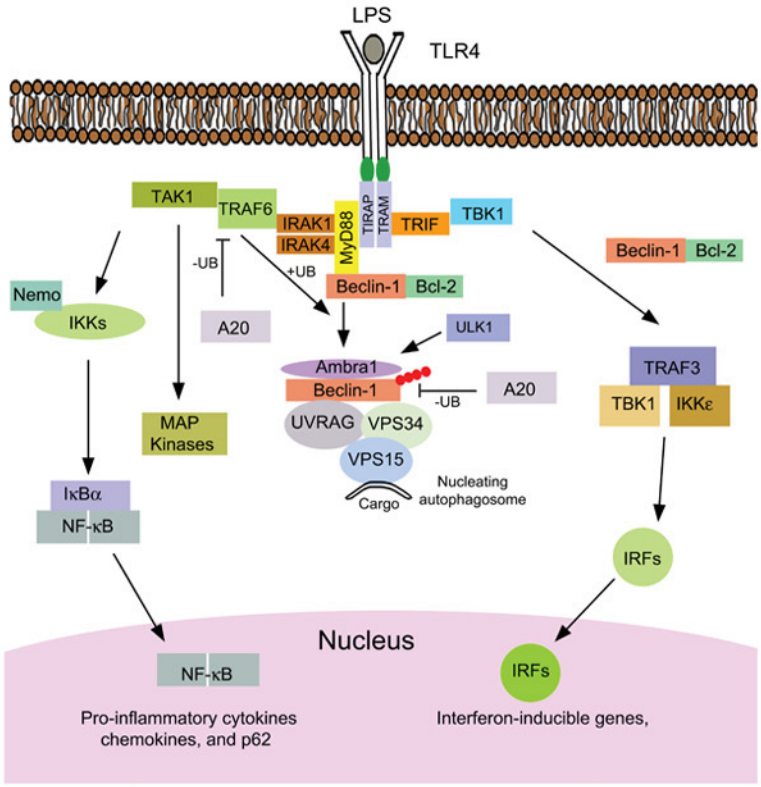
AUTOPHAGY

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

VOLUME 6

EDITED BY

M. A. HAYAT



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Dedication

To:

Julio A. Aguirre-Ghiso, Patrice Codogno, Eduardo Couve, Ana Maria Cuervo, Guido R. Y. De Meyer, Vojo Deretic, Fred J. Dice, William A. Dunn, Jr, Eeva-Lisa Eskelinen, Sharon Gorski, Tomotake Kanki, Daniel J. Klionsky, Guido Kroemer, Beth Levine, Noboru Mizushima, Yoshinori Ohsumi, Brinda Ravikumar, David Rubinsztein, Isei Tanida, Sharon A. Tooze, Herbert W. Virgin, Eileen White, Tamotsu Yoshimori, and others.

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

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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

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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

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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

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Contents

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.

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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.

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CHAPTER

1

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

M.A. *Hayat*

OUTLINE

M.A. *Hayat (ed): Autophagy, Volume 6.*
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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.

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).

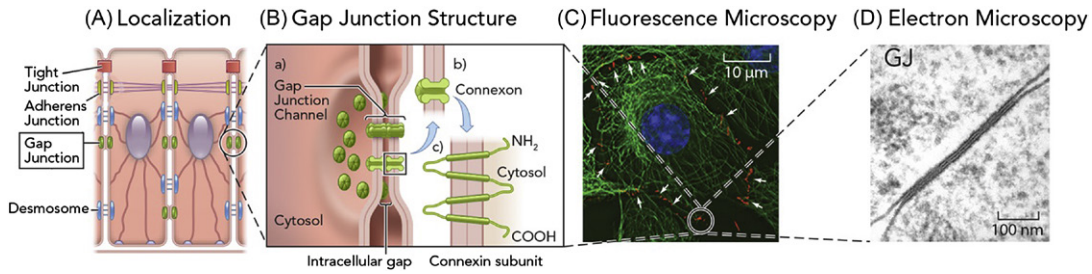

FIGURE 19.1 Cellular location and structure of gap junctions (GJs). (A) GJs are assemblies of double-membrane spanning hydrophilic channels termed "plaques" that bridge the apposing plasma membranes of neighboring cells to provide direct cell-to-cell (or intercellular) communication as shown here for 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

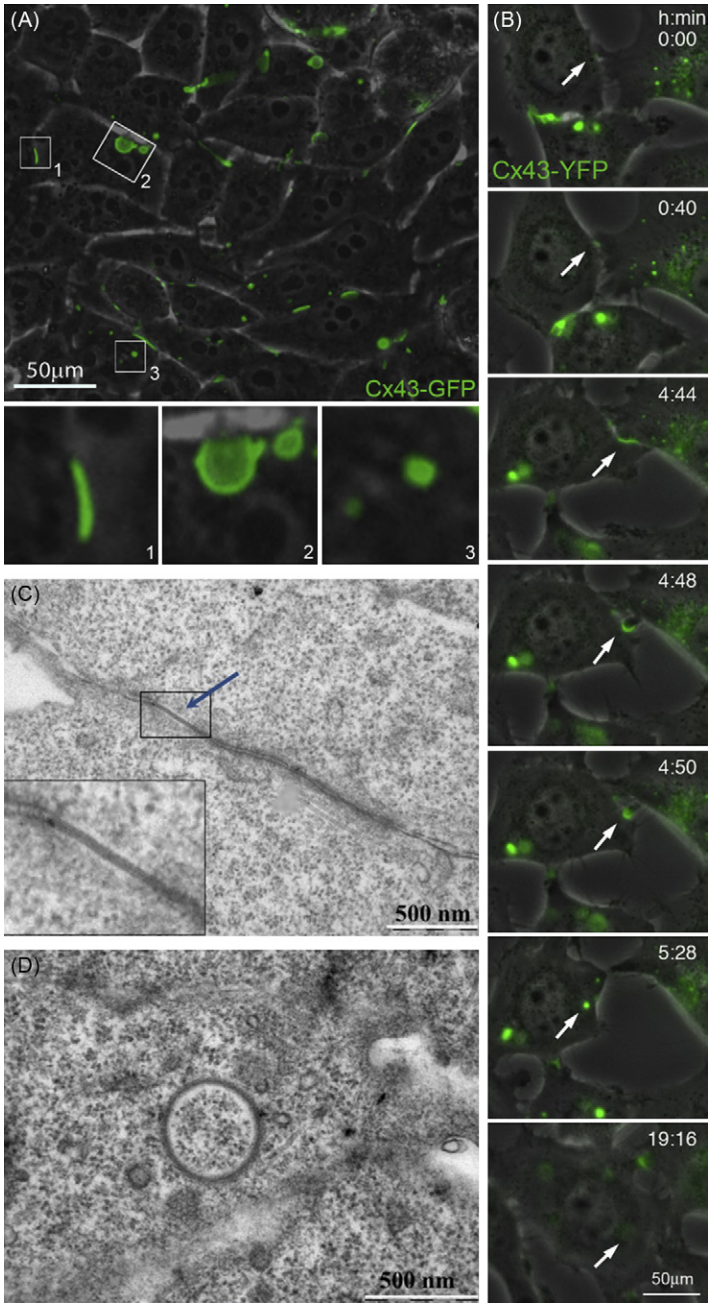

FIGURE 19.2 Gap junctions and endocytosed gap junctions. (A) HeLa cells transfected with Cx43-GFP efficiently express and assemble GJs in the adjacent plasma membranes of transfected cells (visible as green fluorescent lines and puncta such as the one shown in insert 1). Over time, GJs bulge inward (insert 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.

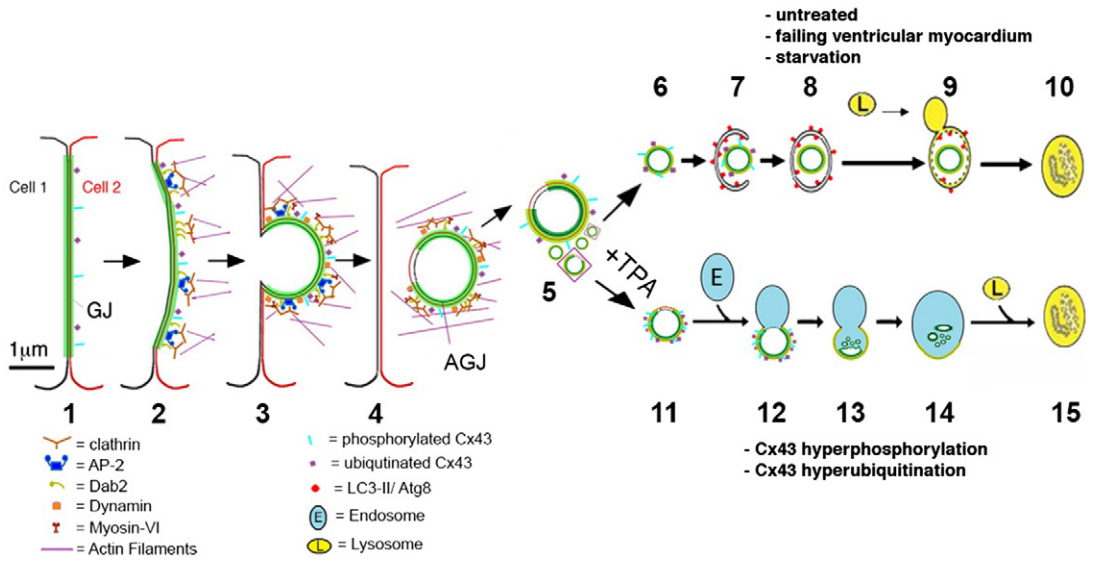

FIGURE 19.3 Mechanisms of gap junction endocytosis and degradation. Schematic representation of proposed steps that lead to GJ internalization (steps 1–3), cytoplasmic AGJ vesicle formation and fragmentation (steps 4, 5), and AGJ vesicle degradation by phago-/lysosomal (steps 6–10) and endo-/lysosomal 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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