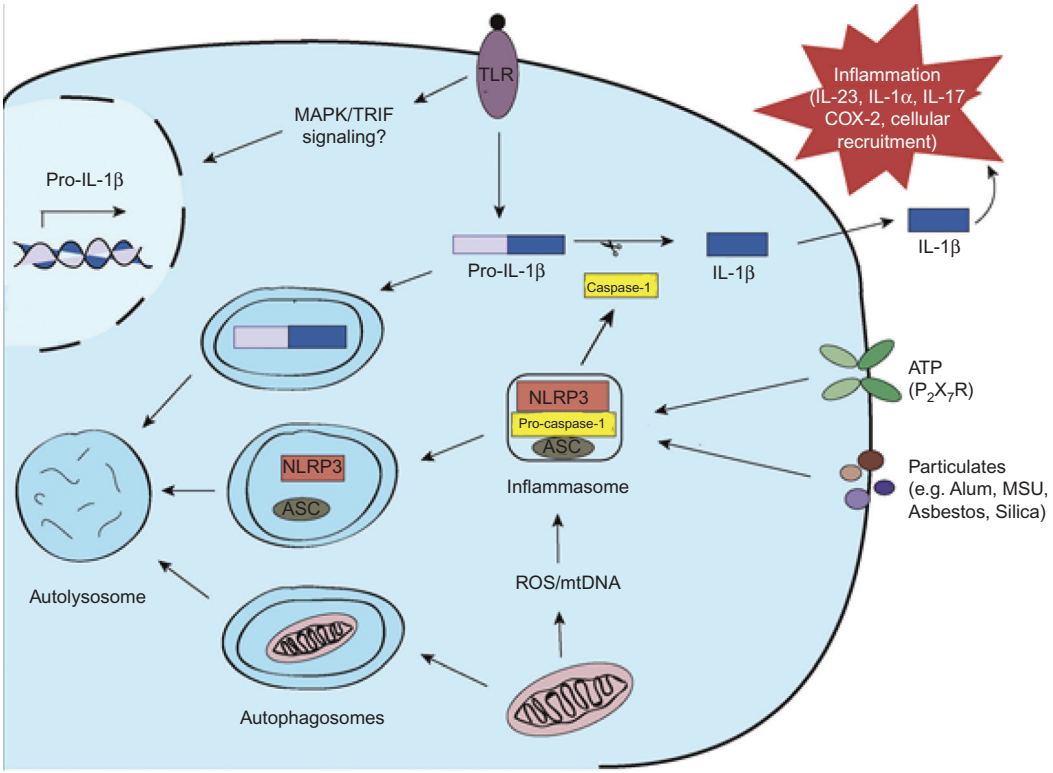

FIGURE 13.1 Regulation of IL-1β by autophagy. IL-1β is a major driver of inflammation and its production and secretion is regulated by autophagy through at least two separate mechanisms. Firstly, in macrophages and dendritic cells, inhibition of autophagy leads to an increased production of IL-1β in response to Toll-like receptor (TLR) ligands. This is because autophagy limits the presence of endogenous inducers of the inflammasome, such as reactive oxygen species (ROS) and/or mitochondrial DNA, in the cytosol. Although in murine cells this process is dependent on TRIF, in human cells it seems to involve MAPK signaling and is exerted at the transcriptional level, rather than at the level of the inflammasome. In addition, autophagy regulates IL-1β secretion through the sequestration and subsequent degradation of pro-IL-1β and the inflammasome components ASC and NLRP3, which are activated by numerous stimuli, including ATP and particulates, such as silica, alum, asbestos and monosodium urate (MSU) crystals.

Autophagy Controls Inflammasome Activation

In macrophages and dendritic cells, loss of autophagy stimulates the processing and secretion of IL-1β in response to TLR agonists. Saitoh *et al.* (2008) showed that Atg16L1 (autophagy-related protein 16-like 1) deficiency causes Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent activation of caspase-1 in LPS-stimulated murine macrophages, leading to an increased production of IL-1β. Atg7-deficient macrophages also produced high levels of IL-1β in response to LPS, as did wild-type peritoneal macrophages after treatment with a chemical inhibitor of autophagy, 3-methyladenine (3-MA). In another study, inhibition of autophagy with 3-MA led to an increased secretion of IL-1β in response to TLR4 and TLR3 ligands that was similarly dependent on TRIF signaling, but only partially dependent on the NLRP3 inflammasome (Harris *et al.*, 2011). These studies demonstrate that autophagy is an important regulator of IL-1β secretion following inflammasome activation.

Other studies have gone on to demonstrate that autophagy regulates inflammasome-mediated innate immune responses and IL-1β secretion through its role in preserving mitochondrial homeostasis. Zhou *et al.* (2011b) demonstrated that blockade of mitophagy, a specialized form of autophagy that removes damaged mitochondria, in human THP-1 monocyte/macrophage-like cells leads to the accumulation of reactive oxygen species (ROS)-producing mitochondria and, in parallel, a dose-dependent secretion of IL-1β. In this case, processing of pro-IL-1β was dependent on the NLRP3 inflammasome, which was activated by mitochondrial ROS. Therefore, mitophagy/autophagy could act as a scavenger of mitochondrial ROS, suppressing NLRP3 activation and inflammasome-dependent IL-1β processing. Further to this, deletion of the autophagic proteins LC3B (microtubule-associated protein 1 light chain 3B) and Beclin-1 in murine macrophages and BMDMs resulted in higher levels of active caspase-1 and increased secretion of IL-1β in response to LPS and ATP (Nakahira *et al.*, 2011). Both effects were a consequence of the accumulation of dysfunctional mitochondria and translocation of mitochondrial DNA to the cytosol, a process found to be dependent on ROS and NLRP3. Together, these studies suggest that autophagy regulates IL-1β production through the control of endogenous inducers of inflammasome activation, such as ROS and/or mitochondrial DNA, in a TRIF-dependent and partially NLRP3-dependent manner. Inhibition of autophagy also enhanced IL-1β production by human peripheral blood mononuclear cells (PBMCs) stimulated with TLR2 and TLR4 ligands. However, this effect was exerted at the transcriptional level, rather than at the level of the inflammasome, and may be dependent on p38 MAPK signaling (Crisan *et al.*, 2011).

IL-1β and Inflammasome Components are Degraded in Autophagosomes

The second mechanism by which autophagy regulates IL-1β secretion is through the sequestration and subsequent degradation of pro-IL-1β and inflammasome components. IL-1β was observed in the autophagosomes of LPS-treated macrophages after 6 h and 24 h, suggesting that IL-1β is sequestered into autophagosomes following stimulation with LPS (Harris *et al.*, 2011). More recently, Shi *et al.* (2012) have demonstrated that activation of AIM2 and NLRP3 inflammasomes triggers autophagy in macrophages, but this did not require complete assembly of the inflammasomes or IL-1β production. In addition, blocking

autophagy with 3-MA resulted in increased inflammasome activation, whereas induction of autophagy with rapamycin or amino acid deprivation limited it. Moreover, AIM2, NLRP3 and ASC co-localized with the autophagy marker LC3 and the lysosomal marker LAMP-1, suggesting that autophagosomes might ultimately degrade inflammasome components following fusion with LAMP-1$^+$ lysosomes. In the same study, polyubiquitinated ASC aggregates co-localized with p62, an autophagic adaptor with a LC3-binding domain that delivers autophagy targets to autophagosomes. These data provide a mechanism by which autophagy degrades inflammasomes via its ubiquitination, which leads to the recruitment of p62 and subsequent delivery to autophagosomes. Thus, autophagy can regulate IL-1β production by direct engulfment of not only pro-IL-1β, but also inflammasome proteins.

Although the previous studies established that inhibition of autophagy using pharmacological approaches or depletion of autophagy proteins enhances IL-1β secretion by macrophages and BMDCs, another study has demonstrated that induction of autophagy can lead to increased IL-1β secretion in response to inflammasome-activating stimuli, including LPS with ATP, silica or nigericin (Dupont *et al.*, 2011). This effect was partially dependent on Atg5 and at least one of the two mammalian Golgi reassembly stacking protein paralogues, GRASP55 and Rab8a. In these experiments, autophagy was induced at the same time as the inflammasome-activating stimulus (ATP, silica, or nigericin) was added. Thus, the role of autophagy in regulating IL-1β secretion may depend on timing and context; in the absence of an inflammasome-activating signal, autophagy may act to remove pro-IL-1β and inflammasome components from the cell, while in the presence of such a signal, autophagy may act as a secretory pathway for IL-1β release.

AUTOPHAGY AND INNATE TH17 IMMUNE RESPONSES

Th17 cells are a subset of T helper cells that produce IL-17A (also referred to as IL-17), IL-17F and IL-22 in response to stimulation with IL-1β and IL-23 (Littman and Rudensky, 2010). In addition, γδ T cells activated by IL-1β and IL-23 are an important innate source of IL-17, independent of TCR stimulation (Sutton *et al.*, 2009). Both cell types are abundant at mucosal surfaces and play a role in immunity to pathogenic bacteria and fungi (Weaver *et al.*, 2007). Th17 cells have also been implicated in different autoimmune and other inflammatory diseases, including multiple sclerosis, rheumatoid arthritis and some forms of colitis (Cua *et al.*, 2003; Koenders *et al.*, 2005; Uhlig *et al.*, 2006). Conversely, defects in Th17 differentiation may predispose to bacterial and fungal infections at mucosal surfaces (Ouyang *et al.*, 2008) and it has been reported that Th17-like cytokines may be important for the efficacy of vaccines against *Streptococcus pneumonia* and *Mycobacterium tuberculosis* (Khader *et al.*, 2007; Moffitt *et al.*, 2011).

A recent study has demonstrated that autophagy plays a role in the modulation of Th17 responses through its regulation of IL-1β and IL-23 production. Peral de Castro *et al.* (2012) demonstrated that inhibition of autophagy pharmacologically or using siRNA against Beclin-1 augmented the secretion of both IL-1β and IL-23 production by human and mouse macrophages and dendritic cells in response to TLR agonists. The effect on IL-23 secretion was found to be dependent on ROS and IL-1. Moreover, supernatants from BMDCs stimulated with LPS and 3-MA induced the secretion of IL-17, IFN-γ and IL-22 by γδ T cells,

showing that by limiting the secretion of IL-1 and IL-23, autophagy is able to further limit inflammatory responses.

More recently, Castillo *et al.* (2012) showed that $CD4^+$ T cells from mice deficient in the autophagy protein Atg5 in the myeloid lineage produce IL-17A upon nonspecific activation. In addition, T cells from Atg5-deficient mice infected with *Mycobacterium bovis* bacillus Calmette-Guérin demonstrated a propensity to IL-17 polarization upon restimulation *ex vivo* with mycobacterial antigens. These data further suggest a link between autophagy and the Th17 response, although in this study regulation of IL-1α may have played a greater role than IL-1β. However, other studies have suggested that IL-1β can drive the secretion of both IL-1α and IL-23 (Fettelschoss *et al.*, 2011; Harris *et al.*, 2008; Peral de Castro *et al.*, 2012). Thus, regulation of IL-1 family members by autophagy can have profound consequences for immunity and inflammation.

AUTOPHAGY AND INFLAMMATORY DISEASES

Inflammatory diseases are classified into autoimmune diseases and autoinflammatory diseases. Autoimmune diseases are disorders of the adaptive immune system with a progressive clinical course that can develop through a combination of hereditary and environmental factors. In comparison, autoinflammatory diseases are disorders of the innate immune system characterized by intense episodes of inflammation, including fever, pain, swelling joints and rashes. Although only some autoinflammatory diseases are due to mutations in proteins that regulate IL-1β activity, many do respond to IL-1β blockade; blocking IL-1β results in a rapid cessation of symptoms and the use of specific inhibitors of caspase-1 reduces the severity of the disease (Dinarello, 2009).

There is increasing evidence that perturbations in the autophagy machinery may contribute to autoinflammatory diseases. The best characterized link to date is between autophagy regulators and Crohn's disease (CD), a common form of inflammatory bowel disease in which a breakdown of clearance or recognition of commensal bacteria, together with altered mucosal barrier function and cytokine production, leads to intestinal inflammation. Polymorphisms in the genes encoding the autophagy-related proteins Atg2a, Atg4a, Atg4d (Brinar *et al.*, 2012), IRGM (Craddock *et al.*, 2010) and ULK-1 (Henckaerts *et al.*, 2011) have been associated with susceptibility to CD. In addition, genome-wide association studies of CD patients have identified the T300A polymorphism in the Atg16L1 gene as a strong susceptibility locus (Rioux *et al.*, 2007), while mice lacking Atg16L1 in hematopoietic cells are more susceptible to dextran sulfate sodium (DSS)-induced acute colitis (Saitoh *et al.*, 2008).

Defects in autophagy pathways may also contribute to the development of autoimmune diseases. Polymorphisms in some autophagy-related genes have been associated with susceptibility to systemic lupus erythematosus (SLE), an autoimmune disease mediated by pathogenic auto-antibodies that cause inflammation and tissue damage. In particular, genome-wide association studies have linked polymorphisms in the IRGM, Atg5 and PRDM1 genes to SLE susceptibility (Han *et al.*, 2009; Ramos *et al.*, 2011; Zhou *et al.*, 2011b). Although the specific role of autophagy in the development and etiology of SLE is not yet understood, there is evidence that autophagy-related abnormalities may be involved in the pathogenesis of the disease. For example, activation of mTOR signaling has emerged

as a key factor in abnormal activation of lymphocytes in SLE and there is increasing interest in the development of agents that can inhibit this kinase. In this regard, rapamycin has been demonstrated as an effective therapeutic treatment, both in animal models of lupus and in patients with SLE (Pierdominici *et al.*, 2012), and is currently undergoing a phase II clinical trial in SLE (NCT00779194). In addition, proinflammatory cytokines, including IL-1β and IL-18, are upregulated in SLE and mediate the inflammatory processes that lead to tissue and organ damage. Given the role that autophagy plays in the regulation of both cytokines, this further suggests a protective role for autophagy in SLE. In the effort to target therapeutically proinflammatory cytokines in SLE, Anakinra, a recombinant version of the human IL-1 receptor antagonist that blocks the biological activity of IL-1, has been used in an open trial on four SLE patients with lupus arthritis (Ostendorf *et al.*, 2005). In this study Anakinra showed safety and efficacy in improving arthritis; however, short-lasting effects were observed in two patients. Anakinra has also been used to treat gout, an inflammatory arthritis caused by monosodium urate (MSU) crystals that stimulate cells of the monocyte/macrophage lineage to release proinflammatory cytokines, including IL-1β. In a pilot study on 10 patients with acute gout, administration of Anakinra reduced pain and inflammation in all cases, confirming the critical role that IL-1β plays in the pathogenesis of this condition (So *et al.*, 2007). More recently, a study has established a link between gout and signaling involved in autophagy and IL-1β activity, which suggests that autophagy may be an additional therapeutic target in this inflammatory condition (Mitroulis *et al.*, 2011).

Other studies suggest that autophagy may play a protective role in sepsis. Sepsis is caused by an excessive immune response to bacteria and other microorganisms, characterized by over-secretion of proinflammatory cytokines, particularly IL-1. Mice lacking the autophagy protein LC3B produce high levels of IL-1β and IL-18 in response to LPS- and cecal ligation and puncture (CLP)-induced sepsis and are more susceptible to LPS-induced lethality (Nakahira *et al.* 2011). Conversely, mice injected intraperitoneally with the autophagy-inducing drug rapamycin showed reduce serum levels of IL-1β after challenge with LPS (Harris *et al.*, 2011) and were protected against cardiac dysfunction following CLP (Hsieh *et al.*, 2011).

Given that autophagy maintains cellular homeostasis and regulates the production of inflammatory cytokines, particularly IL-1β, its importance in preventing the development and pathology of inflammatory diseases is highly plausible. However, further investigations are needed to understand in detail the mechanisms of this contribution and to translate this knowledge into therapeutic approaches.

CONCLUSION

Inflammation is driven by the secretion of proinflammatory cytokines, in particular IL-1β. To date, two separate mechanisms have been described by which autophagy regulates the production and secretion of this cytokine. First, autophagy modulates inflammasome activation. Second, autophagy targets pro-IL-1β and inflammasome components for degradation in autophagosomes.

Through the regulation of IL-1β and other proinflammatory cytokines, such as IL-23, autophagy profoundly influences immunity and inflammation. Thus, it is not surprising

that genome-wide association studies have identified autophagy-related genes that confer susceptibility to various autoinflammatory and autoimmune diseases, including Crohn's disease, SLE and sepsis. Currently, autophagy represents a potent therapeutic target in such immune conditions and, although the effects of modulating autophagy systemically need to be further tested, evidence from animal models and early clinical trials is promising.

References

Brinar, M., Vermeire, S., Cleynen, I., et al., 2012. Genetic variants in autophagy-related genes and granuloma formation in a cohort of surgically treated Crohn's disease patients. J. Crohn's Colitis 6, 43–50.

Castillo, E.F., Dekonenko, A., Arko-Mensah, J., et al., 2012. Autophagy protects against active tuberculosis by suppressing bacterial burden and inflammation. Proc. Natl Acad. Sci. USA 109, E3168–E3176.

Craddock, N., Hurles, M.E., Cardin, N., et al., 2010. Genome-wide association study of CNVs in 16,000 cases of eight common diseases and 3,000 shared controls. Nature 464, 713–720.

Crisan, T.O., Plantinga, T.S., van de Veerdonk, F.L., et al., 2011. Inflammasome-independent modulation of cytokine response by autophagy in human cells. PloS ONE 6, e18666.

Cua, D.J., Sherlock, J., Chen, Y., et al., 2003. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421, 744–748.

Dinarello, C.A., 2009. Immunological and inflammatory functions of the interleukin-1 family. Ann. Rev. Immunol. 27, 519–550.

Dupont, N., Jiang, S., Pilli, M., et al., 2011. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1beta. EMBO J. 30, 4701–4711.

Fettelschoss, A., Kistowska, M., LeibundGut-Landmann, S., et al., 2011. Inflammasome activation and IL-1β target IL-1α for secretion as opposed to surface expression. Proc. Natl Acad. Sci. USA 108, 18055–18060.

Han, J.W., Zheng, H.F., Cui, Y., et al., 2009. Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat. Genet. 41, 1234–1237.

Harris, J., Hartman, M., Roche, C., et al., 2011. Autophagy controls IL-1β secretion by targeting pro-IL-1β for degradation. J. Biol. Chem. 286, 9587–9597.

Harris, K.M., Fasano, A., Mann, D.L., 2008. Cutting edge: IL-1 controls the IL-23 response induced by gliadin, the etiologic agent in celiac disease. J. Immunol. 181, 4457–4460.

Henckaerts, L., Cleynen, I., Brinar, M., et al., 2011. Genetic variation in the autophagy gene ULK1 and risk of Crohn's disease. Inflamm. Bowel Dis. 17, 1392–1397.

Hsieh, C.H., Pai, P.Y., Hsueh, H.W., et al., 2011. Complete induction of autophagy is essential for cardioprotection in sepsis. Ann. Surg. 253, 1190–1200.

Khader, S.A., Bell, G.K., Pearl, J.E., et al., 2007. IL-23 and IL-17 in the establishment of protective pulmonary $CD4^+$ T cell responses after vaccination and during *Mycobacterium tuberculosis* challenge. Nat. Immunol. 8, 369–377.

Koenders, M.I., Lubberts, E., Oppers-Walgreen, B., et al., 2005. Induction of cartilage damage by overexpression of T cell interleukin-17A in experimental arthritis in mice deficient in interleukin-1. Arthritis Rheum. 52, 975–983.

Kono, H., Orlowski, G.M., Patel, Z., et al., 2012. The IL-1-dependent sterile inflammatory response has a substantial caspase-1-independent component that requires cathepsin C. J. Immunol. 189, 3734–3740.

Li, P., Allen, H., Banerjee, S., et al., 1995. Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1 β and resistant to endotoxic shock. Cell 80, 401–411.

Littman, D.R., Rudensky, A.Y., 2010. Th17 and regulatory T cells in mediating and restraining inflammation. Cell 140, 845–858.

Mitroulis, I., Kambas, K., Chrysanthopoulou, A., et al., 2011. Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PloS ONE 6, e29318.

Moffitt, K.L., Gierahn, T.M., Lu, Y.J., et al., 2011. T(H)17-based vaccine design for prevention of *Streptococcus pneumoniae* colonization. Cell Host Microbe 9, 158–165.

Nakahira, K., Haspel, J.A., Rathinam, V.A., et al., 2011. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat. Immunol. 12, 222–230.

Ostendorf, B., Iking-Konert, C., Kurz, K., et al., 2005. Preliminary results of safety and efficacy of the interleukin 1 receptor antagonist anakinra in patients with severe lupus arthritis. Ann. Rheum. Dis. 64, 630–633.
Ouyang, W., Kolls, J.K., Zheng, Y., 2008. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 28, 454–467.
Peral de Castro, C., Jones, S.A., Ni Cheallaigh, C., et al., 2012. Autophagy regulates IL-23 secretion and innate T cell responses through effects on IL-1 secretion. J. Immunol. 189, 4144–4153.
Pierdominici, M., Vomero, M., Barbati, C., et al., 2012. Role of autophagy in immunity and autoimmunity, with a special focus on systemic lupus erythematosus. FASEB J. 26, 1400–1412.
Ramos, P.S., Criswell, L.A., Moser, K.L., et al., 2011. A comprehensive analysis of shared loci between systemic lupus erythematosus (SLE) and sixteen autoimmune diseases reveals limited genetic overlap. PLoS Genet. 7, e1002406.
Rioux, J.D., Xavier, R.J., Taylor, K.D., et al., 2007. Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat. Genet. 39, 596–604.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1β production. Nature 456, 264–268.
Schroder, K., Tschopp, J., 2010. The inflammasomes. Cell 140, 821–832.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Sims, J.E., Smith, D.E., 2010. The IL-1 family: regulators of immunity. Nat. Rev. Immunol. 10, 89–102.
So, A., De Smedt, T., Revaz, S., et al., 2007. A pilot study of IL-1 inhibition by anakinra in acute gout. Arthritis Res. Ther. 9, R28.
Sutton, C.E., Lalor, S.J., Sweeney, C.M., et al., 2009. Interleukin-1 and IL-23 induce innate IL-17 production from γδ T cells, amplifying Th17 responses and autoimmunity. Immunity 31, 331–341.
Uhlig, H.H., McKenzie, B.S., Hue, S., et al., 2006. Differential activity of IL-12 and IL-23 in mucosal and systemic innate immune pathology. Immunity 25, 309–318.
Weaver, C.T., Hatton, R.D., Mangan, P.R., et al., 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Ann. Rev. Immunol. 25, 821–852.
Zhou, R., Yazdi, A.S., Menu, P., et al., 2011a. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225.
Zhou, X.J., Lu, X.L., Lv, J.C., et al., 2011b. Genetic association of PRDM1-ATG5 intergenic region and autophagy with systemic lupus erythematosus in a Chinese population. Ann. Rheum. Dis. 70, 1330–1337.

CHAPTER

14

Role of Autophagy in P2X7 Receptor-Mediated Maturation and Unconventional Secretion of IL-1β in Microglia

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Abstract

Interleukin-1β (IL-1β) is a potent proinflammatory cytokine that is mainly produced by microglia in the central nervous system. It is considered to act as a key mediator of inflammatory processes not only in physiological conditions, but also during various pathological conditions, such as infection, injury, ischemia, and neurodegeneration. The mechanism through which such a leaderless protein is transferred from the cytoplasm to the extracellular space is an important unresolved issue in the study of IL-1β biology. Emerging evidence suggests that autophagy plays an important role in the unconventional secretion of IL-1β. Autophagy might negatively regulate IL-1β expression by lysosomal degradation, while mature IL-1β could be secreted by an autophagy-based Golgi reassembly stacking protein (GRASP)-dependent secretory pathway. We also found that activation of the P2X7 receptor (P2X7R), an ATP-gated cation channel, plays a critical role in the regulation of basal autophagy flux as well as the maturation and unconventional secretion of IL-1β in microglial cells. Taken together, better understanding of the role of the autophagy-lysosomal pathway in the maturation and secretion of IL-1β in microglia might provide a new strategy for targeting neuroinflammation in various pathological conditions.

INTRODUCTION

Interleukin-1β (IL-1β) is a potent proinflammatory cytokine that is secreted during acute and chronic inflammatory responses against microbial infection and injury. In peripheral tissues, IL-1β is produced and released by blood monocytes, macrophages, dendritic cells, and a variety of other nonimmune cells including keratinocytes. In the central nervous system (CNS), IL-1β is mainly produced by microglia, which are the resident macrophages in the brain, although astrocytes and neurons also seem to produce IL-1β, especially in the late phase of excitotoxic neuronal damage. IL-1β is initially synthesized as an inactive 33-kDa pro-cytokine (pro-IL-1β) in response to inflammatory stimuli such as the bacterial endotoxin lipopolysaccharide (LPS), and this inactive form accumulates in the cytoplasm. Secondary stimuli are required for the prompt activation of caspase-1 (formerly called IL-1β processing enzyme), which leads to the processing of pro-IL-1β into the mature 17-kDa form (mIL-1β) and its subsequent secretion into the extracellular milieu. The released mIL-1β then binds to a signaling receptor (IL-1R type-1) expressed on almost all cell types including neurons and astrocytes. The resultant signal is transmitted through downstream components including MyD88, IL-1 receptor-associated kinase (IRAK), and TRAF6, which leads to the activation of NF-κB and its translocation to the nucleus followed by the transcriptional induction of multiple genes encoding inflammatory cytokines and other proteins.

Although the synthesis, biological actions, receptor signaling, and transcription of IL-1β have been extensively characterized, it is still unclear how IL-1β crosses the cell membrane since pro-IL-1β lacks the signal sequence required for secretion via the conventional endoplasmic reticulum (ER)-Golgi secretory pathway. Unconventional secretory pathways are used for the secretion of many proinflammatory alarmins, including high mobility group box 1 protein (HMGB1), IL-1α, and IL-33. Thus, better understanding of the mechanisms through which leaderless proteins are delivered to the extracellular space should be a general goal in the field of inflammation. In this regard, it is worth noting that autophagy might be involved in the secretion of cytoplasmic proteins in addition to its primary role in protein degradation (Dupont *et al.*, 2011). In support of this view, we have shown that P2X7 receptor (P2X7R) activation by ATP modulates autophagic machinery in microglia

(Takenouchi *et al.*, 2009b). Since P2X7R plays a critical role in the unconventional secretion of mIL-1β (Ferrari *et al.*, 2006), here we discuss the possible role of the lysosome-autophagy pathway in P2X7R-mediated innate immune functions in microglial cells.

ROLE OF LYSOSOMES IN THE MATURATION OF IL-1β

Conventional and Secretory Lysosomes

Lysosomes are membrane-enclosed organelles that function as the digestive system of animal cells, serving both to degrade materials taken up from outside the cells and to digest the cells' own worn-out components. They contain about 50 different degradative enzymes, which can hydrolyze proteins, nucleic acids, carbohydrates, and lipids. These enzymes are acid hydrolases and are active at acidic pH (~5), but not at physiological pH; thus, the interior of lysosomes is acidic (pH 4.8) whereas the cytosol is slightly alkaline (pH 7.2). This pH differential is maintained by pumping H^+ ions from the cytosol across the lysosomal membrane using proton pumps. Lysosomes are involved in the digestion of macromolecules during endocytosis, phagocytosis, and autophagy.

A specialized class of lysosomes called "secretory lysosomes" has recently been recognized (Blott and Griffiths, 2002). Secretory lysosomes share features with both conventional lysosomes and secretory granules and are abundant in some cell types, such as hematopoietic cells and melanocytes. Macrophages contain abundant secretory lysosomes and utilize them to exert their innate immune functions. Through the exocytosis of secretory lysosomes, they can secrete not only lysosomal enzymes but also antimicrobial proteins and several cytokines. Microglia also contain an abundance of secretory lysosomes, and we observed that P2X7R activation by ATP induced the secretion of the lysosomal enzyme cathepsin D from microglial cells in an extracellular Ca^{2+}-dependent manner (Takenouchi *et al.*, 2009b, 2011). A recent study demonstrated that secretory lysosomes from microglia contain abundant ATP. In addition, they found that ATP-induced ATP secretion via lysosome exocytosis contributes to the regulation of microglial migration in the brain (Dou *et al.*, 2012).

Involvement of Lysosomal Enzymes in the Processing of Pro-IL-1β

In addition to caspase-1, several proteases, such as proteinase 3, cathepsin G, chymase, collagenase, and elastase, have been proposed to contribute to the processing of pro-IL-1β (Dinarello, 2009). Recent studies have also suggested the involvement of the lysosomal enzyme cathepsin B in the regulatory pathways involved in pro-IL-1β maturation.

Silica and aluminum salt crystals are able to induce the production and secretion of mIL-1β by activating the NALP3 inflammasome in LPS-primed monocytes/macrophages (Hornung *et al.*, 2008). The NALP3 inflammasome is a multiprotein complex formed by the cytoplasmic pattern-recognition receptor NALP3 (also known as cryopyrin or NLRP3), the adaptor protein ASC (apoptosis-associated speck-like protein containing a carboxy-terminal CARD domain), and caspase-1. It mediates the activation of caspase-1, leading to the maturation and secretion of IL-1β (Figure 14.1A) (Ogura *et al.*, 2006). Phagocytosis of these crystals subsequently leads to lysosomal damage and rupturing, which seems to be required for

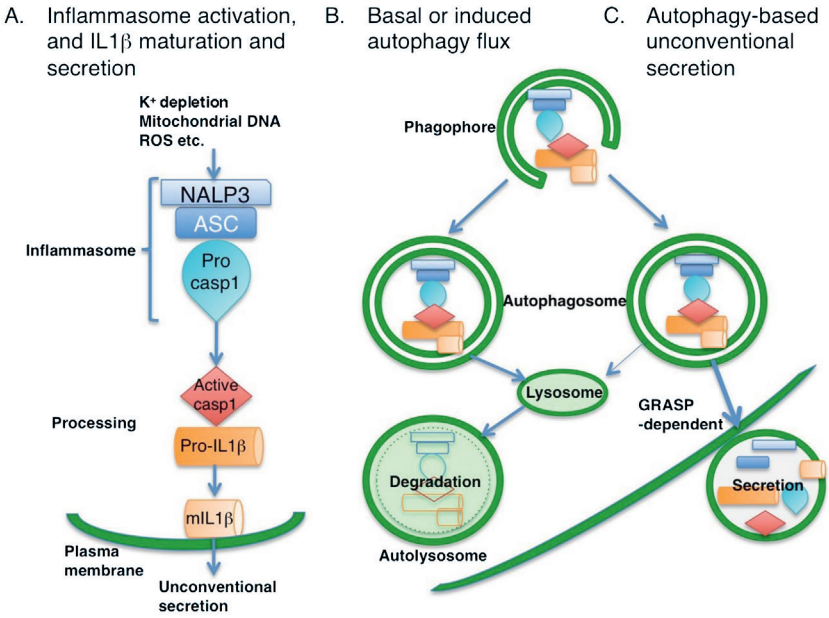

FIGURE 14.1 A schematic representation of the role of autophagy in IL-1β maturation and secretion in monocyte/macrophage lineage cells. The inflammasome, a multiprotein complex consisting of NALP3, ASC, and procaspase-1 (Procasp1), is activated by various stimuli (e.g., K^+ depletion, mitochondrial DNA, and ROS), which leads to the production of activated caspase-1 (Active casp1) followed by the processing of pro-IL-1β to mIL-1β and its subsequent secretion into the extracellular space (A). If basal or induced autophagy flux occurs, the autophagosomes formed after phagophore initiation can sequester pro-IL-1β, mIL-1β, and/or inflammasome components, and contribute to the routine degradation of these proteins via the formation of autolysosomes (B). This process probably plays a negative role in the production of mIL-1β and is considered to be involved in the normal regulation of inflammatory responses. Also, autophagosomal components seem to be directly secreted from the cells in a GRASP-dependent manner (C). This autophagy-based unconventional secretory pathway was recently proposed to explain the unconventional secretion of mIL-1β.

NALP3 inflammasome activation. Since cathepsin B inhibitors can suppress such crystal-induced mIL-1β secretion, it was suggested that crystal-mediated lysosomal destabilization results in the release of cathepsin B into the cytoplasm and that the enzymatic activity of cathepsin B participates in inflammasome activation. Another study reported that fibrillar amyloid β-peptide activates the NALP3 inflammasome in a cathepsin B-dependent manner and causes mIL-1β to be secreted from LPS-primed microglia (Halle *et al.*, 2008). Similar to crystal-induced inflammasome activation in macrophages, the phagocytosis of amyloid β-peptide fibrils and the subsequent induction of lysosomal damage, which is accompanied by the release of cathepsin B into the cytoplasm, have been shown to be necessary for the activation of the NALP3 inflammasome in microglia. The involvement of cathepsin B in the production and secretion of mIL-1β is also observed in microglia stimulated with chromogranin A, an acidic glycophosphoprotein that is found in the secretory granules of neurons and endocrine cells (Terada *et al.*, 2010).

AUTOPHAGY MIGHT REGULATE THE MATURATION AND SECRETION OF IL-1β

Role of Autophagy in the Innate Immune System

Autophagy is a major degradation/recycling system that plays various important roles in maintaining cellular homeostasis in living cells. Autophagy consists of several sequential steps: i.e., the sequestration of a protein or cellular component, its transportation to a lysosome, its degradation, and the utilization of the degradation products. During autophagy, cytoplasmic materials and organelles are randomly sequestered into newly emerging double-membrane vesicles called autophagosomes and delivered for lysosomal degradation via the fusion of the organelle-containing autophagosomes with lysosomes (autolysosomes). Autophagy is subclassified into "basal" and "induced" autophagy, depending on its role (Mizushima, 2007). Basal autophagy constantly occurs at a basal level in most cells, which contributes to the routine turnover of proteins and cytoplasmic components. Induced autophagy can be triggered by changes in environmental or cellular conditions, such as nutrient starvation, ER stress, and mitochondrial dysfunction, and allows a cell to reutilize the digested components or eliminate unfolded proteins and damaged organelles. More than 30 autophagy-related (Atg) proteins have been identified and characterized in yeast and mammals. Numerous studies have shown that autophagy is associated with various physiological functions and disease processes, such as cancer and neurodegenerative diseases.

Recently, the critical role played by autophagy in the innate immune system has been recognized (Saitoh and Akira, 2010). Several ligands for Toll-like receptors (TLR), a type of pattern recognition receptor that initiates innate immune responses, can induce autophagy in macrophages, which leads to the rapid killing and degradation of invading microorganisms (Delgado *et al.*, 2008). It has also been reported that endotoxin-induced inflammasome activation is enhanced in macrophages derived from Atg16L1-deficient and Atg7-knockout mice, in which basal autophagy is impaired (Saitoh *et al.*, 2008). In addition, the depletion of other autophagy-related proteins – e.g., LC3B or Beclin-1 – has been shown to enhance inflammasome activation in mouse macrophages (Nakahira *et al.*, 2011). Since the inflammasome plays a central role in the activation of caspase-1, which leads to the maturation and secretion of IL-1β, these studies suggest that the autophagy system plays a critical involvement in the regulation of inflammatory responses.

Control of the Maturation and Secretion of IL-1β by Autophagy

Recent studies have revealed that autophagy-related proteins can regulate the production and secretion of mIL-1β in macrophages. Atg16L1 is an essential component of the autophagic machinery and has also been identified as a candidate gene for susceptibility to inflammatory bowel disease including Crohn's disease. It was reported that Atg16L1-deficient macrophages produced large amounts of mIL-1β *in vitro* in response to LPS, a ligand for TLR4 (Saitoh *et al.*, 2008). Interestingly, the expression levels of pro-IL-1β mRNA and protein detected in Atg16L1-deficient macrophages after LPS stimulation were comparable to those observed in wild-type cells, which is indicative of abnormal post-translational regulation. In this context, the activation of caspase-1 and induction of reactive oxygen

species (ROS) generation by Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF) have been shown to be correlated with the enhanced production of mIL-1β after LPS stimulation (Figure 14.1A). In this report, the ATP-induced production of mIL-1β was also enhanced in Atg16L1-deficient macrophages primed with LPS. This suggests that the impairment of basal autophagy due to Atg16L1 deficiency affects P2X7R-mediated inflammasome activation in LPS-primed macrophages.

The augmentation of mIL-1β production via the impairment of basal autophagy has been observed in autophagy-related protein deficient settings. For example, the depletion of LC3B and Beclin-1 has been demonstrated to enhance ATP-induced caspase-1 activation and mIL-1β production in LPS-primed macrophages (Nakahira *et al.*, 2011). As for the mechanism responsible for these effects, it was proposed that the depletion of autophagic proteins promotes the release of mitochondrial DNA into the cytoplasm via NALP3 inflammasome activation and ROS generation, which results in the enhanced production of mIL-1β (Figure 14.1A). In addition, the involvement of mitochondrial degradation by autophagy (i.e., mitophagy) in the activation of the NALP3 inflammasome was demonstrated by another study, in which the dysregulation of mitophagy resulted in the cytoplasmic accumulation of mitochondrial DNA and excess ROS generation (Zhou *et al.*, 2011). Collectively, these studies suggest that autophagy flux exerts a suppressive effect on the production of mIL-1β and serves to aid the normal regulation of inflammatory responses in macrophages.

Similar to the results obtained using autophagic protein-deficient mice, the inhibition of autophagy with the type III PI3 kinase inhibitors 3-methyladenine and wortmannin enhanced LPS-induced mIL-1β production and secretion in macrophages (Harris *et al.*, 2011). The latter study suggested that autophagy flux suppresses the production of mIL-1β through at least two separate mechanisms: by targeting pro-IL-1β for lysosomal degradation (Figure 14.1B) and by inhibiting the activation of the NALP3 inflammasome by reducing ROS generation. It has also been reported that the autophagy induced by inflammatory stimuli suppresses the production of mIL-1β by targeting ubiquitinated inflammasome components (e.g., ubiquitinated ASC) for lysosomal degradation (Figure 14.1B) (Shi *et al.*, 2012).

In contrast, Dupont *et al.* (2011) reported the opposite result: i.e., that induced autophagy augments the NALP3 inflammasome-dependent production and secretion of mIL-1β in LPS-primed macrophages. The upregulation of mIL-1β secretion by induced autophagy seems to depend on mammalian Golgi reassembly stacking protein (GRASP) 55 and Rab8a. To explain the discrepancies between the effects of basal and induced autophagy on mIL-1β production and secretion, Deretic *et al.* (2012) proposed that autophagy negatively controls inflammasome activation as well as mIL-1β production via the routine degradation of inflammasome components and/or IL-1β proteins (Figure 14.1B), but positively controls secretory pathways for mIL-1β in a GRASP-dependent manner (Figure 14.1C).

The primary role of autophagosomes is considered to be the delivery of cargos to lysosomes for digestion. However, a growing body of evidence has revealed that autophagosomes are also involved in the pathways responsible for unconventional protein secretion and the trafficking of integral membrane proteins to the plasma membrane (Deretic *et al.*, 2012). In yeast, the unconventional secretion of an acyl coenzyme A-binding protein (Acb1) is reported to require the formation of autophagosomes as well as the expression of Grh1, a yeast homologue of GRASP protein (Duran *et al.*, 2010; Manjithaya *et al.*, 2010). GRASP55 is also known to be required for the autophagy-dependent unconventional secretion of mIL-1β

in mammalian cells (Dupont *et al.*, 2011). Thus, this novel role of autophagy in the regulation of unconventional secretory pathways has attracted much interest.

Studying the membrane biology of autophagosomes might increase our understanding of the connections between autophagic machinery and unconventional protein secretion. Although the origin of autophagosomal membranes is still unclear, at least three models have been proposed (Deretic *et al.*, 2012). Autophagosomes are considered to emerge at least in part from ER membranes, whereas in models highlighting mitochondria, the outer mitochondrial membrane is postulated to participate in autophagosome biogenesis. In addition, plasma membrane-derived Atg16L1 positive vesicles seem to contribute to autophagic membrane growth. These membrane properties might help to explain the links between the autophagy system and unconventional protein secretion or the intracellular trafficking of integral membrane proteins to plasma membranes.

P2X7R-MEDIATED MATURATION AND UNCONVENTIONAL SECRETION OF IL-1β

Functional Expression of P2X7R in Microglia

Extracellular ATP is a stimulator of mIL-1β production and secretion from LPS-primed microglia/macrophages and acts as an endogenous danger signal to alert and activate innate immune cells. Accumulating evidence suggests that P2X7R, a purinoceptor, is critically involved in this event (Ferrari *et al.*, 2006). P2X7R activation by higher concentrations of ATP contributes to the activation of the NALP3 inflammasome, which leads to caspase-1 activation and IL-1β maturation (Figure 14.2A) (Ogura *et al.*, 2006). P2X7R is also involved in the innate immune functions of microglia/macrophages, including the killing of intracellular mycobacteria and the regulation of inflammatory responses (Wiley *et al.*, 2011).

Microglia express various P2 purinoceptors and can receive extracellular signals mediated by ATP in the brain. ATP is released not only from normal neurons and astrocytes, but also from damaged cells in some pathological conditions. P2 purinoceptors are broadly classified into P2X ionotropic and P2Y metabotropic receptors. P2X receptors are ligand-gated ion channels, while P2Y receptors belong to the G protein-coupled receptor family. Among the P2X purinoceptors expressed by microglia, P2X7R is the major form and has been characterized in several studies (North, 2002). P2X7R activation plays important roles in the immune functions of microglia, such as the production of inflammatory cytokines, ROS, and nitric oxide. Activated P2X7R channels become highly permeable to cations, such as Na^+, K^+, and Ca^{2+}, which leads to the activation of multiple intracellular signaling pathways such as the p44/42 extracellular-signal regulated kinase, p38 mitogen-activated protein kinase, c-Jun N-terminal kinase, AMP-activated protein kinase-α, mammalian target of rapamycin/S6 kinase, NF-κB, and nuclear factor of activated T cells signaling pathways (Takenouchi *et al.*, 2010). P2X7R-mediated K^+ efflux is critical for NALP3 inflammasome activation (Figure 14.2A). P2X7R activation also promotes the formation of large nonselective membrane pores, through which hydrophilic molecules of less than 800 Da can pass. The prolonged formation of such membrane pores due to the presence of increased concentrations of ATP ultimately results in necrotic cytolysis.

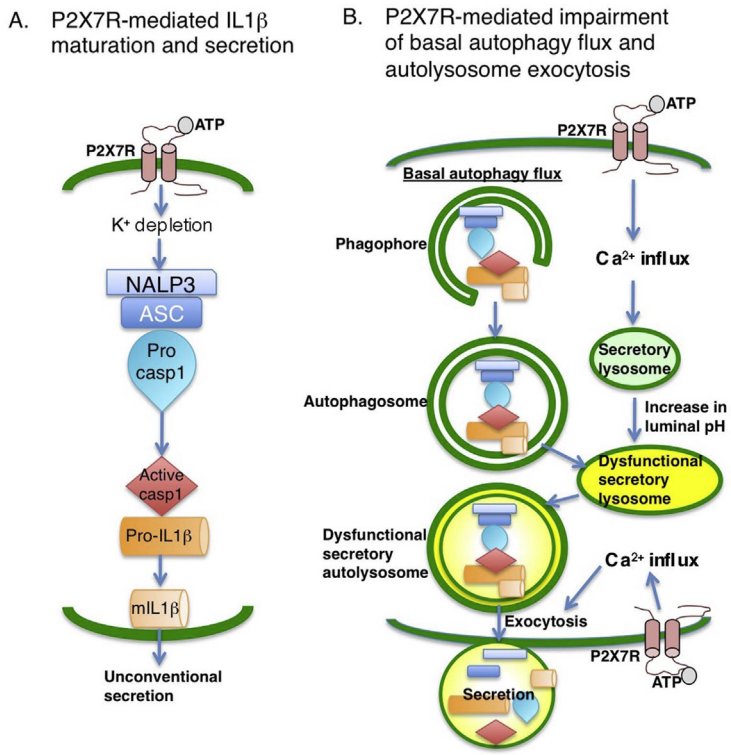

FIGURE 14.2 Schematic model of the putative correlation between P2X7R-mediated impairment of basal autophagy flux and unconventional mIL-1β secretion in LPS-primed microglial cells. The activation of P2X7R channels by ATP elicits K^+ efflux, and the subsequent depletion of cytoplasmic K^+ triggers the activation of the NALP3 inflammasome followed by the activation of caspase-1 and the production and unconventional secretion of mIL-1β (A). At the same time, P2X7R activation induces secretory lysosome dysfunction by increasing lysosomal pH in a Ca^{2+} influx-dependent manner (B). Therefore, pro-IL-1β, mIL-1β, and/or inflammasome components that are sequestered into autophagosomes might remain almost intact, even if autolysosomes are formed by basal autophagy flux (B). Eventually, these proteins could be secreted from the cells through the exocytosis of dysfunctional secretory autolysosomes (B). It is possible that P2X7R-mediated Ca^{2+} influx triggers the exocytosis of such secretory lysosome-related organelles (B).

P2X7R-Mediated Unconventional Secretion Pathway for mIL-1β

At least four models have been proposed to explain the P2X7R-mediated unconventional secretion of mIL-1β from monocyte/macrophage lineage cells (Dubyak, 2012). According to the simplest and oldest model, cytolysis induced by P2X7R activation triggers the activation of caspase-1, causing mIL-1β to be secreted through cytolytic pores. However, emerging evidence has revealed that the pathways leading to mIL-1β release are not explained by simple cell lysis alone. A second model has been proposed in which pro-IL-1β and pro-caspase-1 are transported into lysosomes through unknown transporters, and pro-IL-1β is processed into mIL-1β within lysosomes, before the lysosomal components are secreted from the cells

via exocytosis (Andrei *et al.*, 2004). In the third model, P2X7R stimulation initiates the local accumulation of inflammasome components and pro-IL-1β within the microdomain of the subplasma membrane cytoplasm, and then microvesicles containing the mIL-1β are formed via the evagination of plasma membrane blebs and rapidly shed from the cell surface. As a fourth model, exosomes derived from intraluminal vesicles within multivesicular bodies have been suggested to be involved in the P2X7R-stimulated unconventional secretion of mIL-1β from LPS-primed macrophages. Among these models, microvesicle shedding seems to be the most probable mechanism for the P2X7R-mediated secretion of mIL-1β from LPS-primed microglial cells (Bianco *et al.*, 2005). However, these mechanisms are not mutually exclusive and might represent parallel or intersecting membrane trafficking responses to P2X7R activation (Dubyak, 2012).

Regarding the lysosome-based model, secretory lysosomes have been reported to be involved in the P2X7R-mediated unconventional secretion of mIL-1β in LPS-primed macrophages (Andrei *et al.*, 2004). It has been proposed that pro-IL-1β and pro-caspase-1 are transported into secretory lysosomes in response to P2X7R activation, and that pro-IL-1β is processed by activated caspase-1 within lysosomal compartments, before the resultant mIL-1β is secreted from the cells via the exocytosis of secretory lysosomes. Although unknown transporters are postulated to be involved in the translocation of pro-IL-1β/pro-caspase-1 into the lysosomal lumen, the underlying mechanisms remain elusive. Considering the role of autophagy in the targeting of pro-IL-1β and/or inflammasome components for lysosomal degradation (Harris *et al.*, 2011; Shi *et al.*, 2012), it is easy to speculate that cytoplasmic pro-IL-1β and pro-caspase-1 are sequestered into autophagosomes by basal autophagy and delivered to the lysosomal lumen through autolysosome formation. If lysosomal functions are compromised for some reason (e.g., by P2X7R activation) (Figure 14.2B), the pro-IL-1β and pro-caspase-1 might remain intact and could be used for mIL-1β production within autolysosomes. This idea might partly explain the secretory lysosome-related pathway involved in the unconventional secretion of mIL-1β.

Interestingly, we recently observed that P2X7R activation induces the production and secretion of an unconventional 20-kDa form of IL-1β (p20-IL-1β) in LPS-primed microglia (Takenouchi *et al.*, 2011). We found that acidic extracellular conditions and cathepsin D enzymatic activity are required for the production and secretion of p20-IL-1β. Since extracellular acidosis develops at sites of inflammation and infection, p20-IL-1β might act as a novel modulator of mIL-1β production under pathophysiological conditions.

P2X7R-Mediated Regulation of Autophagy

The P2X7R signaling pathway has been reported to be involved in the regulation of autophagy. The membrane-associated form of LC3 (LC3-II) is often used as a marker of autophagosome formation, while LC3 is a cytoplasmic microtubule-associated protein (LC3-I) (Mizushima and Yoshimori, 2007). Using human THP-1 cells and human monocyte-derived macrophages, Biswas *et al.* (2008) reported that P2X7R activation by ATP increases the expression of LC3-II in an extracellular Ca^{2+}-dependent manner and induces the formation of autophagosome-like structures. They suggested that P2X7R activation induces conventional autophagy and that induced autophagy positively contributes to the ATP-induced rapid killing of intracellular mycobacteria in *Mycobacterium bovis* BCG-infected macrophages.

Similarly, we reported that the expression of LC3-II and the formation of autophagosome-like structures are upregulated by the activation of P2X7R in mouse microglial cells (Takenouchi *et al.*, 2009b). However, we also showed that P2X7R activation induces the dysregulation of lysosomal functions by increasing lysosomal pH in a Ca^{2+} influx-dependent manner and triggers the secretion of components derived from lysosome-related organelles, such as phagolysosomes and autolysosomes, via exocytosis (Figure 14.2B). Based on these findings, we proposed that P2X7R activation results in the impairment of basal autophagy flux via lysosome dysfunction and promotes the secretion of phagolysosomal/autolysosomal components to further eliminate undigested components or bacteria by exposing them to digestion or rapid killing by adjacent neutrophils or macrophages.

The precise reason for the discrepancies between our results and those of Biswas *et al.* is unclear. However, they could be due to the differences between the two experimental systems; i.e., while monocytes/THP-1 cells and live mycobacteria were used by Biswas *et al.*, microglial cells and inactivated *E. coli* were employed in our study. Future studies are required to demonstrate whether the P2X7R activation-based mechanisms regulating lysosomal fusion and exocytotic processes differ between immune cell types and different pathogens.

Role of Autophagy in the P2X7R-Mediated Maturation and Secretion of IL-1β

Since P2X7R plays a key role in the production and secretion of mIL-1β (Figure 14.2A), P2X7R-mediated impairment of basal autophagy flux might participate in the regulatory pathways involved in the ATP-induced production and secretion of mIL-1β in LPS-primed microglial cells (Takenouchi *et al.*, 2009a). As suggested by several recent studies (Harris *et al.*, 2011; Nakahira *et al.*, 2011; Saitoh *et al.*, 2008; Shi *et al.*, 2012), the suppression of basal autophagy flux could have positive effects on the ATP-induced production and secretion of mIL-1β, possibly via the comparative upregulation of pro-IL-1β, inflammasome components, and ROS generation. From another point of view, P2X7R-mediated lysosome dysfunction might result in the accumulation of IL-1β proteins and inflammasome components within autolysosomes (Figure 14.2B). If this is true, mIL-1β could be secreted from the cells via the exocytosis of secretory autolysosomes (Figure 14.2B). This idea is supported by a previous study showing that secretory lysosomes play a key role in the production and unconventional secretion of mIL-1β (Andrei *et al.*, 2004).

On the other hand, according to Biswas *et al.* (2008), it is also considered that induced autophagy plays a role in the promotion of mIL-1β secretion after P2X7R activation. This idea is supported by a recent study showing that induced autophagy positively contributes to the unconventional secretion of mIL-1β (Dupont *et al.*, 2011). Although further experiments will be required to verify these hypotheses, the autophagic pathway could be an important target for the regulation of inflammatory responses through the modulation of the P2X7R-mediated production and secretion of mIL-1β by microglia/macrophages.

P2X7R-Mediated Secretion of IL-1β as a Therapeutic Target in Neurodegenerative Disease

Since IL-1β is a key mediator of inflammatory processes in physiological conditions, the biosynthesis and secretion of its biologically active form are strictly controlled (Dinarello, 2009). However, IL-1β might be harmful in impaired CNS in which

several neurotransmission systems are severely dysregulated. Indeed, the expression of IL-1β is known to be associated with the pathogenesis of various neurological diseases. The increased production of IL-1β is observed in acute neurological disorders including cerebral ischemia, traumatic brain injury, and spinal cord injury, as well as chronic neurodegenerative disorders including Alzheimer's disease and Parkinson's disease. The progression of these neurological disorders seems to be related to the detrimental effects of IL-1β. In line with this, the suppression or modulation of the dysregulated IL-1β responses of microglia might aid the development of therapeutic interventions for neuroinflammatory/neurodegenerative diseases.

This view has led us to speculate that the blockade of P2X7R could be an effective therapeutic strategy for neurodegenerative diseases, such as prion disease. Accordingly, we evaluated the effect of Brilliant Blue G (BBG), a P2X7R antagonist, on a mouse model of prion disease in order to determine its therapeutic potential. The *in vivo* administration of BBG reduced the accumulation of the pathogenic isoforms of prion protein in the brains of mice with prion disease, but did not appear to slow disease progression (Iwamaru *et al.*, 2012). Continued study of BBG as a potential anti-prion compound is warranted since our results suggest that P2X7R plays a complex role in neuronal degeneration in prion diseases.

In conclusion, recent studies have suggested that the autophagy-lysosomal pathway is involved in the regulation of the production and secretion of mIL-1β. In particular, since P2X7R plays a crucial role in mediating the production and secretion of mIL-1β by LPS-primed microglia, further insights into the role of P2X7R-mediated modulation of the autophagy-lysosomal pathway during mIL-1β secretion might aid the development of therapeutic interventions for neuroinflammatory/neurodegenerative diseases.

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References

Andrei, C., Margiocco, P., Poggi, A., et al., 2004. Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: implications for inflammatory processes. Proc. Natl Acad. Sci. USA 101, 9745–9750.

Bianco, F., Pravettoni, E., Colombo, A., et al., 2005. Astrocyte-derived ATP induces vesicle shedding and IL-1 beta release from microglia. J. Immunol. 174, 7268–7277.

Biswas, D., Qureshi, O.S., Lee, W.Y., et al., 2008. ATP-induced autophagy is associated with rapid killing of intracellular mycobacteria within human monocytes/macrophages. BMC Immunol. 9, 35.

Blott, E.J., Griffiths, G.M., 2002. Secretory lysosomes. Nat. Rev. Mol. Cell Biol. 3, 122–131.

Delgado, M.A., Elmaoued, R.A., Davis, A.S., et al., 2008. Toll-like receptors control autophagy. EMBO J. 27, 1110–1121.

Deretic, V., Jiang, S., Dupont, N., 2012. Autophagy intersections with conventional and unconventional secretion in tissue development, remodeling and inflammation. Trends Cell Biol. 22, 397–406.

Dinarello, CA., 2009. Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. 27, 519–550.

Dou, Y., Wu, H.J., Li, H.Q., et al., 2012. Microglial migration mediated by ATP-induced ATP release from lysosomes. Cell Res. 22, 1022–1033.

Dubyak, G.R., 2012. P2X7 receptor regulation of non-classical secretion from immune effector cells. Cell. Microbiol. 14, 1697–1706.
Dupont, N., Jiang, S., Pilli, M., et al., 2011. Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. EMBO J. 30, 4701–4711.
Duran, J.M., Anjard, C., Stefan, C., et al., 2010. Unconventional secretion of Acb1 is mediated by autophagosomes. J. Cell Biol. 188, 527–536.
Ferrari, D., Pizzirani, C., Adinolfi, E., et al., 2006. The P2X7 receptor: a key player in IL-1 processing and release. J. Immunol. 176, 3877–3883.
Halle, A., Hornung, V., Petzold, G.C., et al., 2008. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat. Immunol. 9, 857–865.
Harris, J., Hartman, M., Roche, C., et al., 2011. Autophagy controls IL-1beta secretion by targeting pro-IL-1beta for degradation. J. Biol. Chem. 286, 9587–9597.
Hornung, V., Bauernfeind, F., Halle, A., et al., 2008. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. 9, 847–856.
Iwamaru, Y., Takenouchi, T., Murayama, Y., et al., 2012. Anti-prion activity of Brilliant Blue G. PLoS ONE 7, e37896.
Manjithaya, R., Anjard, C., Loomis, W.F., et al., 2010. Unconventional secretion of *Pichia pastoris* Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. J. Cell Biol. 188, 537–546.
Mizushima, N., 2007. Autophagy: process and function. Genes Dev. 21, 2861–2873.
Mizushima, N., Yoshimori, T., 2007. How to interpret LC3 immunoblotting. Autophagy 3, 542–545.
Nakahira, K., Haspel, J.A., Rathinam, V.A., et al., 2011. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat. Immunol. 12, 222–230.
North, R.A., 2002. Molecular physiology of P2X receptors. Physiol. Rev. 82, 1013–1067.
Ogura, Y., Sutterwala, F.S., Flavell, R.A., 2006. The inflammasome: first line of the immune response to cell stress. Cell 126, 659–662.
Saitoh, T., Akira, S., 2010. Regulation of innate immune responses by autophagy-related proteins. J. Cell Biol. 189, 925–935.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Shi, C.S., Shenderov, K., Huang, N.N., et al., 2012. Activation of autophagy by inflammatory signals limits IL-1β production by targeting ubiquitinated inflammasomes for destruction. Nat. Immunol. 13, 255–263.
Takenouchi, T., Fujita, M., Sugama, S., et al., 2009a. The role of the P2X7 receptor signaling pathway for the release of autolysosomes in microglial cells. Autophagy 5, 723–724.
Takenouchi, T., Nakai, M., Iwamaru, Y., et al., 2009b. The activation of P2X7 receptor impairs lysosomal functions and stimulates the release of autophagolysosomes in microglial cells. J. Immunol. 182, 2051–2062.
Takenouchi, T., Iwamaru, Y., Sugama, S., et al., 2011. The activation of P2X7 receptor induces cathepsin D-dependent production of a 20-kDa form of IL-1β under acidic extracellular pH in LPS-primed microglial cells. J. Neurochem. 117, 712–723.
Takenouchi, T., Sekiyama, K., Sekigawa, A., et al., 2010. P2X7 receptor signaling pathway as a therapeutic target for neurodegenerative diseases. Arch Immunol. Ther. Exp. (Warsz) 58, 91–96.
Terada, K., Yamada, J., Hayashi, Y., et al., 2010. Involvement of cathepsin B in the processing and secretion of interleukin-1beta in chromogranin A-stimulated microglia. Glia 58, 114–124.
Wiley, J.S., Sluyter, R., Gu, B.J., et al., 2011. The human P2X7 receptor and its role in innate immunity. Tissue Antigens 78, 321–332.
Zhou, R., Yazdi, A.S., Menu, P., et al., 2011. A role for mitochondria in NLRP3 inflammasome activation. Nature 469, 221–225.

CHAPTER

15

Autophagy Restricts Interleukin-1β Signaling via Regulation of P62 Stability

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OUTLINE

Abstract

Atg16L1 (autophagy-related 16-like 1), an essential component of autophagy, interacts with the Atg12-Atg5 conjugate for formation of autophagosome. A single nucleotide polymorphism (SNP) in Atg16L1 was identified by genome-wide association studies (GWASs) as a risk factor in Crohn's disease (CD), along with other autophagy-related genes including *LRRK2*, *NOD2*, and *IRGM*. These findings suggest that Atg16L1 exerts anti-inflammatory activities in the gastrointestinal (GI) tract. Atg16L1 in macrophages suppresses post-transcriptional maturation of IL-1β in response to TLR4 stimulation, and it regulates the granule exocytosis pathway in Paneth cells specialized in secreting antimicrobial peptides in the GI tract. In addition, it promotes clearance of phagocytosed bacteria in epithelial cells. We investigated how autophagy regulates the innate response to IL-1β. We found that the IL-1β signal transduction pathways are significantly amplified in Atg16L1- or Atg5-deficient mouse embryonic fibroblasts (MEF). Accumulated p62 in autophagy-deficient cells was solely responsible for the amplified IL-1β signaling. We demonstrated that in addition to the autolysosomal degradative pathway, p62 is also degraded by the ubiquitination proteasomal system (UPS). Our genetic and biochemical analyses revealed that Cullin-3 is the E3 ubiquitin ligase of p62, and that Atg16L1 mediates neddylation of Cullin-3, a critical prerequisite for its activation. Taken together, we provided a novel mechanism by which Atg16L1 suppresses a proinflammatory signal.

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INTRODUCTION

Inflammatory bowel diseases (IBD), i.e., Crohn's disease (CD) and ulcerative colitis (UC), significantly impact quality of life and account for substantial public health burden. The etiology of IBD is complex where genetic and environmental factors together with intestinal microbiota initiate chronic inflammation in the gastrointestinal tract.

Recent genome-wide association studies (GWAS) identified a large number of risk factors: 71 CD-associated genes, 47 UC-associated, and 28 shared by CD and UC (Anderson *et al.*, 2011; Franke *et al.*, 2010). These data suggest that these risk factors are components of several common pathways such as microbial recognition, autophagy, and epithelial barrier function.

Most of the autophagy related genes identified as IBD risk variants [*ATG16L1*, *IRGM*, *NOD2* and *LRRK2* (Hugot *et al.*, 2001; Ogura *et al.*, 2001)] are thought to be CD-specific susceptibility markers. The CD-associated *ATG16L1* variant has a nonsynonymous single nucleotide polymorphism (SNP), a threonine-to-alanine change at position 300 (T300A) in the conserved WD-repeat domain, a protein-to-protein interaction domain. Impaired antibacterial autophagy was observed in epithelial cells line expressing *ATG16L1* T300A (Homer *et al.*, 2010; Kuballa *et al.*, 2008). However, no significant difference was observed in antibacterial autophagy in primary human macrophages or dendritic cells from the donors homozygous for the T300A allele compared to the nonrisk allele (Homer *et al.*, 2010).

Functional deletion of *Atg16L1* in hematopoietic cells in fetal liver chimeric mice caused hyper-production of IL-1β and Il-18 in response to LPS stimulation (Saitoh *et al.*, 2008). The chimeric mice were highly susceptible to dextran sulfate sodium (DSS)-induced colitis mainly due to the high level of IL-1β production. Similarly, overproduction of IL-1β was observed in peripheral blood monocytes from the donors with the *ATG16L1* risk allele (Plantinga *et al.*, 2011).

Atg16L1 hypomorphs with expression of Atg16L1 reduced to about 30% of wild type (WT) did not develop spontaneous colitis (Cadwell *et al.*, 2008). However, *Atg16L1* hypomorphs presented striking abnormalities in Paneth cells. The phenotype, aberrent packaging and exocytosis of granules was reminiscent of that in CD patients homozygous for the *ATG16L1* risk allele. Later, it was found that these abnormalities were triggered by murine norovirus (MNV, CR6) (Cadwell *et al.*, 2010). These Atg16L1 functions are tied to its role in autophagy since *Atg5*-deficient cells exhibit the same phenotype when compared.

P62 (Sequestosome-1, SQSTM1) is a multitasking protein involved in autophagy and signal transduction. P62 acts as a selective autophagy receptor for the ubiquitinated protein aggregates to be degraded by autolysosome (Knaevelsrud and Simonsen *et al.*, 2010; Shaid *et al.*, 2012). It accumulates at a high level in cells with dysfunctional autophagy because it is also a substrate for autophagosome (Bjorkoy *et al.*, 2005). P62 is typically contained in ubiquitinated protein aggregates found in various human diseases, including neurodegenerative, liver, and muscle disorders (Knaevelsrud and Simonsen, 2010). In addition to its role in autophagy, p62 acts as an important scaffold protein in the IL-1β signaling pathway by promoting oligomerization of ubiquitinated TRAF6 (Sanz *et al.*, 2000) and MyD88 (Into *et al.*, 2010), or as an adaptor protein in Nrf2-induced expression of antioxidative response genes (Komatsu *et al.*, 2010).

Protein ubiquitination is carried out by the sequential action of three enzymes, E1, E2, and E3, and the substrate specificity is mainly determined by E3. Cullin-3 is an E3 ubiquitin

ligase for various substrates and conjugation of Nedd8 to Cullin-3 (neddylation) confers conformational changes to Cullin-3 (Liu and Nussinov (2010)), the critical step for its dimerization and activation (Hotton and Callis, 2008; Wimuttisuk and Singer, 2007). P62 interacts with Keap1, a component of Cullin-3 ubiquitin ligase for Nrf2, which inhibits ubiquitination of Nrf2 (Komatsu *et al.*, 2010).

Role of Atg16L1 in TLR Signaling

Toll-like receptors (TLRs) are the major innate immune sensors detecting specific molecular patterns on microbes and thus are critical in maintaining intestinal homeostasis where commensal microbes are abundant (Rakoff-Nahoum *et al.*, 2004). Several TLRs activate autophagy by which phagocytosed bacteria are eliminated (Saitoh *et al.*, 2008; Xu *et al.*, 2007). We investigated how autophagy regulates TLR or proinflammatory cytokine signaling using *Atg16L1*- and *Atg5*-deficient mouse embryonic fibroblasts (MEF). While TLR2, TLR4, or TNF-α signaling pathways were not significantly affected by autophagy deficiency, IL-1β signaling pathways were markedly enhanced in both *Atg16L1*- and *Atg5*-deficient MEF (Lee *et al.*, 2012). The enhanced effect of IL-1β-induced signaling pathways was more pronouced in *Atg16L1*-deficient MEF than in *Atg5*-deficient MEF, though the reason for this difference is unclear. We found that IL-1β also activates autophagy in MEF (Lee *et al.*, 2012). This indicated that autophagy in general suppresses IL-1β-induced signaling pathways.

Regulation of p62 Stability and IL-1β Signal Transduction by Autophagy

Both IL-1β-induced NF-κB and MAP kinase pathways were enhanced in *Atg16L1*-deficient MEF, which indicated that Atg16L1 regulates a molecule(s) downstream to the receptor but upstream to these pathways, namely the MyD88-IRAK-TRAF6-TAK1 pathway. P62 serves as a signaling hub through its ubiquitin binding domain by recruiting and oligomerizing ubiquitinated signaling molecules (Bjorkoy *et al.*, 2005; Moscat and Diaz-Meco, 2009). Indeed, p62 promotes IL-1β-induced signaling pathways by recruiting and oligomerizing both TRAF6 (Sanz *et al.*, 2000) and MyD88 (Into *et al.*, 2010).

As expected, p62 levels in *Atg16L1*- and *Atg5*-deficient MEF were much higher compared to WT MEF. The elevated p62 levels in autophagy-deficient MEF were not due to the increased transcriptional activity. Surprisingly, however, p62 in MEF were not only degraded by autolyosome but also by proteasome. In fact, p62 in WT accumulated at a much higher level upon inhibition of proteasome, suggesting that more p62 is degraded by proteasome than by autolysosome (Lee *et al.*, 2012). Next, we demonstrated that p62 is indeed being ubiquitinated upon inhibition of proteasome, but not of lysosome, in an *Atg16L1*- and *Atg5*-dependent manner. To ensure that p62 ubiquitination is not due to its tight association with ubiquitinated substrates via the ubiquitin binding domain, all assays performed with the samples were boiled in 1% SDS for 10 min right after each treatment. Furthermore, ectopically expressed GFP-p62 was also ubiquitinated, which was dependent on Atg16L1. In addition, expression of ATG16L1 in *Atg16L1*-deficient MEF reconstituted p62 ubiquitination and suppressed the IL-1β signaling pathways. Therefore our data for the first time demonstrated that p62 is downregulated by both autophagy and the ubiquitination proteasomal system (UPS) in MEF.

To test whether the elevated p62 is responsible for the hyperresponsiveness to IL-1β, p62 was silenced in *Atg16L1*-deficient MEF. P62 knockdown (KD) completely reversed the phenotype: activation of NF-κB and MAP kinases by IL-1β decreased concomitantly to the levels of p62. To test the role of p62 in the IL-1β signaling pathways *in vivo*, we injected IL-1β intraperitoneally to WT and p62 KO mice and measured the serum levels of proinflammatory cytokines. The levels of both TNF-α and IL-6 were lower in p62 KO compared to WT mice, confirming the notion that p62 amplifies the IL-1β signal transduction but is not essential (Lee *et al.*, 2012).

Regulation of P62 Ubiquitination by Atg16L1

P62 is a component of Cullin-3 ubiquitin ligase complex containing Keap1 and Nrf2, in which Nrf2 is the substrate (Komatsu *et al.*, 2010). By binding to Keap1, p62 interferes with Nrf2 ubiquitination by Cullin-3 and the stabilized Nrf2 transcriptionally activates antioxidant proteins and detoxification enzymes. Thus in autophagy-deficient cells, p62 accumulation can activate an antioxidative response via Nrf2. As expected, p62 and Cullin-3 formed an immunoprecipitable complex, which was also confirmed by immunofluorescence (Lee *et al.*, 2012).

We next investigated whether p62 itself is a target of ubiquitination by Cullin-3. *Cullin-3* knockdown in WT MEF induced accumulation of p62 with the concomitant suppression of IL-1β signal transduction. Cullin-3 ubiquitinates Nrf2, and Nrf2 activates *p62* transcription (Jain *et al.*, 2010). However, *Cullin-3* knockdown did not significantly increase Nrf2 expression. Furthermore, *Nrf2* knockdown did not affect p62 expression significantly in MEF, excluding the possibility that Cullin-3 regulates p62 via Nrf2. Conversely, overexpression of *Cullin-3* in WT MEF induced p62 ubiquitination and degradation, while enhancing IL-1β signal transduction. However, Cullin-3 overexpression in *Atg16L1*-deficient MEF affected neither p62 expression nor IL-1β signal transduction, demonstrating that Cullin-3 ubiquitinates p62 in an Atg16L1-dependent manner (Lee *et al.*, 2012).

Since Atg16L1 did not affect expression of Cullin-3, we investigated whether it regulates activation of Cullin-3. Neddylation of Cullin-3 induces dimerization and activation of Cullin-3 (Hotton and Callis, 2008; Wimuttisuk and Singer, 2007). Indeed, both constitutive and IL-1β-induced neddylation of Cullin-3 was completely dependent on Atg16L1 (Lee *et al.*, 2012), demonstrating that Atg16L1 promotes the p62 ubiquitination via Cullin-3 by regulating neddylation of Cullin-3.

DISCUSSION

Several TLRs induce autophagy and autophagy regulates TLR signaling. Genomic studies implicated several autophagy-related genes in CD. ATG16L1 has been a focus of investigation since the T300A variant was found to be associated with a high risk for CD. Already several functions of ATG16L1 in inflammation have been described, including its role in IL-1β maturation and secretion, exocytosis function in Paneth cells, and phagocytosis of invading bacteria.

IL-1β is a potent inflammatory cytokine and involved in several inflammatory diseases. Pharmacological inhibition of the IL-1 pathway in rheumatoid arthritis produced only

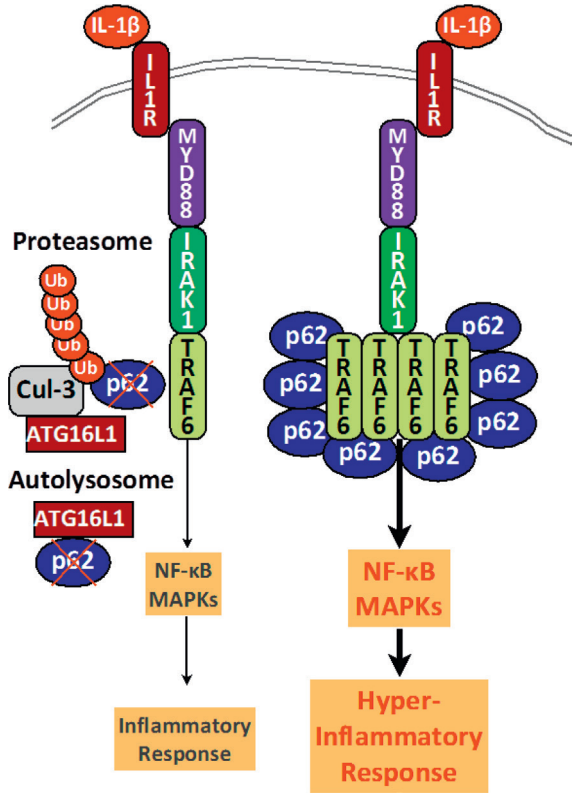

FIGURE 15.1 ATG16L1 is a negative regulator of IL-1β signaling. Constitutive degradation of p62 by the autolysosome and the proteasome in the presence of ATG16L1 restrains the IL-1β signaling cascades and the subsequent inflammatory response. In the absence of ATG16L1, p62 levels are increased. This increase in p62 levels promotes oligomerization and activation of TRAF6 and MyD88, resulting in overactivation of NF-κB and MAPKs upon IL-1β stimulation that leads to a hyperinflammatory response.

modest effects. However, IL-1 antagonists are beneficial in hereditary autoinflammatory conditions associated with excessive IL-1 signaling, such as cryopyrinopathies and IL-1 receptor antagonist (Ra) deficiency (Gabay *et al.*, 2010). Although colonic mucosal IL-1 level is significantly higher in active IBD (Casini-Raggi *et al.*, 1995; Ligumsky *et al.*, 1990), it has yet to be demonstrated that pharmacological inhibition of IL-1 in IBD is beneficial.

While investigating its role in TLR signal transduction in MEF, we found a new mechanism by which Atg16L1 suppresses IL-1β-induced signal transduction (Figure 15.1). Our study demonstrates that Atg16L1, or autophagy in general, does so by downregulating p62. What was most unexpected was that p62 is downregulated not only by autolysosome but by the UPS system. P62 is a receptor for selective autophagy by which misfolded proteins, damaged organelles, and invasive pathogens are being degraded (Kraft *et al.*, 2010). P62 detects polyubiquitinated targets and sequesters them as an aggregate for autophagic clearance. Our data show that p62 itself is a target of selective autophagy. It remains to be

seen whether it also acts as the autophagic receptor for its own clearance, or other receptors such as HDAC6 or NDP52 are involved (Into *et al.*, 2012). P62 is found in neuronal inclusion bodies of individuals with Parkinson's disease (PD) and other neurodegenerative diseases (Wooten *et al.*, 2006). Interestingly, dysfunctional autophagy is a common feature in both PD and CD: LRRK2, a regulator of autophagy, is a risk factor for both PD and CD, although its precise functions are still a mystery. P62 may play an important role in two diseases.

P62 oligomerizes ubiquitinated TRAF6 to enhance IL-1β signal transduction. However, it is not clear why it has minimal impact on other TLRs that utilize TRAF6. It is possible that maybe the difference in the capacity to activate autophagy by different TLRs accounts for this phenomenon.

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References

Anderson, C.A., Boucher, G., Lees, C.W., et al., 2011. Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. Nat. Genet. 43, 246–252.

Bjorkoy, G., Lamark, T., Brech, A., et al., 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. 171, 603–614.

Cadwell, K., Liu, J.Y., Brown, S.L., et al., 2008. A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 456, 259–263.

Cadwell, K., Patel, K.K., Maloney, N.S., et al., 2010. Virus-plus-susceptibility gene interaction determines Crohn's disease gene Atg16L1 phenotypes in intestine. Cell 141, 1135–1145.

Casini-Raggi, V., Kam, L., Chong, Y.J., et al., 1995. Mucosal imbalance of IL-1 and IL-1 receptor antagonist in inflammatory bowel disease. A novel mechanism of chronic intestinal inflammation. J. Immunol. 154, 2434–2440.

Franke, A., McGovern, D.P., Barrett, J.C., et al., 2010. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nat. Genet. 42, 1118–1125.

Gabay, C., Lamacchia, C., Palmer, G., 2010. IL-1 pathways in inflammation and human diseases. Nat. Rev. Rheumatol. 6, 232–241.

Homer, C.R., Richmond, A.L., Rebert, N.A., et al., 2010. ATG16L1 and NOD2 interact in an autophagy-dependent antibacterial pathway implicated in Crohn's disease pathogenesis. Gastroenterology 139, 1630–1641, 1641, e1631–1632.

Hotton, S.K., Callis, J., 2008. Regulation of cullin RING ligases. Annu. Rev. Plant Biol. 59, 467–489.

Hugot, J.P., Chamaillard, M., Zouali, H., et al., 2001. Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411, 599–603.

Into, T., Inomata, M., Niida, S., et al., 2010. Regulation of MyD88 aggregation and the MyD88-dependent signaling pathway by sequestosome 1 and histone deacetylase 6. J. Biol. Chem. 285, 35759–35769.

Into, T., Inomata, M., Takayama, E., et al., 2012. Autophagy in regulation of Toll-like receptor signaling. Cell Signal. 24, 1150–1162.

Jain, A., Lamark, T., Sjottem, E., et al., 2010. p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response element-driven gene transcription. J. Biol. Chem. 285, 22576–22591.

Knaevelsrud, H., Simonsen, A., 2010. Fighting disease by selective autophagy of aggregate-prone proteins. FEBS Lett. 584, 2635–2645.

Komatsu, M., Kurokawa, H., Waguri, S., et al., 2010. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat. Cell. Biol. 12, 213–223.

Kraft, C., Peter, M., Hofmann, K., 2010. Selective autophagy: ubiquitin-mediated recognition and beyond. Nat. Cell. Biol. 12, 836–841.
Kuballa, P., Huett, A., Rioux, J.D., et al., 2008. Impaired autophagy of an intracellular pathogen induced by a Crohn's disease associated ATG16L1 variant. PLoS ONE 3, e3391.
Lee, J., Kim, H.R., Quinley, C., et al., 2012. Autophagy suppresses interleukin-1beta (IL-1beta) signaling by activation of p62 degradation via lysosomal and proteasomal pathways. J. Biol. Chem. 287, 4033–4040.
Ligumsky, M., Simon, P.L., Karmeli, F., et al., 1990. Role of interleukin 1 in inflammatory bowel disease – enhanced production during active disease. Gut 31, 686–689.
Liu, J., Nussinov, R., 2010. Rbx1 flexible linker facilitates cullin-RING ligase function before neddylation and after deneddylation. Biophys. J. 99, 736–744.
Moscat, J., Diaz-Meco, M.T., 2009. P62 at the crossroads of autophagy, apoptosis, and cancer. Cell 137, 1001–1004.
Ogura, Y., Bonen, D.K., Inohara, N., et al., 2001. A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411, 603–606.
Plantinga, T.S., Crisan, T.O., Oosting, M., et al., 2011. Crohn's disease-associated ATG16L1 polymorphism modulates pro-inflammatory cytokine responses selectively upon activation of NOD2. Gut 60, 1229–1235.
Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., et al., 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell 118, 229–241.
Saitoh, T., Fujita, N., Jang, M.H., et al., 2008. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production. Nature 456, 264–268.
Sanz, L., Diaz-Meco, M.T., Nakano, H., et al., 2000. The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. EMBO J. 19, 1576–1586.
Shaid, S., Brandts, C.H., Serve, H., et al., 2012. Ubiquitination and selective autophagy. Cell Death Differ. 20 (1), 21–30.
Wimuttisuk, W., Singer, J.D., 2007. The Cullin3 ubiquitin ligase functions as a Nedd8-bound heterodimer. Mol. Biol. Cell. 18, 899–909.
Wooten, M.W., Hu, X., Babu, J.R., et al., 2006. Signaling, polyubiquitination, trafficking, and inclusions: sequestosome 1/p62's role in neurodegenerative disease. J. Biomed. Biotechnol. 2006 (3), 62079.
Xu, Y., Jagannath, C., Liu, X.D., et al., 2007. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144.

CHAPTER

16

Roles of Autophagy in the Thymic Epithelium

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OUTLINE

Abstract

Epithelial cells have central roles in the development of the thymus and in the selection of T cells in the thymus. Several recent reports have demonstrated active autophagy in thymic epithelial cells. The lipidated form of the autophagy-associated protein LC3 is present in the thymus, and autophagic vesicles are abundant in thymic epithelium. Recombinant fusion of green fluorescent protein to LC3 in a transgenic mouse model has confirmed the high proportion of autophagy-positive thymic epithelial cells. Suppression of autophagy by targeted gene deletion and grafting of thymi from autophagy-deficient embryos into athymic mice led to the development of severe autoimmunity in multiple organs, indicating a failure of autophagy-deficient thymic epithelium to appropriately support the negative selection of autoreactive T cells. Depending on expression levels and intracellular localization of antigens, autophagy appears to be essential for the loading of some but not all self-peptides onto MHC class II molecules within medullary thymic epithelial cells. In spite of these data arguing for a critical direct role of epithelial cells in antigen presentation

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within the thymus, signs of autoimmune disease do not develop in mouse models that lack either one of the autophagy-related genes *Atg5* or *Atg7* in the endogenous thymic epithelium. Thus, autophagy is highly active in normal thymic epithelium and involved in distinct aspects of T cell selection; however, its suppression does not lead to severe autoimmunity.

INTRODUCTION

Thymic Epithelium

The thymus is a lymphoid organ in which T cells mature and undergo selection in a process that is controlled, at least partly, by epithelial cells (Pearse, 2006). Together with dendritic cells, the thymic epithelial cells form the sponge-like stroma through which T cells migrate. The epithelium of the thymus is unique both in its development, shape, and functional properties, some of which likely depend on autophagy.

The human thymus is located in the thorax within the anterior mediastinum and consists of two lobes that are joined by connective tissue and enclosed by a capsule of connective tissue. The two lobes are composed of many lobules of various sizes which contain follicles, each comprising a medulla and a cortex. In the mouse, the lobes of the thymus are not subdivided into lobules but only in the central medulla and a peripheral cortex. In addition, the thymus contains a distinct vasculature. The cortex is stained darkly blue by hematoxylin and eosin (H&E) while the medulla appears lighter.

The stroma of the thymus is largely made up of epithelial cells. The subtypes of thymic epithelial cells comprise cortical and medullary epithelial cells as well as the cells of Hassall's bodies. The latter are located in the medulla and represent corpuscles in which epithelial cells express late differentiation markers of the epidermis such as caspase-14 (Eckhart *et al.*, 2000). The function of Hassall's bodies is only partly understood at present (Watanabe *et al.*, 2005). Other thymic epithelial cells form a reticular meshwork which closely interacts with maturing T cells and dendritic cells (Pearse, 2006).

In embryonic development, the thymus forms prior to all other lymphoid organs. Epithelial cells are derived from precursor cells that express keratins, which are also found in the proliferating cells of the epidermis, i.e., K5 and K14 (Bleul *et al.*, 2006). This fact is important because it provides the basis for investigations of thymic epithelial functions by using mouse models in which distinct genes are knocked out specifically in cells expressing K5 or K14 (Sano *et al.*, 2001). Later in development, cortical epithelial cells express the intermedial filament protein pair K8 and K18, whereas medullary epithelial cells continue to express K5 and K14.

The thymus grows in early life but involutes after the organism has reached sexual maturity. In young mice the thymic epithelium has a high cell turnover rate (Gray *et al.*, 2006). At the age of 4 weeks the turnover time of the thymic epithelial compartment was estimated to be 2 weeks or less, which is a value essentially identical to that of the epidermis (Gray *et al.*, 2006). The turnover rate decreases with age and later the size of the thymic epithelium as well as the overall size of the thymus decreases. The mechanism of thymus involution is not fully understood but a central role of the epithelial compartment has been suggested (Gray *et al.*, 2006).

The main function of the thymus is the maturation and selection of T cells which have central and indispensable roles in adaptive immunity. T cells originate from stem cells in the bone marrow from where they migrate to the thymus. Immature cells undergo a selection process that eliminates essentially all cells that might bind to antigens in the body to initiate harmful immune reactions. It has been estimated that more than 95% of all immature T cells die in the thymus. After this selection mature T cells move to the peripheral tissues.

When immature T cells enter the thymus they first undergo positive selection. Positive selection takes place in the cortex of the thymus where epithelial cells express major histocompatibility complex (MHC) molecules and present self-antigens. The survival of individual T cells requires the productive interaction of their T cell receptor (TCR) with MHC complexes. Failure to bind to the MHC complex on the surface of the epithelial cells of the thymus cortex results in the absence of prosurvival signals and subsequently in apoptosis.

Positively selected T cells migrate to the cortico-medullary junction and the medulla of the thymus to be subjected to negative selection. Epithelial cells as well as dendritic cells of the medulla express MHC class II molecules and present peptides that are derived from self-antigens. The AIRE (autoimmune regulator) protein induces the expression of proteins that have an otherwise tissue-restricted expression pattern (tissue-restricted antigens), in epithelial cells of the medulla. T cells that strongly interact, via their TCR, with self-antigens are potentially dangerous and are driven into apoptosis during negative selection. Both epithelial cells and dendritic cells function as antigen-presenting cells in the thymus medulla. Interestingly, the epithelial cells within Hassall's bodies have been suggested to contribute to T cell selection by cooperating with dendritic cells in the conversion of high-affinity self-reactive T cells into suppressive regulatory T cells (Watanabe *et al.*, 2005).

Although the general mechanisms of T cell development have been clarified to a great extent, there are still uncertainties. One of the open issues is the mechanism by which antigens are loaded on MHC class II molecules in thymic epithelial cells. Recently, autophagy has been implicated in the presentation of at least some self-antigens on the epithelial cells of the thymus (Nedjic *et al.*, 2008).

Autophagy

Autophagy is a mechanism for the degradation of organelles and cytoplasmic components of the cell. At least four types of autophagy, namely macroautophagy, microautophagy, chaperone-mediated autophagy and noncanonical autophagy, can be distinguished in mammalian cells (Mizushima and Levine, 2010; Nishida *et al.*, 2009). As most studies of the thymus have focused on macroautophagy, which is the predominant type of autophagy in eukaryotes (Mizushima and Komatsu, 2011), we will refer to macroautophagy as "autophagy" in this chapter.

The molecular machinery of autophagy is encoded by autophagy-related genes (Atg) that are homologous in humans and mice (Mizushima *et al.*, 2010; Mizushima and Komatsu, 2011; Choi *et al.*, 2013). The steps of the autophagy process are described extensively in other chapters of this book. In brief, organelles, protein complexes and bulk cytoplasm are enclosed by double-membraned autophagosomes which later fuse with lysosomes. The hydrolytic enzymes of the lysosome degrade the content of the autophagosome and allow breakdown products such as amino acids and fatty acids to be recycled. ATG7 and

ATG10 are essential for the conjugation of ATG12 and ATG5 which subsequently cause the attachment of ATG8/LC3 to the autophagosome. ATG7 is also involved in conjugation of LC3 to phosphatidylethanolamine, thereby converting cytoplasmic LC3-I to membrane-associated LC3-II. The two forms of LC3 can be separated by polyacrylamide gel electrophoresis and detected by Western blot analysis in a reaction that is widely used to monitor active autophagy (Mizushima *et al.*, 2010).

Originally, autophagy was considered mainly as a step in intracellular recycling during starvation. However, autophagy is also active in cells that have sufficient supply of extracellular nutrients. Such cells utilize autophagy to remove potentially harmful protein aggregates. In addition, autophagy has important roles in antibacterial and antiviral defense, and it is involved in the control of the important stress response system that depends on the transcription factor Nrf2 (Mizushima and Komatsu, 2011; Sukseree *et al.*, 2013a).

Much of the knowledge about physiological roles of autophagy has been gained in studies using specific inactivation of selected autophagy-related genes. Many of these studies either involved cell type-restricted gene knockouts involving the Cre-loxP system or constitutive gene knockouts in the mouse. Complete deletion of the most widely used autophagy gene targets, i.e., *Atg5* and *Atg7*, results in neonatal lethality (Komatsu *et al.*, 2005; Kuma *et al.*, 2004). This phenotype has been ascribed to a failure in supplying sufficient amounts of amino acids during the neonatal nutrient starvation. However, a recent report about *Atg7* deletion showed that the lethality of *Atg7*-deficient mice can be rescued partially by the homozygous deletion of the protein kinase Chk2 as a means to inhibit the DNA damage response (Lee *et al.*, 2012). Together with other data, this finding suggested that, under conditions of metabolic stress, ATG7 binds to p53 and thereby regulates the expression of the cell cycle inhibitor p21CDKN1A to cause cell cycle arrest. By contrast, the absence of Atg7 is associated with the progression of the cell cycle even under stressed conditions, followed by accumulation of DNA damage, activation of Chk2 and subsequent apoptosis (Lee *et al.*, 2012). Suppression of the Chk2-dependent DNA damage response rescues the defects of several *Atg7*-deficient organs but fails to rescue neurological defects. Importantly, only *Atg7* but not *Atg5* appears to be involved in cell cycle control and the E1-like activity of ATG7 is dispensible for this role (Lee *et al.*, 2012).

EVIDENCE FOR AUTOPHAGY IN THE THYMIC EPITHELIUM

The first line of evidence for the occurrence of autophagy in the thymus comes from ultrastructural investigations. By electron microscopy, extensive autophagic activity was detected in the murine thymic epithelium (Bowen and Lewis, 1980). In another study, transmission electron microscopy was used to detect autophagosomes in the thymus of aging mice (Uddin *et al.*, 2012). Autophagic vacuoles were abundant in cortical and medullary epithelial cells.

For the detection of active autophagy *in vivo*, Mizushima and colleagues have generated transgenic mice in which the recombinant fusion protein, green fluorescent protein (GFP)-microtubule-associated protein light chain 3 (GFP-LC3), is expressed using the chicken actin promoter in essentially all tissues (Mizushima *et al.*, 2004, 2009). While cells without active autophagy show only weak GFP fluorescence on tissue thin sections, active autophagy is

associated with the concentration of GFP-LC3 on autophagosomes that appear as bright green puncta.

Using this mouse model, autophagy was detected in the thymic epithelium (Mizushima *et al.*, 2004; Nedjic *et al.*, 2008; Sukseree *et al.*, 2012). GFP-LC3 puncta were observed in the cortex and medulla, where they co-localized with keratin immunofluorescence, a marker of epithelial cells. The fluorescent puncta can be seen both in the cell bodies and processes of the reticular epithelial cells (Mizushima *et al.*, 2004). Immunogold labeling with anti-GFP antibody and electron microscopy confirmed the localization of GFP-LC3 on autophagosomes of cortical epithelial cells (Mizushima *et al.*, 2004). Notably, a higher abundance of labeled autophagosomes was detected in cortical epithelium than in medullary epithelium (Mizushima *et al.*, 2004; Sukseree *et al.*, 2012). In the latter, the presence of labeled autophagosomes is difficult to assess because of autofluorescence (Mizushima *et al.*, 2004). When cells were isolated from GFP-LC3-transgenic thymi and sorted by fluorescence-activated cell sorting (FACS) into cortical and medullary epithelial as well as dendritic cells, the highest abundance of GFP-labeled autophagosomes was detected in cortical epithelial cells with more than two-thirds of all cells being positive (Nedjic *et al.*, 2008). Interestingly, among medullary epithelial cells the fraction of cells expressing high levels of MHC class II molecules (mature cells) showed more autophagy than cells expressing low levels of MHC class II molecules (immature cells). Importantly, starvation was not necessary to induce the appearance of GFP-LC3 puncta in the thymic epithelium and also did not increase their abundance (Mizushima *et al.*, 2004). Only a few other cell types besides thymic epithelium cells appeared to constitutively activate autophagy *in vivo*. In a later study, epidermal keratinocytes were also found to contain GFP-LC3-labeled autophagosomes under non-starved conditions (Rossiter *et al.*, 2013).

Cells in which autophagy is active do not only contain GFP-LC3-positive autophagosome; they also degrade a significant portion of GFP-LC3 that is produced. This is because the LC3 portion leads to the delivery of the fusion protein to the lysosome for protease-mediated breakdown. When autophagy is suppressed, for example by the deletion of an essential autophagy-related gene, the total amount of GFP-LC3 in the cell increases relative to autophagy-competent cells. This leads to an increase in cellular green fluorescence which can be visualized under the microscope. When *Atg7* was deleted in the thymic epithelium (Sukseree *et al.*, 2012) and in the epidermis (Rossiter *et al.*, 2013) of GFP-LC3 transgenic mice, both epithelia showed an increase in green fluorescence. While in the epidermis the accumulation of GFP-LC3 was restricted to the upper layers of differentiated cells; the increase of GFP fluorescence was observed in essentially all epithelial cells of the cortex and medulla of the murine thymus (Sukseree *et al.*, 2012).

Similar to recombinant GFP-LC3 in transgenic mice, endogenous substrates of autophagy accumulate when *Atg7* or other Atg genes are deleted in cells that normally show active autophagy. To investigate autophagy in the thymic epithelium, the abundance of endogenous LC3 was determined by immunoblot analysis in thymus samples from *Atg5*-floxed K5-Cre and *Atg7*-floxed K14-Cre mice (Sukseree *et al.*, 2012, 2013b). The thymus of fully autophagy-competent mice showed a prominent LC3-II band indicative of active autophagy. Upon suppression of autophagy in the epithelial compartment of the thymus, LC3-I accumulated strongly whereas LC3-II was hardly detectable. High levels of LC3-II and low levels of LC3-I, again suggestive of active autophagy, were observed in thymus

samples of mice of various ages (Uddin *et al.*, 2012). Similarly, LC3-II is highly abundant in cultured mouse thymic epithelial cells and its level can be further increased by protease inhibitors that block LC3 degradation in lysosomes (Kasai and Mizuochi, 2007; Kasai *et al.*, 2009).

Besides LC3, p62/sequestosome 1 (SQSTM1) is a useful marker of autophagic flux. Accordingly, the abundance of p62 was determined by immunoblot in thymus lysates from mice in which epithelial autophagy was suppressed (Sukseree *et al.*, 2012, 2013b). Both *Atg5*-floxed K5-Cre and *Atg7*-floxed K14-Cre mice showed a massive increase of p62 relative to fully autophagy-competent control. Furthermore, p62 was determined by immunofluorescence analysis of Atg7-floxed K14-Cre and control mice (Sukseree *et al.*, 2012). In line with constitutively active autophagy, the genetic suppression of autophagy led to accumulation of p62 in thymic epithelial cells.

EVALUATION OF EPITHELIAL AUTOPHAGY IN T CELL SELECTION

Implication of Autophagy in Delivering Antigens to the MHC Class II Compartment

Thymic epithelial cells are antigen-presenting cells that express MHC class II and contribute to the selection of T cells. It has long been enigmatic how peptides derived from endogenous proteins gain access to the MHC class II compartment, which is considered to receive proteins mainly from endocytosis. Thymic epithelial cells have a particularly low rate of delivering peptides from exogenous proteins to MHC class II molecules (Klein *et al.*, 2001). Nevertheless, the peptidome eluted from MHC class II molecules contains many peptides that have originated from cytosolic and nuclear proteins. A likely explanation has been provided by studies that demonstrated a connection between autophagy and antigen loading onto MHC class II (Nimmerjahn *et al.*, 2003; Schmid *et al.*, 2007). Recently, electron tomography allowed the demonstration that in dendritic cells, which were stimulated with LPS, autophagosomes emerge from MHC class II-positive compartments. Autophagosomes generated by this so-called "endosome-mediated autophagy" contain both MHC class II and LC3. It was proposed that this unconventional and perhaps antigen-presenting cell-specific variant of autophagy delivers cytosolic antigens to the cell surface for presentation on MHC class II (Kondylis *et al.*, 2013). In another report, LC3 was shown, by conventional immunofluorescence double-labeling, to co-localize with MHC class II compartments both in thymic epithelial cells *in vitro* and in thymic epithelium of newborn mice *in vivo* (Kasai *et al.*, 2009).

Thymus Grafts from Autophagy-Deficient Embryos

The physiological relevance of autophagy in delivering peptides to MHC class II molecules for selection of T cells was tested in elegant studies by Klein and colleagues (Aichinger *et al.*, 2013; Klein *et al.*, 2011; Nedjic *et al.*, 2008). In the first study, the thymus of *Atg5*-deficient mouse embryos was grafted under the kidney capsule of adult mice. Both wildtype and autophagy-deficient thymus transplants developed into thymi of apparently

normal morphology. However, grafts from *Atg5* knockout mice were dramatically smaller than those from wildtype mice. The cell number per thymus lobe was reduced by more than 60% (Nedjic *et al.*, 2008). Moreover, the density of MHC class II molecules on cortical epithelial cells was reduced. Nevertheless, the main T cell lineages developed and this model was considered useful to study the role of epithelial autophagy in T cell selection. Indeed, the positive selection of distinct MHC class II-restricted antiforeign TCRs was altered under these conditions, suggesting a role of autophagy in cortical epithelial cells. Importantly, the decrease in the size of thymi developing from autophagy-deficient grafts could be rescued by particular MHC II-restricted TCR specificities in distinct transgenic mouse models (Nedjic *et al.*, 2008).

Wildtype and *Atg5* knockout embryonic thymi were also transplanted to nude mice which lack an endogenous thymus. The frequency of activated $CD4^+$ T cells was determined by FACS in the recipients. Thymus grafts from autophagy-deficient embryos were associated with significantly stronger activation of $CD4^+$ T cells and increased sizes of lymph nodes in the recipient mice. Infiltrates of inflammatory cells were abundant in multiple organs including the lung, liver, and the colon of these mice. Grafting *Atg5* knockout thymi caused macroscopic signs of skin inflammation and abnormalities in many organs such as enlargement of the colon, absence of fat pads, and atrophy of the uterus (Nedjic *et al.*, 2008). Unlike recipients of a wildtype thymus, these mice started to lose weight between 4 and 6 weeks after grafting. The weight loss was attributed to severe autoimmune disease. Nedjic *et al.* (2008) concluded that autophagy in the thymic epithelium is essential for tolerance to self-antigens.

The interpretation of results of thymus transplantation studies requires great caution, especially when the roles of distinct cell types are to be distinguished. In the previously mentioned experiments, both epithelial and hematopoietic cells of the donor thymi were genetically modified to lack autophagy. The grafting procedure is likely to cause severe stress to all thymus cells. Moreover, the tissue environment in the recipient is not normal. Therefore, roles of autophagy in nonepithelial cells and unnatural effects of autophagy deficiency are possible under these conditions.

Investigation of Autophagy in Medullary Epithelial Cells

In another study, the role of autophagy within medullary epithelial cells was investigated more deeply. Aichinger *et al.* (2013) demonstrated that a mitochondrial antigen was delivered to the MHC II compartment via autophagy whereas the antigen targeted to the cell membrane could be presented without an involvement of autophagy. Thereby, they showed that the localization of self-antigens within the cell determines whether autophagy is critical for its presentation on MHC II molecules. When a transgenic antigen was expressed as a fusion to LC3 under the control of the *Aire* gene locus, it was targeted to autophagosomes in the medullary epithelium. This led to efficient antigen presentation on MHC class II molecules and negative selection of reactive T cells. By contrast, when a mutation was introduced into the antigen-LC3 fusion protein in order to prevent targeting to autophagosomes, antigen presentation and T cell selection were abolished (Aichinger *et al.*, 2013). This report confirmed a role of autophagy in negative selection of $CD4^+$ T cells by presentation of self-antigens via MHC II molecules on medullary thymic epithelial cells.

Deletion of Autophagy-Related Genes in Thymic Epithelial Cells

Since the massive autoimmunity induced by grafting of *Atg5* knockout thymi into athymic mice had major implications on the concept of tolerance induction to self-antigens (Nedjic *et al.*, 2008), it was important to test the role of autophagy-related genes in the prevention of autoimmunity in further models. Two studies involving mouse models with epithelium-specific Atg gene knockouts were published. However, they argued against an essential role of thymic epithelial autophagy in the prevention of autoimmunity (Sukseree *et al.*, 2012, 2013b). These studies used mice in which autophagy was suppressed by the Cre-loxP system. Mice in which parts of either one of the two essential autophagy genes, *Atg5* and *Atg7*, were flanked by loxP sites ("floxed") were crossed with mice expressing the Cre recombinase under the control of a promoter active in the precursor of thymic epithelial cells. This led to the excision of the floxed gene segment and, thereby, to gene inactivation in these precursor cells and in their cell progeny. The promoters of the keratin genes *Krt5* and *Krt14* are suitable for gene deletions in the epidermis and in the thymic epithelium.

To inactivate *Atg7* in thymic epithelial cells, mice carrying floxed *Atg7* alleles were crossed with mice expressing K14-Cre (Sukseree *et al.*, 2012). Furthermore, the GFP-LC3 transgene was used as *in situ* reporter for autophagy in a subline of these mice. Immunoblot analysis with LC3-specific antibodies showed that, in the thymus, LC3-II formation was suppressed by epithelial deletion of *Atg7*. The blockade of autophagy led to a strong increase in p62 that was visible on immunoblots and in immunofluorescence. The latter demonstrated that p62 was highly abundant in thymic epithelial cells of Atg7-floxed K14-Cre mice but not in nonepithelial cells. GFP-LC3 appeared in a punctate pattern in thymic epithelial cells of Cre-negative mice but showed a diffuse cytoplasmic accumulation in Cre-positive mice (Sukseree *et al.*, 2012). Together, these observations confirmed efficient and epithelium-specific suppression of autophagy in the thymus of this mouse model.

Next the authors evaluated whether the absence of autophagy was associated with deleterious consequences according to criteria defined in the report about thymus transplant-induced autoimmunity (Nedjic *et al.*, 2008). Thymi of *Atg7*-floxed K14-Cre mice had a normal morphology and compartmentalization into cortex and medulla as well as normal size. The mean body weight of these mice was also not significantly different from that of control mice that lacked the K14-Cre transgene. The ratio of the frequencies of $CD4^+$ T cells to $CD8^+$ T cells was normal, as was the frequency of $CD69^+$ $CD4^+$ T cells in lymph nodes and in the spleen of *Atg7*-floxed K14-Cre mice. Extensive screening for the presence of potentially elevated numbers of infiltrating inflammatory cells in peripheral organs such as liver, lung, uterus, colon, Harderian glands, and the skin did not reveal signs of abnormal tissue inflammation in mice lacking thymic epithelial autophagy. These results suggested that the suppression of autophagy in the thymic epithelium did not compromise the development of tolerance (Sukseree *et al.*, 2012).

The authors of the study summarized here performed a complementary investigation in which a different autophagy-related gene was deleted in the thymic epithelium (Sukseree *et al.*, 2013b). Instead of *Atg7*, the same gene as in the study by Nedjic and colleagues, that is *Atg5* (Nedjic *et al.*, 2008), was deleted by the Cre-loxP system. In this follow-up study, the promoter of the *Krt5* (K5) gene was used to drive expression of Cre. K5 is expressed in

medullary thymic epithelium cells of adult mice but also in a precursor of epithelial cells of the thymus cortex so that a floxed gene is deleted in all thymic epithelial cells. Immunoblot analysis showed autophagy-associated LC3-II in the thymus of control mice and efficient suppression of LC3-II as well as accumulation of LC3-I in the thymus of *Atg5*-floxed K5-Cre mice. The amount of thymic p62 was elevated upon K5-Cre-dependent deletion of *Atg5*. The same differences between genotypes were observed in pure cultures of epithelial cells that expressed K5. However, epithelial cell cultures were not derived from the thymus but from the skin in this study.

Abrogation of *Atg5*-dependent autophagy in the thymic epithelium did not alter the morphology of the thymus cortex and medulla which showed the normal expression pattern of K5 and K8. Screening for signs of tissue inflammation showed the absence of abnormal cell infiltrates in the lung, liver, colon and skin of *Atg5*-floxed K5-Cre mice. Likewise, the preputial glands lacked inflammatory infiltrates but showed a sebocyte differentiation-dependent phenotype (Sukseree *et al.*, 2013b). There was no decrease in the size of fat pads nor an enlargement of the colon that would resemble the observations reported for nude mice receiving *Atg5*-deficient thymus grafts (Nedjic *et al.*, 2008). Furthermore, *Atg5*-floxed K5-Cre mice did not suffer from wasting or form peculiar infections although they were not maintained under specific pathogen-free conditions. Like *Atg7*-floxed K14-Cre mice, *Atg5*-floxed K5-Cre mice did not show elevated mortality.

In summary, two independent mouse models in which autophagy-related genes were deleted in the thymic epithelium did not develop signs of increased autoimmunity. As the investigations of these mice did not include assays that might detect small alterations of T cell responses, only the development of severe autoimmune disease was excluded.

CONCLUSION

The existing literature provides convincing data on the presence of autophagosomes and biochemical markers of active autophagy in thymic epithelial cells. There is also evidence for roles of epithelial autophagy in the control of T cell selection in the thymus. However, autophagy appears to be dispensable for the prevention of severe symptoms of autoimmunity in the mouse. It is important to note that essentially all reports on this topic have focused on macroautophagy in the thymic epithelium. Other modes of autophagy are not well characterized in this tissue yet. Thus, it cannot be excluded that the lack of distinct functions of macroautophagy in Atg gene knockout models is rescued by compensatory upregulation of alternative autophagy mechanisms. Interestingly, epithelial cells of the thymus share the feature of constitutive autophagy with epithelial cells of the skin, pointing to the existence of common physiological roles that have remained elusive so far.

References

Aichinger, M., Wu, C., Nedjic, J., et al., 2013. Macroautophagy substrates are loaded onto MHC class II of medullary thymic epithelial cells for central tolerance. J. Exp. Med. 210, 287–300.

Bleul, C.C., Corbeaux, T., Reuter, A., et al., 2006. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature 441, 992–996.

Bowen, I.D., Lewis, G.H., 1980. Acid phosphatase activity and cell death in mouse thymus. Histochemistry 65, 173–179.
Choi, A.M., Ryter, S.W., Levine, B., 2013. Autophagy in human health and disease. N. Engl. J. Med. 368, 651–662.
Eckhart, L., Declercq, W., Ban, J., et al., 2000. Terminal differentiation of human keratinocytes and stratum corneum formation is associated with caspase-14 activation. J. Invest. Dermatol. 115, 1148–1151.
Gray, D.H., Seach, N., Ueno, T., et al., 2006. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. Blood 108, 3777–3785.
Kasai, M., Mizuochi, T., 2007. Derivation, culture, and characterization of thymic epithelial cell lines. Methods Mol. Biol. 380, 107–123.
Kasai, M., Tanida, I., Ueno, T., et al., 2009. Autophagic compartments gain access to the MHC class II compartments in thymic epithelium. J. Immunol. 183, 7278–7285.
Klein, L., Hinterberger, M., von Rohrscheidt, J., et al., 2011. Autonomous versus dendritic cell-dependent contributions of medullary thymic epithelial cells to central tolerance. Trends Immunol. 32, 188–193.
Klein, L., Roettinger, B., Kyewski, B., 2001. Sampling of complementing self-antigen pools by thymic stromal cells maximizes the scope of central T cell tolerance. Eur. J. Immunol. 31, 2476–2486.
Komatsu, M., Waguri, S., Ueno, T., et al., 2005. Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J. Cell Biol. 169, 425–434.
Kondylis, V., van Nispen Tot Pannerden, H.E., van Dijk, S., et al., 2013. Endosome-mediated autophagy: an unconventional MIIC-driven autophagic pathway operational in dendritic cells. Autophagy 9, 861–880.
Kuma, A., Hatano, M., Matsui, M., et al., 2004. The role of autophagy during the early neonatal starvation period. Nature 432, 1032–1036.
Lee, I.H, Kawai, Y., Fergusson, M.M., et al., 2012. Atg7 modulates p53 activity to regulate cell cycle and survival during metabolic stress. Science 336, 225–228.
Nimmerjahn, F., Milosevic, S., Behrends, U., et al., 2003. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. Eur. J. Immunol. 33, 1250–1259.
Pearse, G., 2006. Normal structure, function and histology of the thymus. Toxicol. Pathol. 34, 504–514.
Mizushima, N., 2009. Methods for monitoring autophagy using GFP-LC3 transgenic mice. Methods Enzymol. 452, 3–23.
Mizushima, N., Komatsu, M., 2011. Autophagy: renovation of cells and tissues. Cell 147, 728–741.
Mizushima, N., Levine, B., 2010. Autophagy in mammalian development and differentiation. Nat. Cell Biol. 12, 823–830.
Mizushima, N., Yamamoto, A., Matsui, M., et al., 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. Mol. Biol. Cell 15, 1101–1111.
Mizushima, N., Yoshimori, T., Levine, B., 2010. Methods in mammalian autophagy research. Cell 140, 313–326.
Nedjic, J., Aichinger, M., Emmerich, J., et al., 2008. Autophagy in thymic epithelium shapes the T cell repertoire and is essential for tolerance. Nature 455, 396–400.
Nishida, Y., Arakawa, S., Fujitani, K., et al., 2009. Discovery of Atg5/Atg7-independent alternative macroautophagy. Nature 461, 654–658.
Rossiter, H., König, U., Barresi, C., et al., 2013. Epidermal keratinocytes form a functional skin barrier in the absence of Atg7 dependent autophagy. J. Dermatol. Sci. 71, 67–75.
Sano, S., Takahama, Y., Sugawara, T., et al., 2001. Stat3 in thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival. Immunity 15, 261–273.
Schmid, D., Pypaert, M., Munz, C., 2007. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. Immunity 26, 79–92.
Sukseree, S., Eckhart, L., Tschachler, E., et al., 2013a. Autophagy in epithelial homeostasis and defense. Front. Biosci. E5, 1000–1010.
Sukseree, S., Mildner, M., Rossiter, H., et al., 2012. Autophagy in the thymic epithelium is dispensable for the development of self-tolerance in a novel mouse model. PLoS ONE 7, e38933.
Sukseree, S., Rossiter, H., Mildner, M., et al., 2013b. Targeted deletion of Atg5 reveals differential roles of autophagy in keratin K5-expressing epithelia. Biochem. Biophys. Res. Commun. 430, 689–694.
Uddin, M.N., Nishio, N., Ito, S., et al., 2012. Autophagic activity in thymus and liver during aging. Age 34, 75–85.
Watanabe, N., Wang, Y.H., Lee, H.K., et al., 2005. Hassall's corpuscles instruct dendritic cells to induce CD4+ CD25+ regulatory T cells in human thymus. Nature 436, 1181–1185.

CHAPTER

17

The Role of Autophagy Receptors in Mitophagy

Mija Marinković and Ivana Novak

OUTLINE

Abstract

Recent discoveries of autophagic receptors that recognize specific cellular cargo have opened a new chapter in the autophagy field. Selective removal of damaged organelles or protein aggregates is essential for proper cellular homeostasis and survival. Until today, autophagy receptors and their actions have been described in several aspects of selective autophagy including xenophagy, aggrephagy, pexophagy and mitophagy. Receptors have been proven indispensable for the initiation and finalization of specific cargo removal by autophagy. Mitophagy receptors, scAtg32, BNIP3 and BNIP3L/NIX, in LIR-dependent manner, interact with Atg8/LC3/GABARAP family proteins and recruit autophagy machinery to damaged mitochondria. Moreover, it has been shown that BNIP3L/NIX mediates mitochondrial clearance during reticulocyte differentiation. Recent discovery of its homologue BNIP3 enlightened the role of mitophagy receptors in cellular fate, where either survival by mitophagy or death by apoptosis is chosen. Autophagic function of the receptors is regulated by phosphorylation but what the trigger is for the phosphorylation and activation of mitophagy is still obscure. Fine-tuning of mitophagy regulation (and general autophagy) is the major challenge for researchers of our time and their answers will allow us to better understand the role of receptors and autophagy in disease development.

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INTRODUCTION

Mitochondrial Dynamics

Mitochondria are highly dynamic organelles with complex structural features, which perform several important roles in cell life. Cellular homeostasis depends on energy produced in mitochondria and it is not surprising that mitochondria are often termed the "powerhouse" of the cell. Not only do they have a key role in ATP synthesis, but also they are pivotal for various cellular processes, including regulation of calcium signaling and storage, metabolite synthesis and the signaling roles in programmed cell death, innate immunity and autophagy (Kroemer *et al.*, 2007). Mitochondrial function is fundamental for cellular survival and dysfunction of these organelles could lead to disruption of crucial functions that could be expressed as disease or even death.

Mitochondria are unusual and unique organelles in many aspects. These double-membrane organelles are, together with the nucleus, the only organelles of the cell that contain their own genome and machinery for RNA and protein synthesis, although the majority of proteins required for mitochondrial function are encoded by nuclear genomes. Similar to the unicellular organisms, mitochondria divide independently of the cell by simple binary fission. Some of these properties are retained from the ancient mitochondrial ancestors. Evolutionarily, mitochondria are thought to have evolved from symbiotic relationships between anaerobic eukaryotic cells and primitive aerobic bacteria capable of oxidative phosphorylation over 1.5 billion years ago. Through this relationship, bacteria evolved into mitochondria and the host cell acquired the ability to metabolically use oxygen, a much more efficient way to produce energy than anaerobic glycolysis (DiMauro and Schon, 2003).

Mitochondria, the energetic organelles, move along the cell, fuse and divide to ensure an adequate mitochondrial function at the appropriate time, but are also strictly regulated. The hundreds of mitochondria in a cell can have a range of morphologies, including small spheres, long tubules and interconnected tubules called the mitochondrial network. This morphological plasticity is based on the ability of mitochondria to undergo continual cycles of fusion (when two mitochondria associate into a single organelle) and fission (the separation of tubular mitochondria into two or more smaller parts) (Chen and Chan, 2009).

Every human cell, except mature erythrocytes, contains mitochondria. The number of mitochondria in a particular cell type is based on the energy needs of that cell. Somatic cells can contain 200 to 2000 mitochondria. Examples of diversity are human germ cells: spermatozoa, which contain a fixed number of 16 mitochondria, in contrast to oocytes which have up to 100,000. Liver and muscle cells with high metabolic activity and high energy needs have a large number of mitochondria and a branchy mitochondrial network. Studies in rat embryos suggest that mitochondrial dynamics are also important during embryonic development (Chen and Chan, 2004).

Mitochondrial dynamics is strictly controlled by a specific set of proteins and many intracellular and extracellular signals such as oxidative stress, membrane potential, mtDNA quality and apoptosis (Kroemer *et al.*, 2007). Fusion and fission processes, crucial for the maintenance of mitochondrial function, are regulated by a series of GTPases. Mitofusins 1 and 2 (MFN1, MFN2) are important for outer mitochondrial membrane fusion, Optic atrophy 1 (OPA1) for inner membrane fusion while Dynamin-related protein 1 (DRP1) and

Fission protein 1 (FIS1) are mandatory for mitochondrial fission. Disruption of this molecular machinery leads to mitochondrial function breakdown and can cause cell death and, more importantly, various diseases. For instance, MFN2 and OPA1 mutations result in defective mitochondrial fusion and cause inherited neurodegenerative disorders: Charcot-Marie-Tooth type 2 neuropathy and autosomal dominant optic atrophy, respectively. Mutations in fission DRP1 protein result in defective neurons with elongated mitochondria unable to maintain normal neurotransmission. Mitochondrial dynamics may affect the occurrence of a wide variety of human diseases through interactions with other cellular processes and many of these diseases are neurodegenerative such as Huntington, Parkinson and Alzheimer disease (Chen and Chan, 2009). Despite the knowledge of important proteins involved in the control of mitochondrial dynamics, their mechanisms of action are still poorly understood, particularly their role in disease development.

Mitochondrial damage is primarily caused by reactive oxygen species (ROS) generated by mitochondria themselves as byproducts of oxidative phosphorylation and it is believed that this damage plays a role in a wide range of seemingly unrelated disorders such as schizophrenia, diabetes, Parkinson disease, cardiomyopathy, chronic fatigue syndrome, hepatitis C and many others (Neustadt and Pieczenik, 2008). ROS are harmful free radicals that circulate throughout the cell, causing DNA damage, lipid peroxidation and protein damage or enzyme inactivation. In comparison to the nuclear DNA (nDNA), mitochondrial DNA (mtDNA) is unprotected by the membrane and histones and is, thus, easily damaged by ROS. It has been estimated that lack of protection results in mutations to mtDNA occurring 10 to 20 times more frequently than mutations to nDNA. Accumulated mutations in mtDNA and misfolding or aggregation of mitochondrial proteins lead to mitochondrial dysfunction, so the maintenance of cellular homeostasis necessarily requires regulated removal of ROS-damaged mitochondria (Kroemer *et al.*, 2007). ROS exposure leads to depolarization of the mitochondrial membrane and triggers activation of cellular mechanisms to remove damaged mitochondria that seems to be a fundamental intracellular catabolic process, important for the elimination of different dysfunctional cellular components during a variety of stress conditions (Scherz-Shouval and Elazar, 2007).

General Autophagy

Cellular homeostasis is established through the proper macromolecule synthesis as well as degradation of damaged, nonfunctional and superfluous cellular parts. Accumulation of these components disturbs cellular homeostasis, which finally may result in disease development. In order to eliminate unnecessary and harmful parts, cells have developed two intracellular degradation mechanisms: the ubiquitin-proteasome degradation pathway (UPS) and the lysosome-mediated degradation pathway known as autophagy.

Proteins destined for degradation are covalently linked to ubiquitin, a small protein responsible for quality control and protein degradation. Ubiquitination serves as the "kiss of death" signal for protein turnover. Proteins with ubiquitin are routed to proteasome, the complex of proteases, where they break down. Proteasome has a limited degradative capacity and can only clean single polypeptide chains but not large protein oligomers, aggregates and entire organelles. In contrast to the proteasome, autophagy has a nearly unlimited

degradation capacity and is not restricted to protein degradation but also breaks down lipids, polysaccharides, DNA and RNA and, more importantly, can target large protein aggregates and organelles.

Autophagy is an evolutionarily well-conserved eukaryotic cellular mechanism. Three types of autophagy have been described in mammals: chaperone-mediated autophagy, microautophagy and macroautophagy. The first mechanism is specific for proteins that expose a KFERQ-like motif. It seems that about 30% of cellular proteins possess this amino acid motif, which can be recognized by specific chaperones and directly targeted to the lysosomes for degradation. Microautophagy is characterized by lysosomal membrane invagination which nonselectively engulfs small parts of the cytoplasm. The third type of autophagy, macroautophagy, is the most studied type of autophagy and usually the term "autophagy" refers to macroautophagy (Cuervo, 2004). Although autophagy has been known for more than 50 years, understanding the molecular basis of this process began 10 years ago with discovery of the ATG genes (autophagy-related genes) in yeast that coordinate and regulate the entire process. Considering that autophagy is evolutionarily conserved from yeast to mammals, homologues of yeast Atg proteins have been identified in higher eukaryotes. Three groups of Atg proteins are important in the autophagosomal formation: (1) complex ULK1/2-Atg13-FIP200, (2) phosphatidylinositol 3-kinase class III (PI3K III) complex of three major proteins Vps34, Vps15 and Beclin-1, and (3) two ubiquitin-like conjugated systems, Atg12 and Atg8/LC3 (Yang and Klionsky, 2010).

Basal and constitutive autophagy, present in all tissues, is important for homeostasis maintenance to eliminate damaged organelles, nonfunctional proteins and protein aggregates. Under normal physiological conditions, the level of basal autophagy is low, but different inductors like starvation, hypoxia, oxidative stress, infection or radiation can increase it significantly. Current research shows that autophagy is implicated in a number of biological processes such as differentiation, erythrocyte and lymphocyte maturation, innate and adaptive immunity, aging and cell death. It is obvious that disturbed autophagy leads to several human diseases or tumor development (Wirawan *et al.*, 2012). Although we have mounting knowledge on autophagy, the mechanisms through which autophagic machinery regulates these diverse processes are not entirely understood.

Selective Autophagy

When autophagy was first discovered, it was described as a general, nonselective degradative process. However, there is ample evidence confirming that autophagy is a highly selective process, present under normal (nutrient-rich) conditions. Compared with classical starvation-induced autophagy, selective autophagy needs to distinguish normal and abnormal cell content and envelope precise cargo into autophagosomes. Selective autophagy was first described in yeast as a Cvt (cytoplasm to vacuole targeting) pathway responsible for the delivery of some vacuolar hydrolases from cytosol to yeast vacuole. Almost any type of cellular cargo can be captured by autophagosomes, including whole organelles, protein aggregates and even invasive intracellular pathogens. Several types of selective autophagy can be categorized depending on specific substrates and are named accordingly: aggrephagy (aggregated proteins), mitophagy (mitochondria), ribophagy (ribosomes), pexophagy (peroxisomes), nucleophagy (parts of the nucleus), lipophagy (lipid droplets), zymophagy

(zymogen granules), glycophagy (glycogen particles) or xenophagy (intracellular pathogens like bacteria and viruses) (Klionsky *et al.*, 2007).

The exact molecular mechanism of selective cargo recognition is still not well understood. Some of the molecular components involved in selective autophagy have been identified, meaning that we begin to understand how selectivity is achieved. Recent studies have revealed a set of specific proteins, called autophagy receptors and adaptors that mediate simultaneous binding of specific cargo and components of the autophagy machinery. Moreover, it becomes obvious that post-translational modifications have an important role in ensuring substrate recognition and selectivity (McEwan and Dikic, 2011). These discoveries gave the idea of universal mechanism of selectivity, which has been confirmed in many types of selective autophagy.

AUTOPHAGY RECEPTORS

Autophagy receptors are the key factors involved in selective autophagy, connecting selected cargo to the autophagic machinery. It is essential that machinery is able to distinguish and package cargo ready for degradation from the nondegradable cargo. Although the same molecular machinery is present in both selective and starvation-induced autophagy, autophagy receptors and adaptors are not required for nonselective autophagy.

Specific cargo-recognizing autophagy receptors and adaptor proteins function together in order to capture the cargo and allow formation of the autophagosomal membrane around the cargo. This is achieved by the ability of autophagy receptors to directly bind both the cargo for degradation and Atg8/LC3 family members for growing the autophagosomal membrane. The Atg8/LC3 family includes several mammalian proteins: LC3A, LC3B, LC3B2, LC3C, GABARAP, GABARAP-L1 and GABARAP-L2/GATE-16 and most autophagy receptors have been found to interact with many of them. Connection between receptors and Atg8/LC3 members is established through a unique motif present on the surface of the receptors, which has a canonical conserved tetrapeptide LC3-interacting region, LIR, ΘxxΓ (where Θ is aromatic and Γ hydrophobic residue) (Pankiv *et al.*, 2007; Rozenknop *et al.*, 2011). Not all LIR-containing proteins are autophagy receptors but most of them are implicated in the autophagy pathway. Due to continual discoveries of new forms of selective autophagy, the list of newly characterized autophagy receptors is expanding. It is interesting that a particular cargo may be recognized by several autophagy receptors (e.g., p62) but some receptors are highly specific (e.g., scAtg32). All receptors found have more than the LIR domain in common, e.g., ubiquitin binding domain, zinc-finger domain, coiled-coil domain or transmembrane domain, that are equally important for selective autophagy induction (Figure 17.1). Their specific roles are discussed in following sections.

p62 and NBR1

Protein aggregation is a continuously ongoing cellular process. Aggregation of some proteins is required for some vital cellular functions, whereas other protein aggregates, resulting from misfolding and caused by various stressors, are prerequisite for many diseases. Toxic aggregates have hydrophobic patches able to interact with other components of the

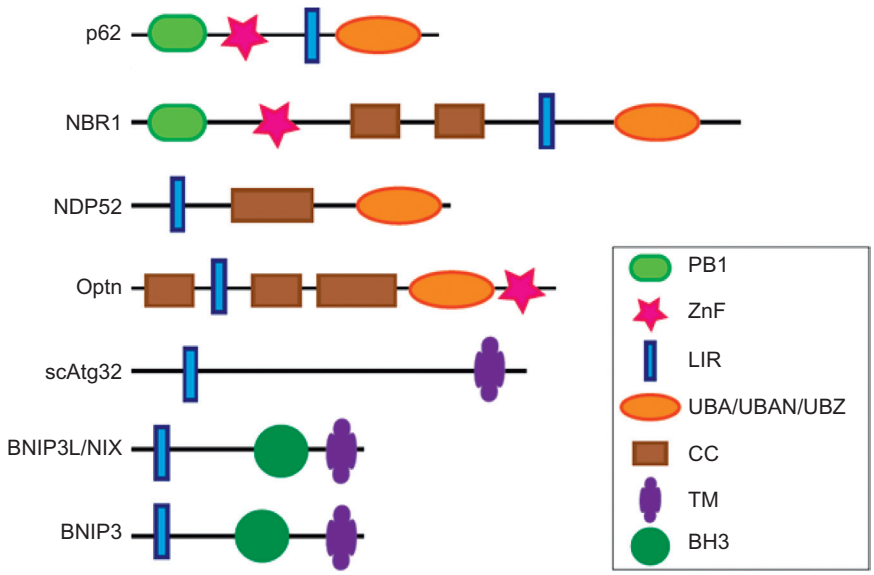

FIGURE 17.1 Domain architecture of autophagy receptors. PB1, Phox and Bem1p; ZnF, zinc finger; LIR, LC3 interacting region; UBA (in p62 and NBR1), UBAN (in OPTN) and UBZ (in NDP52), ubiquitin binding domain; CC, coiled-coil; BH3, BH3-only domain; TM, transmembrane domain.

cell to cause damage. Protein aggregates are successfully removed from cells by autophagosomal degradation or aggrephagy.

Mammalian p62 and NBR1 (neighbor of BRCA1 gene 1) proteins serve as cargo receptors and adaptors for selective autophagic degradation of ubiquitinated intracellular structures (Figure 17.2). Their cooperative actions are important for the selective removal of protein aggregates (Lamark *et al.*, 2009). p62 and NBR1 are structurally quite different in terms of size and primary sequence but have very similar domain structure (Figure 17.1). Both proteins have N-terminal PB1 domain, which mediates homopolymerization of p62 and NBR1 when in complex with ubiquitylated proteins. The PB1 domain-mediated polymerization is essential for both targeting p62 to the autophagosome formation site and ability of p62 to assemble proteins into protein aggregates, all leading to final degradation in autophagosome. p62 and NBR1 share similar LIR motifs to interact with the Atg8/LC3 family. Further, NBR1 can directly bind to p62, and together they act as receptors for polyubiquitinated cargo (Kirkin *et al.*, 2009; Pankiv *et al.*, 2007). Lastly, the third common domain is C-terminal UBA domain that allows binding to mono- or polyubiquitinated proteins. Monoubiquitination, K48 and K63 polyubiquitin chains are implicated in the formation of protein inclusions but only K63 chains participate in aggrephagy (Lamark *et al.*, 2009). It is important to note that p62 and NBR1 also participate in cellular processes that are not related to autophagy. p62 is an important actor in several signaling pathways and many mutations of p62 are associated with defects in signaling. Paget disease of the bone, a chronic metabolic disorder that is a consequence of defective signaling, has been associated with mutations in p62, predominantly its UBA domain (mostly deletions or losses of

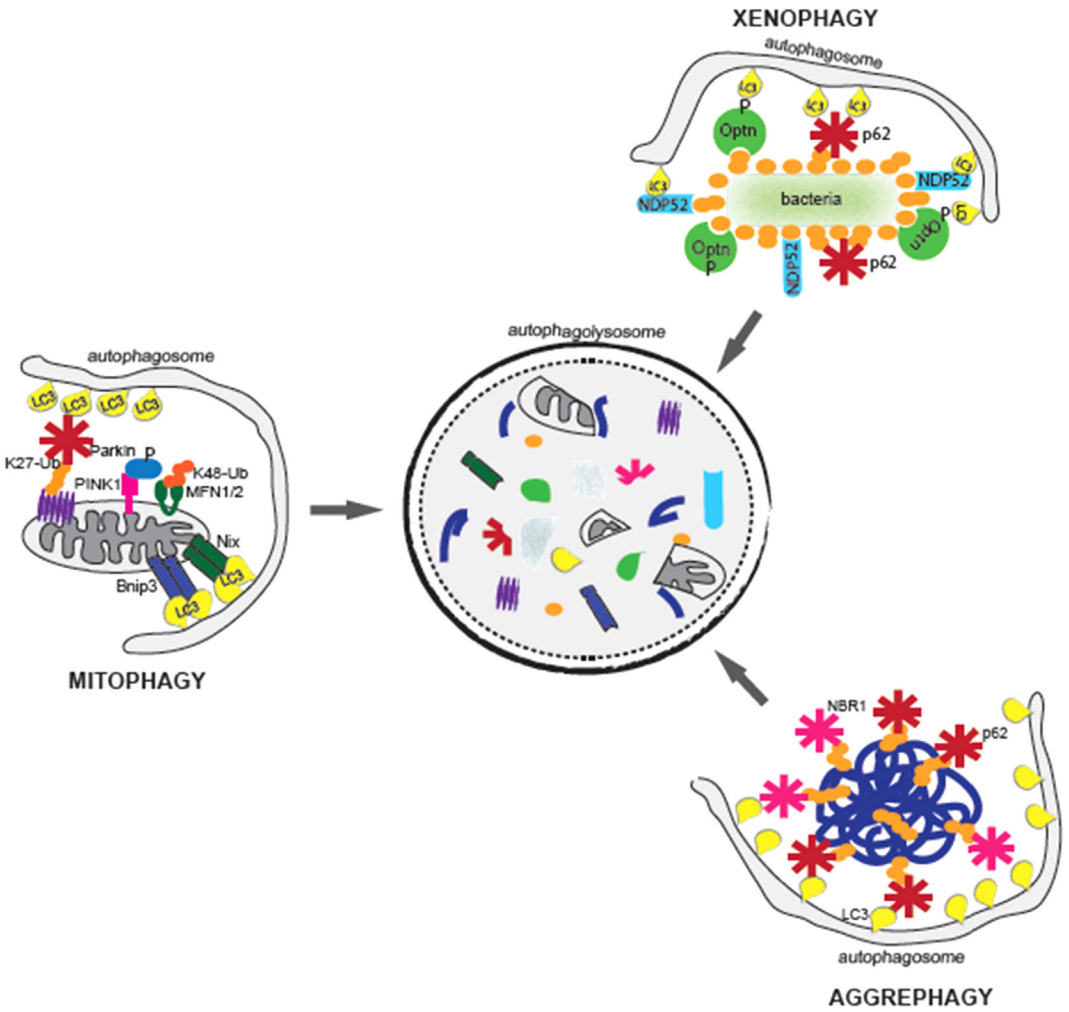

FIGURE 17.2 Selective autophagy. Various autophagy receptors involved in mitophagy, aggrephagy and xenophagy. Cargo, mitochondria, protein aggregates or bacteria, respectively, are degraded in the fully formed autophagosome after its fusion with lysosome.

function in the UBA domain). NBR1 binds sarcomeric protein titin and mutations in titin that disrupt NBR1 binding cause hereditary muscle disease in humans (Shaid *et al.*, 2013).

Further, the role of p62 and NBR1 is seen in some neurodegenerative disorders that are characterized by intracellular protein aggregates and inclusions, i.e., Alzheimer's, Huntington's and Parkinson's diseases. These protein inclusions are mostly highly ubiquitinated and p62 rich. Formation of p62-rich bodies depends on the presence of both p62 and NBR1, indicating that these proteins cooperate in selective autophagic degradation of misfolded proteins (Kirkin *et al.*, 2009). This cooperativity can be explained by the fact that p62

is unique for metazoans while NBR1 protein homologues are found throughout the eukaryotic kingdom. The exact influence of p62 and NBR1 in the development of neurodegenerative diseases is still unknown, but future studies should highlight their importance and possibly give novel drug targeting opportunities.

NDP52 and Optineurin

During infections, autophagy machinery plays a role in the capture and degradation of intracellular pathogens, via a process known as xenophagy. Antibacterial autophagy controls bacterial replication and promotes innate immunity in host cells. Many bacteria, such as *Mycobacteria*, *Salmonella*, *Shigella*, *Listeria*, and *Legionella*, can be targeted for lysosomal destruction but, so far, this has not been proven for viruses. It seems that viruses often affect autophagic signaling complexes rather than being removed by autophagosomes and no virus-specific autophagy receptors or adaptors have yet been found (Levine *et al.*, 2011). Intracellular bacteria in vacuoles or in cytoplasm can be targeted by autophagic machinery. Recognition by host cells is achieved through ubiquitination of bacteria that finally leads to selective removal by autophagy. Ubiquitinylated bacteria are recognized by autophagy receptors including p62, NBR1, NDP52 and optineurin (Lamark *et al.*, 2009; Thurston *et al.*, 2009; Wild *et al.*, 2011). These proteins function as cargo adaptors that connect substrates to the Atg8/LC3 proteins (Shaid *et al.*, 2013) (Figure 17.2).

Among these receptors, two bacteria-specific receptors have been described mediating clearance of *Salmonella enteritica typhimurium* (*S. typhimurium*): NDP52 (Thurston *et al.*, 2009) and optineurin (OPTN) (Wild *et al.*, 2011). When in cytoplasm, this bacterium is usually coated with ubiquitin. Interestingly, both NDP52 and OPTN have ubiquitin-binding domains and LIR motifs. OPTN and NDP52 were shown to localize to common microdomains on the bacteria surface, but these areas do not co-localize with those occupied by p62. It is considered that all three proteins are independently recruited to the same bacterium so their functions are nonredundant and lack of either protein causes propagation of *Salmonella* and impaired xenophagy (Wild *et al.*, 2011). A similar mechanism has been established in some other bacterial species, such as *Mycobacterium tuberculosis*, *Shigella* and *Listeria*, indicating that these mechanisms and protein receptors could be common events in pathogen cargo selection.

MITOPHAGY

Because of multiple roles of mitochondria in cell survival, it is necessary to maintain a sufficient functional mitochondrial population. This includes removal of damaged mitochondrial proteins and repairing damaged and destroying extremely impaired mitochondria. The accumulation of damaged mitochondria is a typical occurrence in some disorders, such as neurodegenerative diseases or tumors (Chen and Chan, 2009). However, partial or complete removal of mitochondria from cells is sometimes necessary for normal cell or tissue function. This occurs during the development and differentiation of certain cell types, e.g., maturation of reticulocytes and lymphocytes, spermatogenesis and development of the eye lens. This selective mitochondrial removal, mitophagy, like general autophagy, includes

autophagosome formation to engulf mitochondria marked for degradation and deliver them to lysosomes. Previous studies confirm that there are at least two mitophagy mechanisms, one for selective removal of damaged mitochondria and the second one for removal of normal mitochondria during differentiation of individual cell types, but molecular mechanisms that selectively recognize mitochondria suitable for elimination are not fully known.

Removal of Damaged Mitochondria

Some of the possible mechanisms underlying recognition and removal of damaged mitochondria emerged from studies of Parkinson's disease (PD). These studies show that mutations in outer mitochondrial membrane PTEN-induced putative kinase 1 (PINK1) and E3-ubiquitin ligase Parkin (or PARK2) are associated with defects in mitophagy and might be involved in the pathogenesis of familial PD (Valente *et al.*, 2004). With PINK1, serine/threonine kinase is expressed in the cytoplasm at very low levels when the mitochondrial membrane potential ($\Delta\psi m$) is intact and rapidly translocates into the mitochondrial inner membrane where it is degraded by mitochondrial inner membrane protease. When the mitochondrial membrane potential is lost, e.g., due to mitochondrial damage, PINK1 import and degradation are blocked and PINK1 accumulates on the outer mitochondrial membrane (Jin *et al.*, 2010). It is not yet clear how PINK1, on the mitochondrial outer membrane, recruits Parkin to mitochondria but possible mechanisms have been suggested. Parkin is predominantly cytosolic protein under basal conditions but, upon loss of $\Delta\psi m$, Parkin rapidly translocates to mitochondria (Narendra *et al.*, 2008). One mechanism suggests that PINK1 directly interacts with Parkin, anchoring it to the defective mitochondria and this interaction does not depend on PINK1 kinase or Parkin E3-ligase activity (Sha *et al.*, 2010). A second model proposes that PINK1 directly phosphorylates Parkin, resulting in activation of its E3 ubiquitin ligase activity, which results in Parkin translocation to mitochondria and its activation (Kim *et al.*, 2008). Once activated, Parkin ubiquitinates proteins on the mitochondrial outer membrane. Four Parkin substrates have been identified in mammalian cells at this time: voltage-dependent anion channel 1 (VDAC1), mitofusin-1 and mitofusin-2 (MFN1 and MFN2) and MIRO. Many studies have shown that an increase in the ubiquitination of a number of mitochondrial proteins correlates with mitophagy induction while mitophagy is reduced upon either PINK1 or Parkin silencing. Mitofusins are essential for formation of the elongated mitochondrial network. Successful mitophagy is achieved through fission events to fragment normal mitochondria that can then more easily be engulfed by autophagosomes (Twig *et al.*, 2008). Therefore, mitofusins need to be removed, which is accomplished by mitofusin polyubiquitination by Parkin, which finally leads to their degradation via proteasome (Gegg *et al.*, 2010). The ubiquitination of MFN1 and MFN2 precedes the mitochondrial clearance and is thus an early event in mitophagy. Degradation of mitofusins serves to switch the balance of mitochondrial dynamics toward fission to facilitate mitophagy (Figure 17.2).

Further, Parkin targets VDAC1 during mitophagy by forming K27 polyubiquitin chains. VDAC1 facilitates the exchange of metabolites and ions across the outer mitochondrial membrane and may regulate mitochondrial functions. This protein also forms channels in the plasma membrane and may be involved in transmembrane electron transport. K27 Ub chains on VDAC1 are not recognized by proteasome. Instead, K27 recruits p62 and

autophagosomal machinery on damaged mitochondria (Geisler *et al.*, 2010) (Figure 17.2). Interestingly, some studies indicate that VDAC1 may not be strictly required for PINK1/Parkin-mediated mitophagy. It seems that two newly discovered proteins, HMGB1 (High mobility group box 1) and HSPB1 (Heat shock protein beta-1), regulate Parkin translocation and VDAC1 ubiquitination during mitochondrial depolarization, but the exact mechanism of this molecular event remains unknown. HMGB1 is an evolutionarily conserved chromatin-associated protein essential for nuclear homeostasis, but it is also a critical regulator of mitochondrial function and dynamics. It plays important cytosolic, nuclear and extracellular roles in maintaining mitochondrial homeostasis and cellular energetic balance. In cytoplasm, HMGB1 binds Beclin-1 protein, a key player in autophagy activation. By binding Beclin-1, HMGB1 causes dissociation of its inhibitory partner, BCL-2 protein, leaving Beclin-1 free for autophagy induction. Extracellular HMGB1 binds the transmembrane RAGE receptor (receptor for advanced glycation end products) which inhibits mTOR and promotes the formation of the Beclin-1-PtdIns3KC3 (phosphatidylinositol-3-kinase class III) complex, which again leads to autophagy induction (Kang *et al.*, 2011). Further, HMGB1 has a wide range of nuclear functions; besides participating in DNA recombination and repair, it serves as a transcriptional factor for HSPB1/HSP27 expression. HSPB1 is an actin cytoskeleton regulator important for intracellular trafficking during autophagy (Tang *et al.*, 2007). It seems that HMGB1 and/or HSPB1 deficiency results in mitochondrial fragmentation, decreased ATP production and defective mitophagy. Thus, HMGB1, and consequently HSPB1 expression, may serve as protective mechanisms that activate autophagic machinery in order to prevent accumulation of damaged mitochondria in cells.

Mitophagy Receptors: Atg32, BNIP3 and BNIP3L/NIX

Alongside PINK1/Parkin-activated mitophagy, the cells also have specific receptors for autophagic removal of mitochondria functioning in a similar fashion as described previously: aggrephagy and xenophagy receptors. The first described selective mitophagy receptor was the budding yeast outer mitochondrial membrane protein scAtg32. As other autophagy receptors, scAtg32 possesses an LIR domain for Atg8 interaction to recruit autophagic machinery to damaged mitochondria. To achieve complete mitophagy, binding to Atg8 is not enough and scAtg32 requires recruitment and interaction with another autophagy protein, scAtg11, requisite for selective autophagy-related pathways (Okamoto *et al.*, 2009). The Atg32-Atg8-Atg11 complex builds an essential platform for the proper mitophagy that is independent of autophagosomal membrane formation. Current evidence indicates that the scAtg32 receptor is activated by phosphorylation of serine 114 and 119 by an unknown kinase. The phosphorylation triggers scAtg11 recruitment and all further sequential autophagy actions essential for mitochondrial removal (Aoki *et al.*, 2011).

Similar phosphorylation events of the receptors have been shown for aggrephagy and xenophagy receptor OPTN. Seemingly, conserved serine residues of OPTN juxtaposed to the LIR are phosphorylated by TANK binding kinase 1 (TBK1) that enhances Atg8/LC3 binding and driving bacteria or ubiquitinated protein aggregates toward the autophagic machinery for elimination (Korac *et al.*, 2013; Wild *et al.*, 2011). Current research is focusing on determination of similar phosphorylation-mediated regulation of other autophagy receptors.

Recently, two mammalian autophagy receptors specialized for mitochondrial removal have been discovered: BNIP3L/NIX and BNIP3 (Novak *et al.*, 2010; Zhu *et al.*, 2013). BNIP3L/NIX was previously known as an outer mitochondrial membrane protein essential for programmed removal of mitochondria in immature reticulocytes (Sandoval *et al.*, 2008; Schweers *et al.*, 2007). These two studies have shown that reticulocytes of BNIP3L/NIX knockout mice are unable to remove their mitochondria, leading to a block in the developmental stage of red blood cells, since they could mature into erythrocytes without complete removal of mitochondria. The researchers at this stage have not defined BNIP3L/NIX as an autophagy receptor since the autophagy in these cells was intact. However, their electron microscopy data illustrated how mitochondria in BNIP3L/NIX-deficient reticulocytes clearly positioned themselves around empty autophagosomes. The following led to detailed investigation of the BNIP3L/NIX role in developmental removal of mitochondria. It has been known that controlled removal of mitochondria occurs during development of some specialized cells, and is essential for correct organ or tissue development. Further, unlike PINK1/Parkin-mediated mitophagy, mitophagy induced during differentiation is not a quality control mechanism for degradation of unhealthy mitochondria, but a programmed complete or almost complete mechanism for elimination of the mitochondrial population. Therefore, BNIP3L/NIX was shown to interact with the autophagic adaptor Atg8/LC3 family proteins to bring the complete population of mitochondria to autophagosomes for degradation in the reticulocytes, the best-studied cell type for differentiation-induced mitophagy (Novak *et al.*, 2010). Direct interaction between two proteins is facilitated through the LC3-interacting region, LIR, located at the cytoplasmic N-terminal end of BNIP3L/NIX, the same consensus tetrapeptide present in other described autophagy receptors. As in other autophagy receptors, LIR is absolutely essential for the proper mitophagy and lack of functional LIR leads to an impaired mitophagy process (Figure 17.2). This was proven by the rescue experiments using a BNIP3L/NIX knockout mouse model where wild-type or LIR mutant BNIP3L/Nix was introduced into a BNIP3L/Nix knockout. Introduction of wild-type BNIP3L/NIX into knockout reticulocytes completely restored mitophagy, while the LIR mutant restored the phenotype only partially (Novak *et al.*, 2010). Together, this suggested the existence of either additional mitochondrial receptors similar to BNIP3L/NIX or alternative receptor-independent mechanisms. Indeed, a newly added mitophagy receptor BNIP3 was identified that, in the same manner as BNIP3L/NIX, recruits autophagic machinery to mitochondria (Zhu *et al.*, 2013). This reveals that possible regulation and activation of the receptors might come from the phosphorylation of the serines adjacent to LIR (Zhu *et al.*, 2013) (Figure 17.2). Moreover, the study has demonstrated that phosphorylation of serines in LIR of BNIP3 (similar to Optn) enhances mitochondrial elimination by more robust recruitment of autophagosomal membranes and also determines the cellular fate: survival through mitophagy or death through apoptosis.

Both mitophagy receptors BNIP3 and BNIP3L/NIX permanently reside in mitochondria through the C-terminal transmembrane domain and that specifically marks them as exclusive receptors for mitochondria, in contrast to p62, NBR1, NDP52 and OPTN which bear cargo-binding domain (ubiquitin binding domains to recognize polyubiquitinated cargo) which recognizes both ubiquitinated protein aggregates and bacteria. The transmembrane domain seems to be another essential part of the receptors, both to be able to recruit mitochondria and to strengthen the receptor's potential to serve as a receptor through

homodimerization (Figure 17.2) since it cannot function as a mitophagy receptor without being anchored to the mitochondrial membrane (Novak *et al.*, unpublished results).

The major questions to be put under investigation are how mitochondria become a target, and what sensors activate mitophagy. Further studies will have to address how damaged mitochondria are recognized and what regulates phosphorylation of the LIR. This might be solved if kinases and phosphatases that target these specific serines are defined. There are indications that other mechanisms might be involved in regulation of mitophagy, one being the intertwining of mitophagy receptor actions and the PINK1/Parkin pathway.

DISCUSSION

It is evident that mitophagy plays a central role in the regulation of mitochondrial dynamics and homeostasis, together with fusion and fission events, since the health status of all cells depends on the quality of mitochondria. Discovery of selective autophagy receptors enlightened the importance of highly regulated autophagy to ensure removal of redundant, damaged or toxic organelles, protein aggregates or intracellular pathogens. Growing evidence of the function of particular autophagy receptors indicates that most of them are often working together in the same selective autophagy pathway, such as aggrephagy, xenophagy and mitophagy. The mitophagy receptor's mechanisms are the least known and, thus, the lessons from xenophagy are directing future research towards better understanding of how mitophagy is activated and regulated. After all, this is not surprising because there is a well-accepted endosymbiotic theory that connects bacteria and mitochondria, where mitochondria are bacterial evolutionary descendants. A primary interest is to decipher the role of phosphorylation of receptors and their interacting partners. Further, novel results on triggers that mark mitochondria for autophagic elimination are essential for better understanding of this process, because the knowledge of how damaged mitochondria are selected from healthy ones is still very obscure. It should be kept in mind that PINK1/Parkin and mitophagy receptor mechanisms might work conjointly as suggested by recent discoveries (for more details, see Novak, 2012). Future studies analyzing individual components of mitophagy might decipher the complex network that will help us understand the physiological and, more importantly, pathological conditions (e.g., Parkinson's disease) and allow us to manipulate mitophagy to fight diseases.

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References

Aoki, Y., Kanki, T., Hirota, Y., et al., 2011. Phosphorylation of Serine 114 on Atg32 mediates mitophagy. Mol. Biol. Cell. 22 (17), 3206–3217.

Chen, H., Chan, D.C., 2004. Mitochondrial dynamics in mammals. Curr. Top. Dev. Biol. 59, 119–144.

Chen, H., Chan, D.C., 2009. Mitochondrial dynamics – fusion, fission, movement, and mitophagy – in neurodegenerative diseases. Hum. Mol. Genet. 18 (R2), R169–R176.

Cuervo, A.M., 2004. Autophagy: many paths to the same end. Mol. Cell. Biochem. 263 (1–2), 55–72.
DiMauro, S., Schon, E.A., 2003. Mitochondrial respiratory-chain diseases. N. Engl. J. Med. 348 (26), 2656–2668.
Gegg, M.E., Cooper, J.M., Chau, K.Y., et al., 2010. Mitofusin 1 and mitofusin 2 are ubiquitinated in a PINK1/parkin-dependent manner upon induction of mitophagy. Hum. Mol. Genet. 19 (24), 4861–4870.
Geisler, S., Holmstrom, K.M., Skujat, D., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell. Biol. 12 (2), 119–131.
Jin, S.M., Lazarou, M., Wang, C., et al., 2010. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J. Cell. Biol. 191 (5), 933–942.
Kang, R., Livesey, K.M., Zeh, H.J., et al., 2011. Metabolic regulation by HMGB1-mediated autophagy and mitophagy. Autophagy 7 (10), 1256–1258.
Kim, Y., Park, J., Kim, S., et al., 2008. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem. Biophys. Res. Commun. 377 (3), 975–980.
Kirkin, V., McEwan, D.G., Novak, I., et al., 2009. A role for ubiquitin in selective autophagy. Mol. Cell. 34 (3), 259–269.
Klionsky, D.J., Cuervo, A.M., Dunn Jr., W.A., et al., 2007. How shall I eat thee? Autophagy 3 (5), 413–416.
Korac, J., Schaeffer, V., Kovacevic, I., et al., 2013. Ubiquitin-independent function of optineurin in autophagic clearance of protein aggregates. J. Cell. Sci. 126 (Pt 2), 580–592.
Kroemer, G., Galluzzi, L., Brenner, C., 2007. Mitochondrial membrane permeabilization in cell death. Physiol. Rev. 87 (1), 99–163.
Lamark, T., Kirkin, V., Dikic, I., et al., 2009. NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell Cycle 8 (13), 1986–1990.
Levine, B., Mizushima, N., Virgin, H.W., 2011. Autophagy in immunity and inflammation. Nature 469 (7330), 323–335.
McEwan, D.G., Dikic, I., 2011. The three musketeers of autophagy: phosphorylation, ubiquitylation and acetylation. Trends Cell. Biol. 21 (4), 195–201.
Narendra, D., Tanaka, A., Suen, D.F., et al., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell. Biol. 183 (5), 795–803.
Neustadt, J., Pieczenik, S.R., 2008. Medication-induced mitochondrial damage and disease. Mol. Nutr. Food Res. 52 (7), 780–788.
Novak, I., 2012. Mitophagy: a complex mechanism of mitochondrial removal. Antioxid. Redox. Signal. 17 (5), 794–802.
Novak, I., Kirkin, V., McEwan, D.G., et al., 2010. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO Rep. 11 (1), 45–51.
Okamoto, K., Kondo-Okamoto, N., Ohsumi, Y., 2009. Mitochondria-anchored receptor Atg32 mediates degradation of mitochondria via selective autophagy. Dev. Cell. 17 (1), 87–97.
Pankiv, S., Clausen, T.H., Lamark, T., et al., 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 282 (33), 24131–24145.
Rozenknop, A., Rogov, V.V., Rogova, N.Y., et al., 2011. Characterization of the interaction of GABARAPL-1 with the LIR motif of NBR1. J. Mol. Biol. 410 (3), 477–487.
Sandoval, H., Thiagarajan, P., Dasgupta, S.K., et al., 2008. Essential role for Nix in autophagic maturation of erythroid cells. Nature 454 (7201), 232–235.
Scherz-Shouval, R., Elazar, Z., 2007. ROS, mitochondria and the regulation of autophagy. Trends Cell. Biol. 17 (9), 422–427.
Schweers, R.L., Zhang, J., Randall, M.S., et al., 2007. NIX is required for programmed mitochondrial clearance during reticulocyte maturation. Proc. Natl Acad. Sci. USA 104 (49), 19500–19505.
Sha, D., Chin, L.S., Li, L., 2010. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum. Mol. Genet. 19 (2), 352–363.
Shaid, S., Brandts, C.H., Serve, H., et al., 2013. Ubiquitination and selective autophagy. Cell. Death. Differ. 20 (1), 21–30.
Tang, D., Kang, R., Xiao, W., et al., 2007. Nuclear heat shock protein 72 as a negative regulator of oxidative stress (hydrogen peroxide)-induced HMGB1 cytoplasmic translocation and release. J. Immunol. 178 (11), 7376–7384.
Thurston, T.L., Ryzhakov, G., Bloor, S., et al., 2009. The TBK1 adaptor and autophagy receptor NDP52 restricts the proliferation of ubiquitin-coated bacteria. Nat. Immunol. 10 (11), 1215–1221.
Twig, G., Elorza, A., Molina, A.J., et al., 2008. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J. 27 (2), 433–446.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., et al., 2004. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 304 (5674), 1158–1160.

Wild, P., Farhan, H., McEwan, D.G., et al., 2011. Phosphorylation of the autophagy receptor optineurin restricts *Salmonella* growth. Science 333 (6039), 228–233.

Wirawan, E., Vanden Berghe, T., Lippens, S., et al., 2012. Autophagy: for better or for worse. Cell. Res. 22 (1), 43–61.

Yang, Z., Klionsky, D.J., 2010. Mammalian autophagy: core molecular machinery and signaling regulation. Curr. Opin. Cell. Biol. 22 (2), 124–131.

Zhu, Y., Massen, S., Terenzio., M., et al., 2013. Modulation of serines 17 and 24 in the LC3-interacting region of Bnip3 determines pro-survival mitophagy versus apoptosis. J. Biol. Chem. 288 (2), 1099–1113.

CHAPTER

18

The Role of Parkin and PINK1 in Mitochondrial Quality Control

Matthew Y. Tang and Thomas M. Durcan

OUTLINE

Abstract

Within the brain, mitochondria serve as the primary producers of ATP to meet the high energy requirements of individual neurons. Through its electron transport chain (ETC), mitochondria generate most of this ATP in an oxygen-dependent manner, with toxic reactive oxidative stress (ROS) also released from the same process. Over time an accumulation of this ROS can severely damage the mitochondrial population within the neuron, ultimately causing apoptosis of the affected neurons. Mitochondrial dysfunction is often implicated in disorders of the brain, in particular Parkinson's disease (PD), an incurable movement disorder caused by the progressive neurodegeneration of dopaminergic neurons (DA).

Compared to other neurons, DA neurons are more vulnerable to ROS due to their intrinsic pacemaking ability. As a consequence, these neurons are under constant oxidative stress that can cause irreparable damage to mitochondria. To cope with this stress, surveillance mechanisms exist to eliminate dysfunctional

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mitochondria and prevent a domino effect of damage spreading to neighboring mitochondria. Central to this surveillance process are PINK1 and Parkin, two proteins encoded by genes associated with early onset PD. In this chapter, we describe the removal of damaged or dysfunctional mitochondria in detail, outlining the structures and function of both PINK1 and Parkin, and how this pathway may be implicated in mitochondrial quality control within neurons.

INTRODUCTION

Individual neurons in the brain have a high demand for energy in order to sustain ionic gradients across the cell membranes and for neurotransmission. Mitochondria serve as the primary producers of ATP, with much of this ATP generated via the electron transport chain (ETC) through a stepwise reaction that consumes oxygen. Any impairment in the ability of the mitochondria to generate ATP makes it increasingly difficult for a neuron to survive and function normally. Toxic byproducts generated as the consequence of mitochondrial energy production make the mitochondria a major site for generating cellular reactive oxidative species (ROS), which over time can severely damage mitochondria, causing an accumulation of dysfunctional mitochondria, an increase in the generation of ROS, and ultimately apoptosis of the affected neurons. Mitochondrial dysfunction is therefore often implicated in disorders of the brain, including Parkinson's disease (PD).

PD is an incurable movement disorder that primarily involves the progressive neurodegeneration of dopaminergic neurons (DA) within the substantia nigra pars compacta (SNpc) of the brain, though neuronal loss can also occur in other regions. While the majority of PD cases are idiopathic, a number of genes that encode proteins with essential functions at the mitochondria and in mitochondrial quality control pathways have been implicated in familial forms of PD. As the second most common neurodegenerative disease, PD is a chronic age-related disorder, affecting more than 1% of the population over the age of 65, and 4% over the age of 85. However, PD is not only observed in older patients, as cases have been reported with patients presenting with PD symptoms at a much earlier onset between the ages of 20–40 years old. As the loss of dopaminergic neurons progresses, motor symptoms start to manifest that include resting tremor, bradykinesia, gait disturbance and postural instability. Nonmotor symptoms such as loss of olfaction, constipation, and rapid eye movement (REM) sleep disorders are also common in PD and often precede the motor symptoms described above.

Although many neuronal types are affected in PD, a major hallmark of PD is the progressive loss of dopaminergic neurons within the SNpc and other brainstem regions. Yet, why are DA more vulnerable relative to other neuronal cell types in PD? DA neurons possess intrinsic pacemaking ability and are constantly taking up and releasing calcium to drive their basal activity. Excessive calcium can be toxic to neurons, and to prevent calcium from accumulating to toxic levels, mitochondria sequester and promote the release of calcium through an ATP-dependent mechanism. However, generating ATP comes at a cost to the neurons, as superoxide and ROS are byproducts, thereby increasing the vulnerability of these neurons to mitochondrial oxidant stress (Guzman *et al.*, 2010). If stress levels become too high, defects within one or more mitochondria can arise, placing an undue burden on the healthy mitochondrial population. As defective mitochondria can be deleterious to

neighboring mitochondria by producing yet more ROS in a "vicious cycle" of oxidative damage, surveillance mechanisms have been developed within the cell to promote the removal of damaged mitochondria. The inability to eliminate dysfunctional mitochondria can cause a domino effect with damage spreading to neighboring mitochondria and may therefore contribute to the pathogenesis of PD.

PARKINSON'S DISEASE AND MITOCHONDRIAL DYSFUNCTION

The first compelling evidence for a link between PD and mitochondrial dysfunction came in the 1980s, when intravenous drug users developed symptoms similar to those of advanced PD in a matter of weeks. It was discovered that they were accidentally exposed to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine), a contaminant byproduct in the synthetic preparation of MPPP (1-methyl-4-phenyl-4-propionoxypiperidine), an analogue of the synthetic opioid meperidine. Once present in the bloodstream, MPTP was metabolized to MPP+ (1-methyl-4-phenylpyridinium), enabling it to cross the blood-brain barrier, where it could be freely taken up by DA neurons via the dopamine transporter. Within the DA neurons, MPP+ accumulates in mitochondria, inhibiting complex I (NADH ubiquinone oxidoreductase) of the electron transport chain, thereby resulting in an acute parkinsonian syndrome that differs from the slow, progressive nature of PD. Since the discovery that exposure to MPTP causes acute PD, other complex 1 inhibitors including rotenone (an insecticide) and paraquat (a herbicide) have also been implicated as causal agents in PD.

A direct association between mitochondrial dysfunction and PD was first established when postmortem autopsies of PD patients revealed a reduced activity of complex 1 in the SNpc and frontal cortex (Schapira *et al.*, 1989). Furthermore, complex 1 subunits derived from mitochondrial preparations from PD patients were found to be oxidatively damaged (Keeney *et al.*, 2006). To determine whether this defect was due to an environmental toxin or a genetic alteration within the mitochondrial or nuclear DNA, a cytoplasmic hybrid technique was utilized. Hybrid cells (called cybrids) were generated by isolating mitochondria from the platelets of a PD patient and transferring them into cells depleted of mitochondrial DNA. The PD cybrid cells were found to have a decrease in complex 1 activity that coincided with increased ROS production and a decrease in their overall respiratory capacity, all features consistent with defects in the mitochondrial ETC being involved in PD. Intriguingly, the mitochondria in the PD cybrid cells were found to be enlarged with disrupted cristae. Moreover, between PD and control samples, differences were observed in the expression of many proteins implicated in mitochondrial functions and cellular responses to oxidative stress. Thus, evidence from these studies strongly suggests an involvement for the mitochondria in PD.

Following the cybrid studies, the identification of Parkin (*PARK2*) and PTEN-induced putative kinase 1 (*PINK1*) as genes mutated in a familial form of early onset PD has led to many seminal studies highlighting essential roles for the products of both genes at the mitochondria. The first compelling evidence that Parkin and PINK1 might regulate mitochondrial function emerged from studies in *Drosophila melanogaster*. Relative to other vertebrate systems, fruitflies possess a small number of neuronal and glial cells in their central nervous system. However, they share the same types of GABAinergic and dopaminergic

neurotransmitter systems and are able to accomplish a number of complex behaviors. Thus, considering the conservation between genes and signaling pathways in flies and vertebrates, and the ease with which genes in the flies can be manipulated, *Drosophila* models provided a valuable starting point for understanding the normal functions of Parkin and PINK1.

PARKIN AND PINK1 MUTANT FLIES

Immense progress has been achieved in understanding the contribution of Parkin and PINK1 to mitochondrial pathology using *Drosophila* models of PD. Parkin functions as an E3 ubiquitin (Ub) ligase to modify and target substrates destined for degradation through the assembly of ubiquitin chains. Parkin is highly conserved between vertebrates and invertebrates with high levels of Parkin expression detected in the CNS of both. To first elucidate its biological function, mutant flies were generated in which Parkin expression had been abolished. Interestingly, these mutants were viable, albeit with a reduced life span, and at the same time displaying deficits in locomotor activity and male sterility. Intriguingly, the locomotor deficits related to defects in the mitochondria correlated with the widespread apoptotic degeneration in muscle fibers (Greene *et al.*, 2005). Moreover, the sterility in Parkin null males was also caused by defects in mitochondrial function. Consistent with a role at the mitochondria, Parkin was also demonstrated to partially localize to the mitochondria.

The importance of Parkin at the mitochondria was further underlined by the discovery of a genetic interaction with another PD-associated gene, PINK1. Like Parkin, PINK1 is also highly expressed in the fly brain and testes, and flies lacking PINK1 phenocopy the defects observed in the Parkin null flies. Interestingly, PINK1 contains a mitochondrial targeting signal (MTS) that directs it towards the mitochondria. Using different combinations of Parkin and PINK1 null flies, Parkin was demonstrated to function downstream of PINK1, with Parkin overexpression rescuing the mitochondrial defects found in PINK1 null flies. Conversely, PINK1 overexpression had no effect on the Parkin phenotype. Furthermore, knockdown of both genes in double mutant flies caused the same phenotype as single mutants alone. From these findings, PINK1 and Parkin appear to function in the same pathway, with PINK1 acting upstream of Parkin.

Mitochondrial fission and fusion processes are critical for maintaining a healthy mitochondrial network. Fission is important for creating new mitochondria and the isolation of damaged segments of mitochondria for autophagic degradation, whereas fusion results in the mixing of mitochondrial matrices thereby mitigating cellular stress and maximizing its oxidative capacity. In both Parkin and PINK1 null mutant flies, defects in mitochondrial morphology are observed, suggesting a potential role for PINK1, Parkin or both in the regulation of mitochondrial dynamics. Consistent with this idea, manipulating flies to promote fission in mitochondria suppresses the PINK1 phenotype. When mitochondrial fission was enhanced, defects in mitochondrial morphology were rescued in PINK1 mutants. In addition, the muscle defects observed in PINK1 and Parkin mutant flies were mitigated when mitochondrial fission was increased. Conversely, decreased fission had the opposite effect, enhancing the PINK1 null phenotype. Given that flies lacking PINK1 and Parkin are still

viable suggests that these are not core components in mitochondrial fission/fusion, since mutations in essential factors are normally lethal. Thus, the PINK1/Parkin pathway may regulate additional aspects of mitochondrial morphology.

What then is the biological function for Parkin and PINK1 at the mitochondria? The first clue came from a recent study in which PINK1 was demonstrated to phosphorylate Parkin at Thr 175, thereby promoting its relocalization from the cytoplasm onto the mitochondria (Kim *et al.*, 2008). Moreover, when overexpressed in neuroblastoma cells, Parkin remained in the cytosol and only localized onto the mitochondria when PINK1 was co-expressed. If levels of PINK1 were reduced or a kinase-dead form of PINK1 was overexpressed, Parkin again failed to relocalize from the cytoplasm. Remarkably, when *Drosophila* was used to validate the findings from cells, expression of a modified Parkin carrying a mitochondrial targeting signal bypassed PINK1 and rescued the PINK1/Parkin double mutant, independent of phosphorylation (Kim *et al.*, 2008). In addition, experiments using mammalian cells demonstrated that treatment with chemical inhibitors of mitochondrial function, such as the mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenyl hydrazone) or paraquat, results in the recruitment of Parkin onto mitochondria that have lost their membrane potential ($\Delta\Psi m$) (Narendra *et al.*, 2008). Thus, Parkin appears to play a protective role, by directing these damaged mitochondria for clearance via the autophagy pathway (called mitophagy) in a PINK1-dependent manner. Taken together, a scenario can be envisaged in which PINK1 acts to promote the recruitment of Parkin onto mitochondria that are destined for clearance.

STABILIZATION OF PINK1 ON MITOCHONDRIA

Before mutations in PINK1 were implicated in autosomal recessive PD, PINK1 was first described in a study on ovarian tumor tissues in which its expression was abolished. To date, close to 50 mutations in the PINK1 gene have been associated with PD, with mutations identified in approximately 1–3% of early onset PD cases of European ancestry; 2.5% of causes from ethnic Chinese, Malays, and Indians; and as high as 8.9% in a cohort of Japanese patients presenting with early onset PD.

PINK1 is a ubiquitously expressed 581 amino acid serine/threonine kinase that contains an N-terminal mitochondrial targeting sequence (MTS), a serine/threonine kinase domain, and a C-terminal domain whose function is unclear (Figure 18.1). Evidence from many studies has demonstrated PINK1 to function at the mitochondria, controlling mitochondrial morphology, regulating the mitochondrial recruitment of Parkin, while also playing a role in mitochondrial maintenance. As a kinase, recombinant PINK1 can phosphorylate itself, and several artificial substrates. Although there is no current crystal model for PINK1, its kinase domain has been demonstrated to be most similar to calmodulin-dependent kinases and the majority of mutations associated with PD are found within this kinase domain.

Under normal untreated conditions, levels of endogenous PINK1 are constitutively low. Intriguingly, when cells are treated with CCCP or other uncouplers that abolish the mitochondrial membrane potential ($\Delta\Psi$), the full-length form of PINK1 accumulates on the outer membrane of depolarized mitochondria. Following this increase in full-length PINK1, Parkin is recruited onto mitochondria lacking $\Delta\Psi$ (Narendra *et al.*, 2010b). Accumulation of

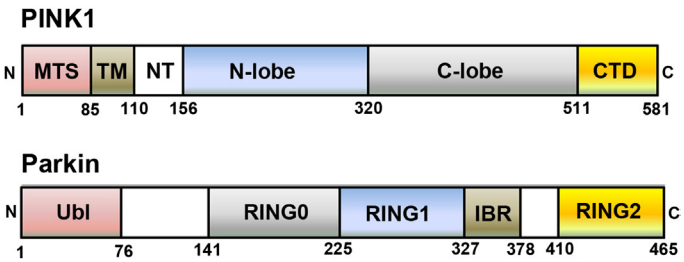

FIGURE 18.1 **The domain boundaries of full-length human PINK1 and Parkin.** The domains indicated for PINK1 are the mitochondrial targeting sequence (MTS), transmembrane helix (TM), N-terminal regulatory region (NT), N- and C-terminal lobes and the C-terminal domain (CTD). The domains indicated for Parkin are the N-terminal Ub-like domain (Ubl), the REP (Repressor) domain, the RING0, RING1 and RING2 zinc binding domains, and the In-Between RING (IBR) domain. Numbers represent amino acid position.

PINK1 on mitochondria, and not necessarily a loss in membrane potential, is essential for Parkin recruitment. For example, the overexpression of PINK1 is sufficient to redirect Parkin from the cytoplasm onto mitochondria that have an intact $\Delta\Psi$ (Narendra *et al.*, 2010b). Moreover, the kinase activity of PINK1 is also required, as an accumulation of a kinase-dead form of PINK1 on the outer surface of depolarized mitochondria fails to elicit the relocalization of Parkin from the cytoplasm (Geisler *et al.*, 2010; Narendra *et al.*, 2010b). Thus, the accumulation of PINK1 on mitochondria lacking $\Delta\Psi$ is an essential first step in this mitophagy pathway, yet how does this loss in membrane potential cause PINK1 to accumulate on the mitochondrial surface?

In untreated cells, three forms of PINK1 can be detected by Western blot analysis: a 64 kDa full-length form and two cleaved fragments (60 and 52 kDa) (Greene *et al.*, 2012; Jin *et al.*, 2010). As a result of its N-terminal MTS, PINK1 is constitutively imported into the mitochondria via the Translocase of outer membrane (TOM) and Translocase of inner membrane (TIM) complex. Once imported, the MTS of full-length PINK1 is cleaved generating a 60 kDa cleaved isoform. This first cleavage step is associated with binding of PINK1 to the mitochondrial processing peptidase (MPP). Next, the 60 kDa fragment of PINK1 is further cleaved by a combination of Presenilin-associated Rhomboid protease (PARL) and AFG3L2, giving rise to a 52 kDa processed fragment that is exported to the cytosol where it is rapidly degraded by the proteasome (Greene *et al.*, 2012; Jin *et al.*, 2010). It is speculated that this form of PINK1 may play a role at the mitochondria, although no function has yet been described for it. Through this pathway of successive proteolytic cleavages, PINK1 levels are kept intrinsically low to prevent mitophagy of healthy mitochondria (Figure 18.2). Since the import of PINK1 into the mitochondria requires an active proton gradient, depolarization of mitochondria now prevents the import of PINK1 into the mitochondria through the TOM/TIM complex, thereby leading to a build-up of PINK1 on the outer surface of depolarized mitochondria (Lazarou *et al.*, 2012). In the absence of mitochondrial depolarization, overwhelming the import channels by overexpressing PINK1 can also lead to its accumulation on mitochondria, coinciding with Parkin recruitment (Narendra *et al.*, 2010b). More recently,

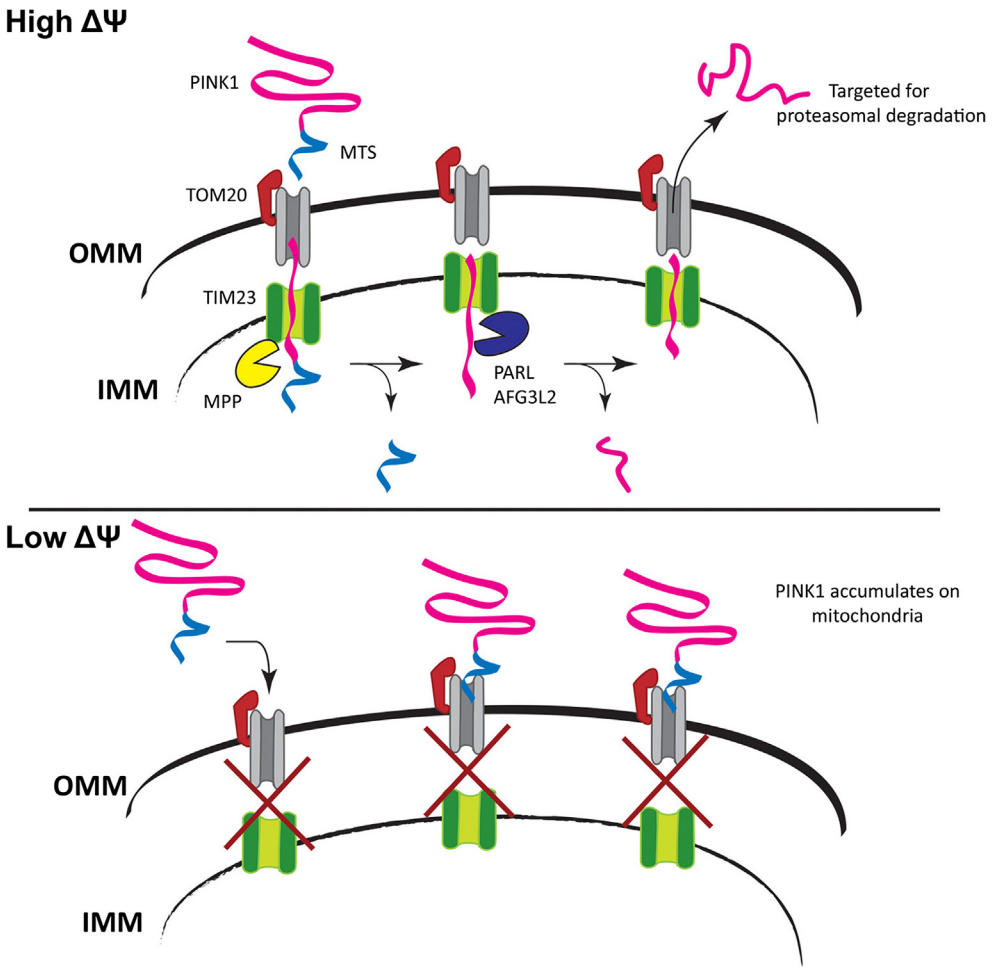

FIGURE 18.2 **Model for PINK1 import and export through the mitochondria.** Through its N-terminal mitochondrial targeting sequence (MTS), PINK1 is directed towards the mitochondria. In healthy mitochondria containing an intact membrane potential (ΔΨm), PINK1 translocates through the TOM:TIM complex where in the mitochondrial matrix, its MTS is cleaved by the MPP mitochondrial protease. Next, PARL and AFG3L3 cooperate together to cleave PINK1 generating a 52 kDa fragment. This fragment is actively transported out of the mitochondria where it is degraded by the proteasome. In mitochondria where (ΔΨm) is abolished, PINK1 can no longer be imported via the TOM:TIM complex, leading to its accumulation on the outer surface of the mitochondria.

the knockdown of specific mitochondrial proteases mentioned above for cleaving PINK1 was shown to be sufficient to promote the accumulation of PINK1 on healthy mitochondria, which in turn initiated Parkin recruitment and Parkin-mediated mitophagy (Greene *et al.*, 2012; Jin *et al.*, 2010). Thus, the presence of PINK1 on the outer mitochondrial surface is essential for Parkin to be recruited.

PINK1 ACTIVITY ON THE MITOCHONDRIA

Once present on the outer mitochondrial surface, PINK1 signals for Parkin to relocalize from the cytoplasm onto the damaged mitochondria. For Parkin recruitment to occur, PINK1 must be catalytically active, as accumulation of the kinase-dead form of PINK1 was unable to recruit Parkin (Geisler *et al.*, 2010). When PINK1 accumulates on the surface of the mitochondria following mitochondrial depolarization, its kinase domain faces into the cytoplasm, enabling it to phosphorylate both mitochondrial and cytosolic proteins (Zhou *et al.*, 2008). PINK1 autophosphorylation has been suggested to play a key role in the recruitment of Parkin onto mitochondria. As it accumulates on the outer mitochondrial surface, PINK1 phosphorylates itself directly at Ser 228, with Ser 402 in the activation loop essential for this to occur (Okatsu *et al.*, 2012). When the constitutively active form of PINK1 (S228D/S402D) was expressed in PINK1 null MEFs, Parkin was readily recruited onto mitochondria, whereas the inactive form of PINK1 (S228A/S402A) had no effect. In addition to these two sites, a separate study identified Thr 257 as a third site on PINK1 that was phosphorylated, although mutating this residue had no effect on Parkin recruitment (Kondapalli *et al.*, 2012). In concert with its ability to autophosphorylate, oligomerization of PINK1 has been associated with Parkin recruitment, with PINK1 demonstrated to form a 700 kDa complex with the TOM complex following mitochondrial depolarization (Lazarou *et al.*, 2012).

As a kinase, PINK1 has the potential to phosphorylate both cytosolic and mitochondrial proteins, yet the identity of these substrates remains unclear. There are two proteins that have been identified to be substrates of PINK1: the mitochondrial protein TRAP1 (Pridgeon *et al.*, 2007) and the axonal transport protein Miro (Wang *et al.*, 2011). However, PINK1-mediated phosphorylation of Parkin remains controversial. A recent study reported that PINK1 is unable to phosphorylate Parkin *in vitro* (Vives-Bauza *et al.*, 2010), whereas a separate study reported that PINK1 was able to phosphorylate Parkin both *in vitro* and *in vivo* (Sha *et al.*, 2010). In support of the idea that PINK1 could phosphorylate Parkin, a later study identified Ser 65 as a unique site within Parkin that is phosphorylated by PINK1 (Kondapalli *et al.*, 2012), with phosphorylation at this site necessary for the efficient recruitment of Parkin onto mitochondria. Taken together, it appears likely that PINK1-mediated phosphorylation of Parkin is required for its recruitment onto mitochondria, although the molecular basis for this remains unclear.

PARKIN: A PD-ASSOCIATED E3-UBIQUITIN LIGASE

While PINK1 serves to initiate mitophagy, the activity of Parkin is required to direct the damaged mitochondria for clearance. Parkin is encoded by the *PARK2* gene, the first gene to be associated with early onset (20–45 years of age) autosomal recessive PD. To date, over 120 mutations within the *Parkin* gene are associated with a familial form of early onset PD, with point mutations found in every domain of the Parkin protein (Kitada *et al.*, 1998; Mata *et al.*, 2004). These loss-of-function mutations impair the normal activity of the Parkin protein as an E3 ubiquitin-ligase, thus impeding its normal function in a variety of neuroprotective pathways, including mitochondrial quality control.

As the protein product of the *PARK2* gene, the 52 kDa Parkin protein functions as a RING1-InBetweenRING-RING2 (RBR)-type E3 ubiquitin-ligase that functions to mediate the covalent attachment of ubiquitin (Ub) onto lysine residues within target proteins. Ub is a highly conserved 76 amino acid protein that contains seven lysine residues and is typically attached via a covalent bond between its carboxy-terminus and a lysine residue within the substrate protein. A single Ub moiety can be attached to the substrate in a process designated monoubiquitylation. Alternatively, individual Ub proteins can be attached at multiple sites within the substrate (multiubiquitylation), or a single ubiquitin can serve as the attachment point for a chain of ubiquitin molecules (polyubiquitylation) with each ubiquitin attached to a lysine within a subsequent ubiquitin protein.

Since Ub contains seven lysines, the lysine through which each Ub is linked in a polyUb chain influences how it regulates the function of the substrate when attached. Best characterized are chains linked via lysine at position 48 of ubiquitin (K48) targeting proteins for proteasomal degradation, whereas chains assembled using lysine 63 have functions in signal transduction and DNA repair. In contrast, proteins modified with Ub conjugates linked through one of the other lysines or simply with an individual Ub moiety (monoubiquitination) escape proteasomal destruction and mediate important functions in a variety of other processes (Newton *et al.*, 2008).

From early studies, Parkin has been shown to ubiquitinate a variety of substrates and has the ability to ubiquitinate itself in a process called autoubiquitination (Durcan *et al.*, 2011). However, it is only with the recent studies elucidating the crystal structure of Parkin that we now have a better understanding as to how Parkin functions as an E3-ligase (Trempe *et al.*, 2013; Wauer and Komander, 2013). From these structural studies, Parkin was found to contain an N-terminal Ub-like domain and four distinct zinc-finger domains (Figure 18.1). The final three zinc fingers in Parkin form a distinct RBR domain, placing Parkin in a distinct group of 13 RBR-type E3s that also include HHARI, Ariadne, HOIL and HOIP.

Until recently, RBR-type E3s were thought to act like RING-type E3s, with the RING domain acting to direct the Ub from the E2 onto the substrate. From a recent study on HHARI, this model was disproved, as RBR-type E3s were demonstrated to act like a RING-HECT like hybrid E3s (Wenzel *et al.*, 2011). In this new model, RBR proteins such as HHARI and Parkin interact with the E2, with the E2 transferring the Ub onto the catalytic site, thereby forming an E3-Ub thioester intermediate. In the case of Parkin, the Ub is transferred onto its active site cysteine at site 431 (Lazarou *et al.*, 2013; Riley *et al.*, 2013). This step is transient, with the E3 rapidly transferring onto a lysine within a substrate protein for the RBR-type E3, which can also include the E3 itself.

In addition to a deeper understanding into how Parkin functions as an E3, these studies also provided the first evidence that Parkin exists in an autoinhibited state. The structure of Parkin shows that this inhibition is caused by two distinct features: (1) the repressor element of Parkin (REP) that binds the RING1 domain and thus prevents binding of E2 ubiquitin-conjugating enzymes and (2) a RING0-RING2 interaction that blocks access to the Cys 431 catalytic site in Parkin. Indeed, the introduction of mutations into Parkin to abolish either of these interactions (W403A, F463A, F146A, RING0 deletion) cause a dramatic increase in Parkin autoubiquitination (Trempe *et al.*, 2013). In particular, the W403A mutation facilitates the binding of the E2 enzyme UbcH7, which leads to faster recruitment of Parkin onto depolarized mitochondria (Trempe *et al.*, 2013). In contrast, the T240R mutation in the E2-binding

site on RING1 abolishes UbcH7 binding and autoubiquitination, strongly impairing Parkin recruitment to mitochondria and Parkin-dependent mitophagy (Geisler *et al.*, 2010; Matsuda *et al.*, 2010; Trempe *et al.*, 2013). Thus, the binding of E2 enzymes to Parkin is crucial for its biological activity.

PINK1-MEDIATED RECRUITMENT OF PARKIN ONTO MITOCHONDRIA

Structural studies strongly imply that Parkin exists in an autoinhibited state requiring a specific signal(s) to become activated. In the mitochondrial quality control pathway, recruitment of PINK1 onto depolarized mitochondria coincides with a dramatic increase in Parkin self-ubiquitination (Matsuda *et al.*, 2010), pointing to PINK1-mediated phosphorylation as one such signal that can activate Parkin ligase activity. Indeed, there is strong evidence that phosphorylation of Parkin by PINK1 is required for Parkin activation during the early stages of mitophagy. Firstly, PINK1 kinase activity is required for Parkin to be recruited onto depolarized mitochondria. Specifically, phosphorylation of Parkin at Ser 65 by PINK1 was found to increase Parkin E3 Ub-ligase activity (Kondapalli *et al.*, 2012). When this site was mutated, Parkin was no longer recruited onto mitochondria, implying a crucial role for this site in priming Parkin for activation. Secondly, findings from FLIM studies are suggestive of Parkin and PINK1 being in close proximity on the mitochondria (Vives-Bauza *et al.*, 2010), with immunoprecipitation assays further demonstrating the formation of a complex between PINK1 and Parkin (Sha *et al.*, 2010; Vives-Bauza *et al.*, 2010). This is consistent with the notion of PINK1 interacting directly with Parkin. However, these findings remain controversial as BLUE-native experiments and side-exclusion chromatography failed to detect PINK1 and Parkin as a complex, thereby countering the notion that PINK1 and Parkin interact at the mitochondria (Lazarou *et al.*, 2012). Although PINK1 drives Parkin self-association and recruitment onto mitochondria (Lazarou *et al.*, 2013), further experiments are required to fully elucidate the molecular basis through which PINK1 signals for Parkin activation.

PARKIN-MEDIATED UBIQUITINATION OF MITOCHONDRIAL PROTEINS

Once present on mitochondria, a robust increase in the activity of Parkin is observed with Parkin ubiquitinating a wide range of substrates, including itself (Lazarou *et al.*, 2013; Matsuda *et al.*, 2010). Such activity is necessary for mitophagy to occur, with Parkin first ubiquitinating a range of outer mitochondrial proteins that in turn recruits specific adaptor proteins that direct the mitochondria into newly forming autophagosomes (Figure 18.3).

Two proteins that are first targeted by Parkin following its recruitment are mitofusin 1 and 2, which serve as GTPases that are centrally involved in mitochondrial fusion. Both are rapidly degraded following the recruitment of Parkin onto the mitochondria, with Parkin forming K48-linked Ub conjugates that direct both substrates for degradation via the proteasome (Chan *et al.*, 2011). Following their degradation, mitochondrial fusion is now greatly

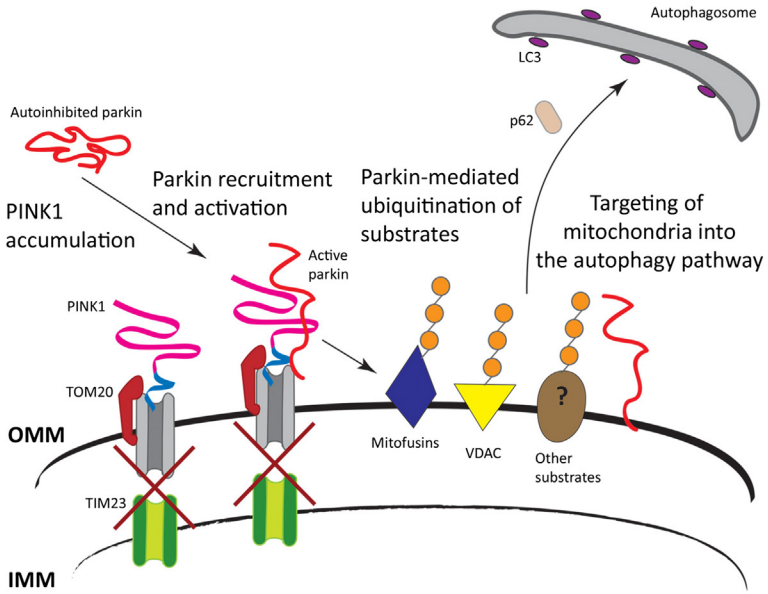

FIGURE 18.3 **Model for PINK1-Parkin mediated mitophagy.** Following the loss of membrane potential, PINK1 is no longer actively imported via the TOM:TIM complex, leading to its accumulation on the outer surface of the mitochondria. PINK1 signals directly or indirectly for Parkin to be relocated from the cytoplasm onto the outer surface of the mitochondria. Once present on the mitochondria, activated Parkin now ubiquitinates VDAC1, the mitofusins and a range of other proteins. The presence of polyubiquitin chains signals them for degradation. Signal adaptor proteins such as p62/SQSTRM1 have high affinity for polyubiquitin chains and facilitate binding to LC3 found on autophagosomes. Mitochondria are subsequently directed to the autophagosome for autophagic degradation.

diminished, leading to a greater number of smaller mitochondrial fragments. Although proteasomal degradation of the mitofusins has been one of the first steps described for Parkin, its clearance is not sufficient for the initiation of mitophagy. Inhibition of proteasome degradation in mitofusin null cells prevents mitophagy, indicating that additional targets do exist. Indeed, from ubiquitylome studies, hundreds of substrates ubiquitinated by Parkin were identified following treatment with mitochondria uncouplers (Sarraf *et al.*, 2013), underlining the wide diversity of substrates that Parkin must act upon to successfully initiate and complete mitophagy.

In addition to Parkin ubiquitinating a wide diversity of substrates, Parkin has also been demonstrated to form distinct Ub conjugates on its substrate. For the mitofusins, Parkin primarily forms K48-linked conjugates that direct them towards the proteasome for degradation (Chan *et al.*, 2011). However, Parkin can also mediate the formation of Ub conjugates linked in a noncanonical manner. In the case of VDAC1, Parkin forms both K27- and K63-linked Ub conjugates on this component of the permeability transition pore, and this appears to initiate a signaling cascade that initiates mitophagy (Geisler *et al.*, 2010). However, these findings are controversial as a second study found that Parkin-mediated ubiquitination of VDAC1 was not necessary for the successful completion of mitophagy

(Narendra *et al.*, 2010a). Yet, this does not eliminate the possibility that an accumulation of ubiquitinated substrates on the mitochondria is acting as a signal to direct mitochondria into the mitophagy pathway. Considering the diversity of proteins ubiquitinated by Parkin, further work is needed to elucidate the molecular basis through which Parkin-mediated ubiquitination promotes mitophagy.

PARKIN/PINK1-MEDIATED MITOPHAGY

Following ubiquitination of a diverse array of mitochondrial substrates, PINK1 and Parkin must now redirect the mitochondria designated for clearance into the autophagy pathway. Yet, how do they direct mitochondria into this pathway and are they interacting with specific autophagic proteins? The adaptor protein p62/SQSTRM1 is believed to be one such protein that can link the two pathways. P62/SQSTRM1 has been demonstrated to have a high affinity for polyubiquitin chains and LC3, an autophagic protein found on the outer membrane of newly formed autophagosomes. P62/SQSTRM1 binds to ubiquitinated substrates on the mitochondria and directs them into newly forming autophagic vesicles by binding LC3 (Geisler *et al.*, 2010; Narendra *et al.*, 2010a). Whether or not p62/SQSTRM1 is essential for mitophagy remains to be determined as mitophagy has been observed in its absence.

Moreover, it also raises the question as to how Parkin might stimulate the formation of isolation membranes for the newly forming autophagosomes. From recent studies, Parkin has been demonstrated to mediate the recruitment of Ambra1 onto mitochondria (Van Humbeeck *et al.*, 2011). Ambra1 present on the mitochondrial interacts with the Beclin-1 PI3K complex, resulting in the formation of pre-autophagosomal membrane around the damaged mitochondria (Michiorri *et al.*, 2010). Taken together, Parkin plays a central role in clearing damaged mitochondria, ubiquitinating mitochondrial proteins and recruiting Ambra1, events that ultimately lead to the autophagic clearance of the damaged mitochondria.

MITOPHAGY AND NEURONS

While studies on the Parkin-PINK1 quality control mechanism have relied heavily on the use of chemical uncouplers in a variety of mammalian cells, evidence for this pathway in neurons has remained elusive. When Parkin was investigated in neurons, surprisingly recruitment failed to occur following mitochondrial depolarization. Moreover, Parkin failed to mediate the clearance of damaged mitochondria, raising concerns that findings in cells may not translate into neurons.

One striking difference between cells in culture and neurons is that neurons are unable to switch to glycolysis as an exclusive means of ATP production, and are therefore dependent on mitochondrial ETC for generating energy. Whereas cultured cells can survive following the removal of its entire mitochondrial network, neurons are unable to survive large-scale clearance of its mitochondrial population. As a result, treatment of neurons with uncouplers that disrupt the mitochondrial ETC culminates in a bioenergetic

crisis within the neuron that may be responsible for preventing Parkin and PINK1 from mediating mitophagy.

Considering the discrepancy between findings from neurons and cells, is Parkin even recruited onto depolarized mitochondria? If Parkin is not recruited, does it mediate mitophagy in a manner analogous to findings observed in immortalized cells? One pitfall in studying Parkin in neurons is the high degree of variability between studies. For example, two studies have demonstrated Parkin recruitment in neurons, albeit using different culture conditions. In the first study, Parkin recruitment was observed when inhibitors of apoptosis (the caspase inhibitor Z-VAD-FMK) were used in combination with chemical uncouplers (Cai *et al.*, 2012). Thus, by preventing neuronal cell death, Parkin recruitment could now occur. However, one caveat to this study is that the presence of apoptotic inhibitors within the media may be masking the normal physiological response of the neurons to mitochondrial stress. Remarkably, it was the second study that showed the highest percentage of Parkin recruitment onto mitochondria, caused when antioxidants were omitted from the neuronal media (Joselin *et al.*, 2012). In particular, the B-27 antioxidant was absent from the neuron cell culture media. It must be noted, however, that Parkin translocation was much slower (12–24 h) when B-27 was absent, relative to studies when it is present (4 h). Taken together, these studies suggest that Parkin localizes to mitochondria when uncouplers are present, albeit dependent on the culture conditions under which neurons are maintained.

One shortcoming from these studies is an absence of quantification for Parkin-mediated mitophagy following the recruitment of Parkin onto mitochondria. Although Parkin is recruited onto mitochondria in neurons, does its presence now signal for the clearance of mitochondria via the mitophagy pathway? Considering the limitations of cultured neurons, one particular study has attempted to address this question using the MitoPark mouse (Sterky *et al.*, 2011), in which the mitochondrial transcription factor A has been ablated in dopaminergic neurons. The MitoPark mice develop symptoms of progressive Parkinsonism and mitochondrial abnormalities, making it an ideal mouse model for PD. In this study, the MitoPark mice were crossed with wild-type or Parkin knockout mice. Remarkably, neither mouse line demonstrated an increased accumulation of damaged mitochondria, implying that Parkin is playing no role in directing the clearance of dysfunctional mitochondria. However, it must also be noted that the defects observed in MitoPark mice have yet to be fully characterized. Moreover it is possible that mitochondria in these mice, although damaged, are not sufficiently depolarized to stabilize PINK1 on its surface to promote Parkin recruitment. In the context of studies from cells, neurons and mouse models, it is clear that, while a great deal is known about the PINK1-Parkin pathway, further work is essential to fully elucidate the mechanisms at play in this pathway and whether Parkin and PINK1 are indeed required within the neurons for mitochondrial quality control.

DISCUSSION

Since their discovery as PD-associated genes, Parkin and PINK1 have been implicated as key players in mitochondrial quality control. The fact that two PD-linked genes cooperate with each other to remove damaged mitochondria further underlines the involvement of mitochondrial dysfunction in the pathogenesis of PD. Although much has been learned

about the Parkin-PINK1 pathway in the past decade, many critical questions still remain unanswered. Firstly, and most importantly, do the findings in immortalized cells translate into neurons and *in vivo* mouse models? While several studies in neurons demonstrate that Parkin recruitment to the mitochondria can occur, it remains to be established whether or not mitophagy is occurring. Thus, a lot more work is required to demonstrate whether Parkin and PINK1 are needed to maintain a healthy mitochondrial population within the neuron.

In addition to establishing a role for PINK1 and Parkin in neurons, further work is required to address several key questions. These include: (1) understanding how PINK1 promotes the relocalization of Parkin onto depolarized mitochondria; (2) establishing why Parkin ubiquitinates a specific subset of mitochondrial proteins; and (3) elucidating the molecular mechanism through which Parkin directs damaged mitochondria into the autophagy pathway. To date, findings with Parkin and PINK1 have been robust and reproducible, and have the potential to open up new avenues for the development of new treatments in PD. However, before therapies can be explored, it is imperative that the molecular basis for this pathway is further established. Moreover, it is crucial that we understand the physiological role for both Parkin and PINK1 in neurons and within the context of the brain as whole.

References

Cai, Q., Zakaria, H.M., Simone, A., et al., 2012. Spatial parkin translocation and degradation of damaged mitochondria via mitophagy in live cortical neurons. Curr. Biol. 22, 545–552.

Chan, N.C., Salazar, A.M., Pham, A.H., et al., 2011. Broad activation of the ubiquitin-proteasome system by Parkin is critical for mitophagy. Hum. Mol. Genet. 20, 1726–1737.

Durcan, T.M., Kontogiannea, M., Thorarinsdottir, T., et al., 2011. The Machado-Joseph disease-associated mutant form of ataxin-3 regulates parkin ubiquitination and stability. Hum. Mol. Genet. 20, 141–154.

Geisler, S., Holmstrom, K.M., Skujat, D., et al., 2010. PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. Nat. Cell Biol. 12, 119–131.

Greene, A.W., Grenier, K., Aguileta, M.A., et al., 2012. Mitochondrial processing peptidase regulates PINK1 processing, import and Parkin recruitment. EMBO Rep. 13, 378–385.

Greene, J.C., Whitworth, A.J., Andrews, L.A., et al., 2005. Genetic and genomic studies of *Drosophila* parkin mutants implicate oxidative stress and innate immune responses in pathogenesis. Hum. Mol. Genet. 14, 799–811.

Guzman, J.N., Sanchez-Padilla, J., Wokosin, D., et al., 2010. Oxidant stress evoked by pacemaking in dopaminergic neurons is attenuated by DJ-1. Nature 468, 696–700.

Jin, S.M., Lazarou, M., Wang, C., et al., 2010. Mitochondrial membrane potential regulates PINK1 import and proteolytic destabilization by PARL. J. Cell Biol. 191, 933–942.

Joselin, A.P., Hewitt, S.J., Callaghan, S.M., et al., 2012. ROS-dependent regulation of Parkin and DJ-1 localization during oxidative stress in neurons. Hum. Mol. Genet. 21, 4888–4903.

Keeney, P.M., Xie, J., Capaldi, R.A., et al., 2006. Parkinson's disease brain mitochondrial complex I has oxidatively damaged subunits and is functionally impaired and misassembled. J. Neurosci. 26, 5256–5264.

Kim, Y., Park, J., Kim, S., et al., 2008. PINK1 controls mitochondrial localization of Parkin through direct phosphorylation. Biochem. Biophys. Res. Commun. 377, 975–980.

Kitada, T., Asakawa, S., Hattori, N., et al., 1998. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392, 605–608.

Kondapalli, C., Kazlauskaite, A., Zhang, N., et al., 2012. PINK1 is activated by mitochondrial membrane potential depolarization and stimulates Parkin E3 ligase activity by phosphorylating Serine 65. Open Biol. 2, 120080.

Lazarou, M., Jin, S.M., Kane, L.A., et al., 2012. Role of PINK1 binding to the TOM complex and alternate intracellular membranes in recruitment and activation of the E3 ligase Parkin. Dev. Cell 22, 320–333.

Lazarou, M., Narendra, D.P., Jin, S.M., et al., 2013. PINK1 drives Parkin self-association and HECT-like E3 activity upstream of mitochondrial binding. J. Cell Biol. 200, 163–172.
Mata, I.F., Lockhart, P.J., Farrer, M.J., 2004. Parkin genetics: one model for Parkinson's disease. Hum. Mol. Genet. 13 Spec No 1, R127–R133.
Matsuda, N., Sato, S., Shiba, K., et al., 2010. PINK1 stabilized by mitochondrial depolarization recruits Parkin to damaged mitochondria and activates latent Parkin for mitophagy. J. Cell Biol. 189, 211–221.
Michiorri, S., Gelmetti, V., Giarda, E., et al., 2010. The Parkinson-associated protein PINK1 interacts with Beclin1 and promotes autophagy. Cell Death Differ. 17, 962–974.
Narendra, D., Kane, L.A., Hauser, D.N., et al., 2010a. p62/SQSTM1 is required for Parkin-induced mitochondrial clustering but not mitophagy; VDAC1 is dispensable for both. Autophagy 6, 1090–1106.
Narendra, D., Tanaka, A., Suen, D.F., et al., 2008. Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. J. Cell Biol. 183, 795–803.
Narendra, D.P., Jin, S.M., Tanaka, A., et al., 2010b. PINK1 is selectively stabilized on impaired mitochondria to activate Parkin. PLoS Biol. 8, e1000298.
Newton, K., Matsumoto, M.L., Wertz, I.E., et al., 2008. Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell 134, 668–678.
Okatsu, K., Oka, T., Iguchi, M., et al., 2012. PINK1 autophosphorylation upon membrane potential dissipation is essential for Parkin recruitment to damaged mitochondria. Nat. Commun. 3, 1016.
Pridgeon, J.W., Olzmann, J.A., Chin, L.S., et al., 2007. PINK1 protects against oxidative stress by phosphorylating mitochondrial chaperone TRAP1. PLoS Biol. 5, e172.
Riley, B.E., Lougheed, J.C., Callaway, K., et al., 2013. Structure and function of Parkin E3 ubiquitin ligase reveals aspects of RING and HECT ligases. Nat. Commun. 4, 1982.
Sarraf, S.A., Raman, M., Guarani-Pereira, V., et al., 2013. Landscape of the PARKIN-dependent ubiquitylome in response to mitochondrial depolarization. Nature 496, 372–376.
Schapira, A.H., Cooper, J.M., Dexter, D., et al., 1989. Mitochondrial complex I deficiency in Parkinson's disease. Lancet 1, 1269.
Sha, D., Chin, L.S., Li, L., 2010. Phosphorylation of parkin by Parkinson disease-linked kinase PINK1 activates parkin E3 ligase function and NF-kappaB signaling. Hum. Mol. Genet. 19, 352–363.
Sterky, F.H., Lee, S., Wibom, R., et al., 2011. Impaired mitochondrial transport and Parkin-independent degeneration of respiratory chain-deficient dopamine neurons in vivo. Proc. Natl Acad. Sci. USA 108, 12937–12942.
Trempe, J.F., Sauve, V., Grenier, K., et al., 2013. Structure of parkin reveals mechanisms for ubiquitin ligase activation. Science 340, 1451–1455.
Van Humbeeck, C., Cornelissen, T., Hofkens, H., et al., 2011. Parkin interacts with Ambra1 to induce mitophagy. J. Neurosci. 31, 10249–10261.
Vives-Bauza, C., Zhou, C., Huang, Y., et al., 2010. PINK1-dependent recruitment of Parkin to mitochondria in mitophagy. Proc. Natl Acad. Sci. USA 107, 378–383.
Wang, X., Winter, D., Ashrafi, G., et al., 2011. PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. Cell 147, 893–906.
Wauer, T., Komander, D., 2013. Structure of the human Parkin ligase domain in an autoinhibited state. EMBO J. 32, 2099–2112.
Wenzel, D.M., Lissounov, A., Brzovic, P.S., et al., 2011. UBCH7 reactivity profile reveals parkin and HHARI to be RING/HECT hybrids. Nature 474, 105–108.
Zhou, C., Huang, Y., Shao, Y., et al., 2008. The kinase domain of mitochondrial PINK1 faces the cytoplasm. Proc. Natl Acad. Sci. USA 105, 12022–12027.

CHAPTER

19

Autophagy Degrades Endocytosed Gap Junctions

Matthias M. Falk

OUTLINE

Abstract

Four principal categories of cell-cell junctions connect cells in vertebrates and form the basis for shaping distinct tissues and organs. Gap junctions (GJs), one of the four junction types, provide direct cell-to-cell communication by mediating passive diffusion of small hydrophilic signaling molecules between neighboring cells. Gap junction mediated intercellular communication (GJIC) has been shown to play a crucial role for all aspects of multicellular life, including embryonic development, tissue function, and cellular homeostasis; and mutations in the GJ forming proteins, connexins (Cxs), have been linked to severe human diseases that include inherited and sporadic nonsyndromic hearing loss, neuropathies, eye lens cataracts, cardiac diseases, craniofacial malformations, and a number of acute skin disorders. Clearly, biosynthesis and degradation significantly contribute to GJ function and need to be controlled precisely. We have previously shown that GJs are removed from the plasma membrane via the internalization of entire GJ plaques (or portions thereof) in a cellular process that resembles clathrin-mediated endocytosis. GJ endocytosis results in

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the formation of double-membrane vesicles (termed annular gap junctions [AGJs] or connexosomes) in the cytoplasm of one of the coupled cells. A set of recent independent studies consistent with earlier ultrastructural analyses demonstrate the degradation of endocytosed AGJs by autophagy. Some other reports, however, describe AGJ degradation by endo-/lysosomal pathways in cells that were treated with TPA. Here, I summarize evidence that supports the concept that autophagy serves as the principal cellular degradation pathway for internalized GJs under physiological and pathological conditions.

INTRODUCTION

Gap Junction Structure and Function

Cells in vertebrates including humans are linked together by four principal types of cell-cell junctions to form tissues and organs. Each type of cell-cell junction is considered to fulfill a special function (Figure 19.1A). Tight junctions (TJs) form a net-like belt of branched ridges of transmembrane proteins (claudins, occludins, tricellulin) around cells that tightly link cells together to separate apical from baso-lateral membrane domains, or (in case of epithelia and vascular endothelia) to separate outside from inside, or the lumen of blood vessels from the surrounding body, respectively. Desmosomes and adherens junctions (AJs) form patchy cell-cell contacts that connect cytoskeletal elements (intermediate and actin filaments, respectively) of neighboring cells to provide tissue strength, aid in tissue morphogenesis during development, and to maintain proper tissue organization. Gap junctions (GJs) consist of clusters of double-membrane spanning hydrophilic channels that provide direct cell-to-cell communication by allowing the passage of signaling molecules, ions, and electrical currents. Epithelia and endothelia, sheets of polarized single-cell layers that coat the outside and inside surface of organs such as the intestine, liver, kidneys, or the vasculature, are particularly rich in cell-cell junctions and exhibit a well-organized hierarchical architecture of these structures (Figure 19.1A).

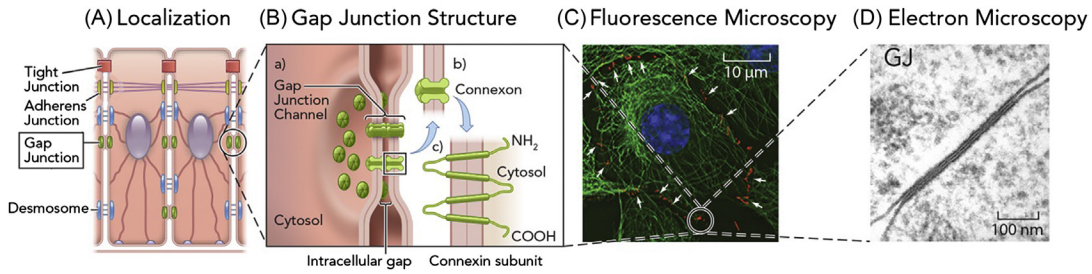

FIGURE 19.1 Cellular location and structure of gap junctions (GJs). (A) GJs are assemblies of double-membrane spanning hydrophilic channels termed "plaques" that bridge the apposing plasma membranes of neighboring cells to provide direct cell-to-cell (or intercellular) communication as shown here for epithelial cells. (B) GJ channels form by the head-on docking of two hemi-channels or "connexons" each assembled and trafficked to the plasma membrane by one of the two contacting cells. Connexons are assembled from six four-pass trans-membrane proteins termed "connexins" (Cxs). (C) GJs can be detected by immunofluorescence light microscopy when stained with fluorescence-tagged antibodies, such as the ones shown here in T51B liver cells assembled from endogenously expressed Cx43 protein. (D) GJs also appear as structures with unique morphology in ultrathin sections when examined by electron microscopy (EM).

Direct cell-to-cell communication is a pivotal cellular function of multicellular organisms. It is established by GJ channels, which bridge apposing plasma membranes of neighboring cells. Typically, tens to thousands of GJ channels cluster into densely packed two-dimensional arrays, termed GJ plaques, that can reach several micrometers in diameter (Figure 19.1B). GJ channels are assembled from a ubiquitously expressed class of four-pass trans-membrane proteins, termed connexins (Cxs), with connexin 43 (Cx43) being the most abundantly expressed Cx type. Six Cx polypeptides oligomerize into a ring to form a hexameric trans-membrane structure with a central hydrophilic pore, called a hemi-channel or connexon. Once trafficked to the plasma membrane, two connexons, one provided by each of two neighboring cells, dock head-on in the extracellular space to form the complete double-membrane spanning GJ channel that is completely sealed off to the extracellular space (Thévenin *et al.*, 2013) ((Figures 19.1, 19.2). Recruitment of additional GJ channels along the outer edge enlarges the GJ plaques, while simultaneous removal of older channels from plaque centers balances GJ channel turnover (Falk *et al.*, 2009; Gaietta *et al.*, 2002; Lauf *et al.*, 2002).

RESULTS

Gap Junction Endocytosis Generates Cytoplasmic Double-Membrane Vesicles

Goodenough and Gilula (1974), and Ghoshroy *et al.* (1995) found that connexons, once docked, appear inseparable under physiological conditions (Ghoshroy *et al.*, 1995; Goodenough and Gilula, 1974), suggesting that cells may endocytose and degrade GJ plaques in whole. Indeed, we found that cells endocytose their GJs as complete double-membrane structures via a combined endo-/exocytic process (endocytic for the receiving cell, exocytic for the donating cell) (Baker *et al.*, 2008; Falk *et al.*, 2009; Gilleron *et al.*, 2008; Gumpert *et al.*, 2008; Piehl *et al.*, 2007) (Figure 19.3, steps 1–5). Internalization was found to occur preferentially into one of two coupled cells, indicating a highly regulated process (Falk *et al.*, 2009; Piehl *et al.*, 2007). Further analyses indicated that GJ internalization utilizes well-known components of the clathrin-mediated endocytosis (CME) machinery, including the classical endocytic coat protein clathrin, the clathrin-adaptors AP-2 and Dab2, the GTPase dynamin2, the retrograde actin motor myosin VI (myo6), as well as the process of actin polymerization (Gumpert *et al.*, 2008; Piehl *et al.*, 2007) (Figure 19.3, steps 1–4). A recent analysis from our lab revealed that two AP-2 binding sites are present in the C-terminus of Cx43 that cooperate to mediate GJ endocytosis (Fong *et al.*, 2013), suggesting a mechanistic model for clathrin's ability to internalize these large plasma membrane structures.

GJ internalization generates characteristic cytoplasmic double-membrane GJ vesicles, termed annular GJs (AGJs) or connexosomes (Figures 19.2, 19.3). Note that the outer membrane of the generated AGJ vesicles corresponds to the plasma membrane of the host cell, while the inner membrane and the vesicle lumen correspond to plasma membrane and cytoplasm of the neighboring donor cell (Figures 19.2, 19.3, steps 1–5). Extensive further analyses revealed that cells turn over their GJs constitutively (Falk *et al.*, 2009; Piehl *et al.*, 2007), and efficiently after treatment with inflammatory mediators such as thrombin and endothelin (Baker *et al.*, 2008); mitogens such as EGF and VEGF (Fong and Falk, and Nimlamool and Falk, unpublished); in response to treatment with the nongenomic

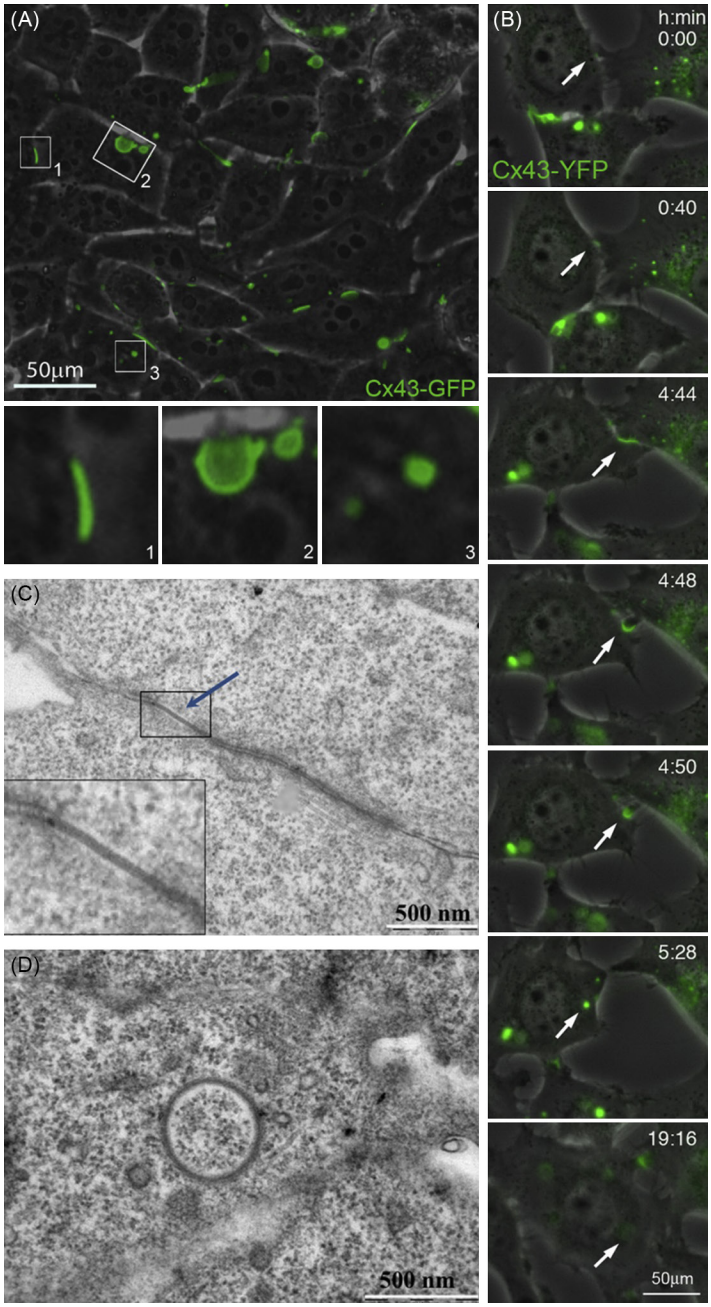

FIGURE 19.2 Gap junctions and endocytosed gap junctions. (A) HeLa cells transfected with Cx43-GFP efficiently express and assemble GJs in the adjacent plasma membranes of transfected cells (visible as green fluorescent lines and puncta such as the one shown in insert 1). Over time, GJs bulge inward (insert 2), detach from the plasma membrane and form endocytosed cytoplasmic annular gap junction (AGJ) vesicles or connexosomes (insert 3). (B) Selected still images of a time-lapse recording of stably transfected Cx43-YFP expressing HeLa cells showing the formation of a GJ, its endocytic internalization into the cytoplasm of one of the previously coupled cells, and final degradation of the generated AGJ vesicle, indicated by the loss of its fluorescence (marked with arrows). Combined phase contrast and fluorescence images are shown in (A) and (B). Transmission electron micrographs of a gap junction (C) and an annular gap junction (D) in mouse embryonic stem cells.

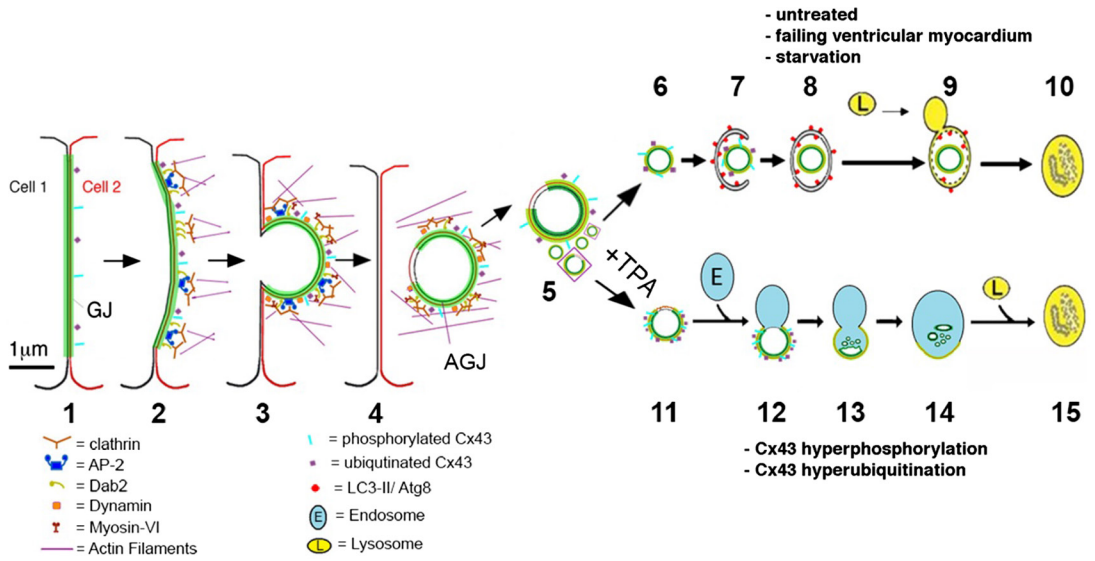

FIGURE 19.3 Mechanisms of gap junction endocytosis and degradation. Schematic representation of proposed steps that lead to GJ internalization (steps 1–3), cytoplasmic AGJ vesicle formation and fragmentation (steps 4, 5), and AGJ vesicle degradation by phago-/lysosomal (steps 6–10) and endo-/lysosomal pathways (steps 11–15) based on the previous work by others and us. Note the proposed nonjunctional membrane domains missing the green GJ label (shown in steps 4, 5, 11, 12), and the increased phosphorylation and ubiquitination on AGJ vesicles that fuse with endosomes (steps 11, 12 versus 6, 7).

carcinogen lindane (Gilleron *et al.*, 2008); and under pathological conditions such as in the failing canine ventricular myocardium (Hesketh *et al.*, 2010). Constitutive and acute endocytosis of GJ plaques correlates with the described short half-life of connexins of only 1–5 hours (Beardslee *et al.*, 1998; Berthoud *et al.*, 2004; Falk *et al.*, 2009; Fallon and Goodenough, 1981; Gaietta *et al.*, 2002).

Endocytosed Gap Junctions are Degraded by Autophagy

Four recent studies by Hesketh *et al.* (2010), Lichtenstein *et al.* (2011), Fong *et al.* (2012), and Bejarano *et al.* (2012) report the degradation of endocytosed AGJ vesicles via autophagy (Figure 19.3, steps 6–10). Hesketh *et al.* (2010) report loss of GJs from the plasma membrane, and GJ endocytosis and AGJ degradation by autophagy in pacing-induced failing canine ventricular myocardium. Lichtenstein *et al.* (2011) report that autophagy contributes to the degradation of endogenously (NRK cells, mouse embryonic fibroblasts) and exogenously (HeLa cells) expressed Cx43 protein, and of wild-type and cataract-associated mutant Cx50 proteins in both un-induced cells and in cells in which autophagy was induced by starvation (Lichtenstein *et al.*, 2011). Fong *et al.* (2012) report the autophagic degradation of AGJ vesicles in normal, untreated HeLa cells that express exogenous fluorescently tagged Cx43; and in primary porcine pulmonary artery endothelial cells (PAECs) endogenously

expressing Cx43. Bejarano *et al.* (2012) report the Nedd4-mediated ubiquitin-dependent autophagic degradation of internalized GJs *in situ* (mouse liver) as well as in starved and fed cultured cells expressing Cx43 endogenously and exogenously (mouse embryonic fibroblasts, NIH3T3, COS7, and NRK cells).

In all four studies cytoplasmic AGJ vesicles were detected inside phagophores by ultrastructural analyses. Autophagosomes exhibit a highly characteristic, clearly recognizable double-membrane structure on ultra-thin sections (Figure 19.2D), making conventional electron microscopy a very reliable technique for the characterization of autophagosomes (Mizushima, 2004). Also, in all studies AGJs were observed to co-localize with the autophagy marker protein, LC3-II/Atg8, known to be one of the most useful generic marker proteins for the characterization of autophagosomes (Kabeya *et al.*, 2000). Microtubule-associated protein light chain 3 (LC3, the mammalian homologue of the yeast autophagic protein Atg8) is an abundant soluble cytoplasmic protein. It is proteolytically processed by the removal of a few N-terminal amino acid residues shortly after translation that generates LC3-I. LC3-I is recruited to developing phagophores, is covalently conjugated to phosphatidyl-ethanolamine (PE) of the phagophore membrane (termed LC3-II), and remains on autophagosomes for most of their lifetime (Kabeya *et al.*, 2000; Mizushima, 2004).

While the Lichtenstein *et al.* and Bejarano *et al.* studies were aimed more broadly at a potential role of autophagy contributing to Cx and GJ degradation in general, the Fong *et al.* and the Hesketh *et al.* studies were aimed specifically at investigating the fate of internalized AGJ vesicles that others and we had characterized previously (Baker *et al.*, 2008; Gumpert *et al.*, 2008; Jordan *et al.*, 2001; Piehl *et al.*, 2007). To further support their findings, Lichtenstein *et al.* and Bejarano *et al.* knocked down the autophagy-related proteins Atg5 and Atg7 in cells expressing either endogenous or exogenous Cx43, and used the drugs chloroquine and 3MA to inhibit autophagy. Fong *et al.* knocked down expression of the autophagy related proteins Beclin-1 (Atg6), LC3 (Atg8), LAMP-2 and p62/sequestosome 1 (SQSTM1), and used the drugs 3MA, Wortmannin, and Bafilomycin A1 in Cx43-GFP expressing HeLa cells.

As mentioned previously in the Lichtenstein *et al.*, Fong *et al.*, and Bejarano *et al.* studies the ubiquitin-binding protein p62/SQSTM1 was identified as a protein that targets internalized GJs to autophagic degradation. Knocking down p62/SQSTM1 protein levels as performed by Fong *et al.* resulted in a significantly increased accumulation of cytoplasmic AGJs (av. 55%, n = 4) and a significantly reduced co-localization (av. 69.5%, n = 3) of AGJs with autophagosomes. In summary, all four complementary studies (Bejarano *et al.*, 2012; Fong *et al.*, 2012; Hesketh *et al.*, 2010; Lichtenstein *et al.*, 2011) compellingly show that under physiological and pathological conditions GJ plaques are endocytosed from the plasma membrane, and that the generated AGJ vesicles are degraded by autophagy.

Structural Elements Warrant the Autophagic Degradation of Endocytosed Gap Junctions

Since cytoplasmic vesicles normally can fuse with endosomes, at first glance, autophagic degradation of AGJ vesicles might not appear intuitive. However, considering the GJ internalization process that generates double-membrane vesicles in which both membranes are tightly linked to each other (not single membrane vesicles that typically are formed by the

endocytosis of cargo molecules on the plasma membrane), the structural organization of AGJ vesicles (multiprotein complexes with paracrystalline surface packing), and their cytoplasmic location, autophagic degradation emerges as the most apparent cellular degradation pathway. Finally, the unique structural composition of AGJ vesicles with lumen and inner membrane derived from the neighboring cell (being foreign to the AGJ-receiving host cell) may further direct AGJs to autophagic degradation. Taken together, the structural and functional characteristics of AGJ vesicles, along with the fact that autophagy serves as the generic degradation pathway for cytoplasmically localized structures (organelles and protein aggregates), renders autophagic degradation the most obvious cellular AGJ degradation pathway.

Potential Other Degradation Pathways for Endocytosed Gap Junctions

Interestingly, a recent paper by Leithe *et al.* (2009) reports that in TPA-treated cells (a structural analogue of the secondary messenger molecule diacylglycerol [DAG]), internalized GJs may be degraded by the endo-/lysosomal and not the autophagosomal pathway (Figure 19.3, steps 11–15). Recently, the Leithe lab identified the protein Smurf2 (the HECT E3 ubiquitin ligase smad ubiqitination regulatory factor-2) as a critical factor that regulates GJ internalization and endo-/lysosomal targeting in TPA-treated cells (Fykerud *et al.*, 2012). DAG is a known potent activator of protein kinase C (PKC), and PKC is known to phosphorylate and promote ubiquitination of Cx43 (Leithe *et al.*, 2009; Leithe and Rivedal, 2004b; Postma *et al.*, 1998). Based on these and our own results, it is tempting to speculate that cells might be able to regulate by which pathway (endo-/lysosomal versus phago-/lysosomal) specific cargo is sequestered and processed (e.g., endo-/lysosomal and phago-/lysosomal pathways might process internalized GJs in different ways). Furthermore, the level of cargo-phosphorylation and/or ubiquitination might determine which of these pathways is ultimately chosen (basic phosphorylation/ubiquitination signaling autophagic AGJ vesicle degradation; elevated phosphorylation/ubiquitination signaling endo-/lysosomal AGJ vesicle degradation) (see Figure 19.3, steps 6–10 versus 11–15).

Endo-/lysosomal degradation of AGJs as observed in TPA-treated cells by Leithe *et al.* (2009) of course raises an important question: How is it structurally possible for a double-membrane vesicle that consists of tightly bonded membrane layers and densely packed GJ channels to fuse with a single-membrane endosome? The Rivedal and Leithe laboratories suggest that subsequent to GJ internalization and AGJ formation, the inner AGJ membrane splits and peels away from the outer AGJ membrane, generating a single-membraned cytoplasmic AGJ vesicle that then can fuse with a single-membraned endosome (Kjenseth *et al.*, 2010, 2012; Leithe *et al.*, 2009, 2012). However, since docked GJ channels cannot split into undocked connexons under physiological conditions (Ghoshroy *et al.*, 1995; Goodenough and Gilula, 1974) – which appears to be the apparent reason for double-membrane GJ endocytosis – it is not clear how membrane separation could be initiated in the AGJ vesicles shortly after their generation. Clearly low pH, a characteristic of late endosomes and lysosomes, and a potential initiator of GJ splitting, can be excluded because AGJ vesicle membrane-separation needs to occur before AGJ/endosome fusion.

Interestingly, by electron microscopic (EM) examination, we found that AGJ vesicles examined by electron microscopy (EM) appear to include a small region where the two membranes are void of GJ channels and are not docked or linked to each other (Falk *et al.*, 2012;

Piehl *et al.*, 2007) (shown schematically in Figure 19.3, steps 4, 5, 11 and 12). Similar small AGJ membrane separations were also observed in classical ultrastructural analyses of GJs and AGJ vesicles (see, e.g., Mazet *et al.*, 1985). Possibly, these nonjunctional membrane domains consist of plasma membrane that is derived from both neighboring cells, and we postulated that these areas might originate from plasma membrane regions that were located immediately adjacent to the GJ plaques and were internalized as well. To gain further support for this hypothesis, we incubated inducible stably Cx43-YFP expressing HeLa cells for 2–4 hours with a fluorescently tagged lectin, Alexa594-wheat germ agglutinin (WGA), and examined AGJ vesicles by high-resolution fluorescence microscopy. WGA binds specifically to sialic acid and N-acetylglucosaminyl carbohydrate moieties commonly found on extracellular-exposed carbohydrate side-chains of plasma membrane proteins. Due to its relatively large size (~38 kDa), WGA is not able to traverse the plasma membrane in living cells. However, WGA will bind to and label the extracellular surface of plasma membranes, and subsequently will be endocytosed and then will also label intracellular membrane compartments. Interestingly we found that a significant portion of AGJ vesicles (~50%, n = 80; the ones that likely were generated during the WGA-incubation period), exhibited red-fluorescent WGA-puncta (Falk *et al.*, 2012). These results support our hypothesis that the undocked membrane domains we detected by EM indeed represent plasma membrane areas that were located in the immediate vicinity of GJ plaques and were concomitantly internalized in the AGJ endocytosis process. It is very likely that these nonjunctional membrane domains provide the single membrane areas that allow double-membrane AGJ vesicles to fuse with single-membrane endosomes.

Signals that Prime Gap Junctions for Endocytosis and Direct them to Autophagic Degradation

Post-translational modification of proteins is a widespread mechanism to fine-tune the structure, function, and localization of proteins. One of the most versatile and intriguing protein modifications is the covalent attachment of ubiquitin (Ub) or Ub-like modifications to target proteins. Ub is a small, 76-amino acid protein, and either single or multiple Ub moieties can be conjugated to lysine amino acid residues of target proteins. An incredible diversity of mono- and poly-Ub chains (in which Ub moieties can be linked to each other via the Ub residues Met1-, Lys6-, Lys11-, Lys27-, Lys29-, Lys33-, Lys48-, and Lys63-) conjugated to target proteins have been characterized that can range in function from protein activation to protein degradation (Fushman and Wilkinson, 2011). Multiple mono-Ubs, and Lys48- and Lys63-linked poly-Ubs, have been recognized as important signals for protein degradation. For example, conjugation of Ub moieties to proteins has been recognized as a signal for both proteasomal targeting (addition of Lys48-linked poly-Ub chains) and more recently as a sorting signal for internalized vesicles of the late endocytic pathway. This is achieved through the addition of multiple mono-Ub moieties or of Lys63-linked poly-Ub chains, which ultimately lead to degradation by lysosomes (Hicke, 2001; Hicke and Dunn, 2003; Schnell and Hebert, 2003). In addition, Lys-63-linked polyubiquitination can act as an internalization signal for clathrin-mediated endocytosis (CME) (Belouzard and Rouille, 2006; Geetha *et al.*, 2005). Lys63-polyubiquitinated target proteins are recognized by specific CME machinery protein components that associate with a subset of Ub-binding proteins, specifically Epsin1 and Eps15 (Barriere *et al.*, 2006; Hawryluk *et al.*, 2006; Madshus, 2006). Further

work has shown that the Ub-binding protein p62/SQSTM1 recognizes and interacts via its UBA-domain with polyubiquitinated proteins (Ciani *et al.*, 2003; Seibenhener *et al.*, 2004) and delivers polyubiquitinated (Lys63-linked) oligomeric protein complexes to the autophagic degradation pathway (Bjorkoy *et al.*, 2005; Pankiv *et al.*, 2007). Ubiquitination of Cx43-based GJs has been described previously (Catarino *et al.*, 2011; Girao *et al.*, 2009; Leithe *et al.*, 2009; Leithe and Rivedal, 2004b). The findings that Cx43-based GJs can become ubiquitinated (e.g., Lys63-polyubiquitinated; Kells and Falk, unpublished), the known affinity of p62/SQSTM1 for ubiquitinated protein complexes, its co-localization with plasma membrane GJs in HeLa, COS7, and PAE cells (Bejarano *et al.*, 2012; Fong *et al.*, 2012; Lichtenstein *et al.*, 2011), and its apparent involvement in targeting AGJ vesicles to autophagic degradation (Fong *et al.*, 2012) suggest that ubiquitination of Cx43 (and at least Cx50), besides serving as a likely signal for GJ internalization, may also serve as the signal for targeting AGJ vesicles to autophagic degradation. Future research will be required to determine the potentially numerous types (multiple mono-Ubs, Lys48- and Lys63-linked poly-Ubs, etc.) and functions of connexin ubiquitination (see Kjenseth *et al.*, 2010; Leithe *et al.*, 2012; Su and Lau, 2012 for recent reviews that discuss Cx-ubiquitination). Very recently, Kjenseth *et al.* (2012) described an additional, Ub-like post-translational modification of Cx43, SUMOylation (SUMO, small ubiquitin-like modifier) that appears to be involved in regulating GJ stability and turnover. The small Ub-like protein SUMO was found to be conjugated to lysines 144 and 237 of the Cx43-C-terminal domain, further widening the role of Ub and Ub-like signals in the maintenance and degradation of GJs.

DISCUSSION

Cells have developed three principal degradation pathways: the proteasomal, the endo-/lysosomal, and the phago-/lysosomal system (termed macroautophagy or simply autophagy), and all three have been implicated previously at various steps in the regulation of GJ stability and Cx degradation (Hesketh *et al.*, 2010; Laing *et al.*, 1997; Leach and Oliphant, 1984; Leithe and Rivedal, 2004a; Musil *et al.*, 2000; Pfeifer, 1980; Qin *et al.*, 2003). While the two latter ones utilize the lysosome for final degradation and are designed for the degradation of protein aggregates, multiprotein complexes and cytoplasmic organelles, the proteasomal system is designed for the degradation of single polypeptide chains that require unfolding to be inserted into the tubular core of the cytoplasmically located proteasome. Since AGJ vesicles are highly complex multi-subunit protein assemblies, their degradation by the proteasome is highly unlikely, and no evidence appears to exist that would suggest a proteasome-mediated degradation of GJs or AGJ vesicles. Similarly, lysosomal inhibitors such as leupeptin, chloroquine, NH_4Cl, and E-64, which previously have been used to gain evidence for endo-/lysosomal degradation of GJs (Berthoud *et al.*, 2004; Laing *et al.*, 1997; Musil *et al.*, 2000; Qin *et al.*, 2003), will also inhibit autophagic GJ degradation, and thus obtained results may not have been interpreted correctly. Experimental approaches that specifically target the autophagosomal degradation pathway that were used by others and us compellingly demonstrate that endocytosed GJs are degraded by autophagy.

Historically, autophagy has been known as a lysosomal degradation pathway that becomes essential to cell survival following nutrient depletion. However, substantial research over

the past decade has indicated that autophagy, besides its well-known function in organelle degradation during starvation, represents a much more common and highly conserved autonomous lysosome-based cellular degradation pathway that is specifically designed to remove and degrade protein aggregates, multiprotein complexes, organelles, and invading pathogens from the cytoplasm (Bjorkoy *et al.*, 2005; Hung *et al.*, 2009; Pohl and Jentsch, 2009; Ravikumar *et al.*, 2008). Recent studies have further shown that protein aggregates, such as the ones formed by huntingtin and β-amyloid protein, and cellular structures such as the midbody ring, a mitotic cytokinesis leftover multiprotein complex, are all degraded by autophagy (Bjorkoy *et al.*, 2005; Hung *et al.*, 2009; Pohl and Jentsch, 2009; Ravikumar *et al.*, 2008). Clearly, these cellular structures are degraded by autophagy independent of starvation. In addition, autophagosomal degradation of membranous/vesicular organelles, as for example malfunctioning mitochondria, is common. Since the catabolic activity of lysosomes is used in this process, degradation-prone structures first need to be separated from the cytoplasm. This is necessary due to the destructive activity of lysosomal enzymes, which cannot be released directly into the cytoplasm. Thus, cytoplasmic structures targeted for degradation are first engulfed in double-membrane vesicles (autophagosomes) that allow lysosomal fusion, degradation, and subsequent recycling of the phagosome cargo and the phagosome membrane.

CONCLUSION

In this article, I have summarized recent experimental results and discussed structural and functional considerations that all support the concept that autophagy serves as the default degradation pathway for endocytosed GJs. Indeed, in several classical ultrastructural analyses of various cells and tissues *in situ* including heart, dermis, and liver (Leach and Oliphant, 1984; Mazet *et al.*, 1985; Pfeifer, 1980; Severs *et al.*, 1989), autophagic degradation of GJs had been suggested. However, surprisingly back then not much attention was attributed to this evidently fundamental GJ degradation pathway. Autophagic degradation of GJs plays a significant role in the regulation of GJ function, as inhibition of cellular autophagy increases GJIC, prevents internalization of GJs, slows down the degradation of Cxs, and causes cytoplasmic accumulation of internalized GJ vesicles *in situ*, and in cells that either express endogenously or exogenously connexin proteins (Bejarano *et al.*, 2012; Fong *et al.*, 2012; Lichtenstein *et al.*, 2011). Hence, it is likely that certain disease-causing mutations in Cx proteins will impair physiological levels of GJ endocytosis and autophagosomal turnover, and that this will cause a detrimental misregulation of GJ function. Future research also will need to address the signals that specifically modify the Cx proteins to initiate GJ endocytosis and degradation. Post-translational modifications, such as phosphorylation, ubiquitination, and acetylation, the binding/release of regulatory proteins (e.g., ZO-1), and specific conformational changes of the Cx43-C-terminus that regulate access of modifying enzymes are all enticing possibilities.

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References

Baker, S.M., Kim, N., Gumpert, A.M., et al., 2008. Acute internalization of gap junctions in vascular endothelial cells in response to inflammatory mediator-induced G-protein coupled receptor activation. FEBS Lett. 582, 4039–4046.

Barriere, H., Nemes, C., Lechardeur, D., et al., 2006. Molecular basis of oligoubiquitin-dependent internalization of membrane proteins in mammalian cells. Traffic 7, 282–297.

Beardslee, M.A., Laing, J.G., Beyer, E.C., et al., 1998. Rapid turnover of connexin43 in the adult rat heart. Circ. Res. 83, 629–635.

Bejarano, E., Girao, H., Yuste, A., et al., 2012. Autophagy modulates dynamics of connexins at the plasma membrane in a ubiquitin-dependent manner. Mol. Biol. Cell 23, 2156–2169.

Belouzard, S., Rouille, Y., 2006. Ubiquitylation of leptin receptor OB-Ra regulates its clathrin-mediated endocytosis. EMBO J. 25, 932–942.

Berthoud, V.M., Minogue, P.J., Laing, J.G., et al., 2004. Pathways for degradation of connexins and gap junctions. Cardiovasc. Res. 62, 256–267.

Bjorkoy, G., Lamark, T., Brech, A., et al., 2005. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. J. Cell Biol. 171, 603–614.

Catarino, S., Ramalho, J.S., Marques, C., et al., 2011. Ubiquitin-mediated internalization of connexin43 is independent of the canonical endocytic tyrosine-sorting signal. Biochem. J. 437, 255–267.

Ciani, B., Layfield, R., Cavey, J.R., et al., 2003. Structure of the ubiquitin-associated domain of p62 (SQSTM1) and implications for mutations that cause Paget's disease of bone. J. Biol. Chem. 278, 37409–37412.

Falk, M.M., Baker, S.M., Gumpert, A.M., et al., 2009. Gap junction turnover is achieved by the internalization of small endocytic double-membrane vesicles. Mol. Biol. Cell 20, 3342–3352.

Falk, M.M., Fong, J.T., Kells, R.M., et al., 2012. Degradation of endocytosed gap junctions by autophagosomal and endo-/lysosomal pathways: a perspective. J. Membrane Biol. 245, 465–476.

Fallon, R.F., Goodenough, D.A., 1981. Five-hour half-life of mouse liver gap-junction protein. J. Cell Biol. 90, 521–526.

Fong, J.T., Kells, R.M., Falk, M.M., 2013. Two tyrosine-based sorting signals in the Cx43 C-terminus cooperate to mediate gap junction endocytosis. Mol. Biol. Cell 24, 2834–2848.

Fong, J.T., Kells, R.M., Gumpert, A.M., et al., 2012. Internalized gap junctions are degraded by autophagy. Autophagy 8, 794–811.

Fushman, D., Wilkinson, K.D., 2011. Structure and recognition of polyubiquitin chains of different lengths and linkage. F1000 Biol. Rep. 3, 26.

Fykerud, T.A., Kjenseth, A., Schink, K.O., et al., 2012. Smad ubiquitination regulatory factor-2 controls gap junction intercellular communication by modulating endocytosis and degradation of connexin43. J. Cell Sci. 125, 3966–3976.

Gaietta, G., Deerinck, T.J., Adams, S.R., et al., 2002. Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 503–507.

Geetha, T., Jiang, J., Wooten, M.W., 2005. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. Mol. Cell 20, 301–312.

Ghoshroy, S., Goodenough, D.A., Sosinsky, G.E., 1995. Preparation, characterization, and structure of half gap junctional layers split with urea and EGTA. J. Membrane Biol. 146, 15–28.

Gilleron, J., Fiorini, C., Carette, D., et al., 2008. Molecular reorganization of Cx43, ZO-1 and Src complexes during the endocytosis of gap junction plaques in response to a non-genomic carcinogen. J. Cell Sci. 121, 4069–4078.

Girao, H., Catarino, S., Pereira, P., 2009. Eps15 interacts with ubiquitinated Cx43 and mediates its internalization. Exp. Cell Res. 315, 3587–3597.

Goodenough, D.A., Gilula, N.B., 1974. The splitting of hepatocyte gap junctions and zonulae occludentes with hypertonic disaccharides. J. Cell Biol. 61, 575–590.

Gumpert, A.M., Varco, J.S., Baker, S.M., et al., 2008. Double-membrane gap junction internalization requires the clathrin-mediated endocytic machinery. FEBS Lett. 582, 2887–2892.

Hawryluk, M.J., Keyel, P.A., Mishra, S.K., et al., 2006. Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein. Traffic 7, 262–281.

Hesketh, G.G., Shah, M.H., Halperin, V.L., et al., 2010. Ultrastructure and regulation of lateralized connexin43 in the failing heart. Circ. Res. 106, 1153–1163.

Hicke, L., 2001. Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. 2, 195–201.
Hicke, L., Dunn, R., 2003. Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. Annu. Rev. Cell Dev. Biol. 19, 141–172.
Hung, S.Y., Huang, W.P., Liou, H.C., et al., 2009. Autophagy protects neuron from Abeta-induced cytotoxicity. Autophagy 5, 502–510.
Jordan, K., Chodock, R., Hand, A.R., et al., 2001. The origin of annular junctions: a mechanism of gap junction internalization. J. Cell Sci. 114, 763–773.
Kabeya, Y., Mizushima, N., Ueno, T., et al., 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19, 5720–5728.
Kjenseth, A., Fykerud, T., Rivedal, E., et al., 2010. Regulation of gap junction intercellular communication by the ubiquitin system. Cell Signal. 22, 1267–1273.
Kjenseth, A., Fykerud, T.A., Sirnes, S., et al., 2012. The gap junction channel protein connexin43 is covalently modified and regulated by SUMOylation. J. Biol. Chem. 287, 15851–15861.
Laing, J.G., Tadros, P.N., Westphale, E.M., et al., 1997. Degradation of connexin43 gap junctions involves both the proteasome and the lysosome. Exp. Cell Res. 236, 482–492.
Lauf, U., Giepmans, B.N., Lopez, P., et al., 2002. Dynamic trafficking and delivery of connexons to the plasma membrane and accretion to gap junctions in living cells. Proc. Natl Acad. Sci. USA 99, 10446–10451.
Leach, D.H., Oliphant, L.W., 1984. Degradation of annular gap junctions of the equine hoof wall. Acta Anat. (Basel) 120, 214–219.
Leithe, E., Kjenseth, A., Sirnes, S., et al., 2009. Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. J. Cell Sci. 122, 3883–3893.
Leithe, E., Rivedal, E., 2004a. Epidermal growth factor regulates ubiquitination, internalization and proteasome-dependent degradation of connexin43. J. Cell Sci. 117, 1211–1220.
Leithe, E., Rivedal, E., 2004b. Ubiquitination and down-regulation of gap junction protein connexin-43 in response to 12-O-tetradecanoylphorbol 13-acetate treatment. J. Biol. Chem. 279, 50089–50096.
Leithe, E., Sirnes, S., Fykerud, T., et al., 2012. Endocytosis and post-endocytic sorting of connexins. Biochim. Biophys. Acta 1818, 1870–1879.
Lichtenstein, A., Minogue, P.J., Beyer, E.C., et al., 2011. Autophagy: a pathway that contributes to connexin degradation. J. Cell Sci. 124, 910–920.
Madshus, I.H., 2006. Ubiquitin binding in endocytosis – how tight should it be and where does it happen? Traffic 7, 258–261.
Mazet, F., Wittenberg, B.A., Spray, D.C., 1985. Fate of intercellular junctions in isolated adult rat cardiac cells. Circ. Res. 56, 195–204.
Mizushima, N., 2004. Methods for monitoring autophagy. Int. J. Biochem. Cell Biol. 36, 2491–2502.
Musil, L.S., Le, A.C., VanSlyke, J.K., et al., 2000. Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. J. Biol. Chem. 275, 25207–25215.
Pankiv, S., Clausen, T.H., Lamark, T., et al., 2007. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. 282, 24131–24145.
Pfeifer, U., 1980. Autophagic sequestration of internalized gap junctions in rat liver. Eur. J. Cell Biol. 21, 244–246.
Piehl, M., Lehmann, C., Gumpert, A., et al., 2007. Internalization of large double-membrane intercellular vesicles by a clathrin-dependent endocytic process. Mol. Biol. Cell 18, 337–347.
Pohl, C., Jentsch, S., 2009. Midbody ring disposal by autophagy is a post-abscission event of cytokinesis. Nat. Cell Biol. 11, 65–70.
Postma, F.R., Hengeveld, T., Alblas, J., et al., 1998. Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-Src. J. Cell Biol. 140, 1199–1209.
Qin, H., Shao, Q., Igdoura, S.A., et al., 2003. Lysosomal and proteasomal degradation play distinct roles in the life cycle of Cx43 in gap junctional intercellular communication-deficient and -competent breast tumor cells. J. Biol. Chem. 278, 30005–30014.
Ravikumar, B., Imarisio, S., Sarkar, S., et al., 2008. Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. J. Cell Sci. 121, 1649–1660.
Schnell, D.J., Hebert, D.N., 2003. Protein translocons: multifunctional mediators of protein translocation across membranes. Cell 112, 491–505.

Seibenhener, M.L., Babu, J.R., Geetha, T., et al., 2004. Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. Mol. Cell Biol. 24, 8055–8068.

Severs, N.J., Shovel, K.S., Slade, A.M., et al., 1989. Fate of gap junctions in isolated adult mammalian cardiomyocytes. Circ. Res. 65, 22–42.

Su, V., Lau, A.F., 2012. Ubiquitination, intracellular trafficking, and degradation of connexins. Arch Biochem. Biophys. 524, 16–22.

Thévenin, A.F., Kowal, T.J., Fong, J.T., et al., 2013. Proteins and mechanisms regulating gap junction assembly, internalization and degradation. Physiology 28, 93–116.

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