Regulation of Mammalian Autophagy in Physiology and Pathophysiology


Department of Medical Genetics, University of Cambridge, Cambridge Institute for Medical Research, Addenbrooke's Hospital, Cambridge, United Kingdom

I. Introduction
II. Autophagy Machinery
   A. Initiation of autophagosome formation
   B. Elongation
   C. Maturation and fusion
III. Selective Autophagy
IV. Cross-Talk Between the Ubiquitin-Proteasome System, Chaperone-Mediated Autophagy, and Autophagy
   A. Ubiquitin-proteasome system and autophagy
   B. ERAD and autophagy
   C. CMA and autophagy
V. Signaling Pathways Regulating Mammalian Autophagy
   A. mTOR pathway
   B. PI3K pathway
   C. Other protein kinases
   D. mTOR-independent pathways
   E. Other mTOR-independent autophagy regulators
   F. Ceramides and other bioactive sphingolipids regulate autophagy
   G. Reactive oxygen species and autophagy
VI. Autophagy, Metabolism, Cell Death, Development, and Ageing
   A. Autophagy, autophagic cell death, and apoptosis
   B. Autophagy in development
   C. Erythropoiesis
   D. Autophagy and ageing
   E. Autophagy and lipid metabolism
VII. Autophagy and Immunity
   A. Autophagy, antigen processing, and presentation
   B. Autophagy and infectious diseases
   C. Autophagy and Crohn's disease
   D. Autophagy and parasites
VIII. Diseases Associated With Defective Autophagosome Substrate Degradation
   A. Lysosomal storage disorders
   B. Autophagy deficiency: a secondary disease mechanism in neurodegeneration
IX. Autophagy and Aggregate-Prone Proteins
   A. Autophagy induction for various neurodegenerative diseases
   B. Alzheimer's disease
   C. Autophagy and α1-antitrypsin deficiency
X. Other Diseases Associated With Changes in Autophagy
   A. Autophagy in muscle atrophy, myopathy, and cardiac disease
   B. Autophagy and pancreatitis
   C. Autophagy and stroke
   D. Autophagy and cancer
   E. Autophagy and type 2 diabetes
XI. Concluding Comments
I. INTRODUCTION

The word autophagy is derived from the Greek roots “auto” (self) and “phagy” (eating) and broadly refers to the cellular catabolic processes in which cytoplasmic target material is transported to lysosomes for degradation. Christian de Duve, who was awarded the Nobel Prize for his work on lysosomes, first used the term autophagy in 1963. He used the word to describe the phenomenon associated with single- or double-membraned vesicles that contained cytoplasm, including organelles, at various stages of digestion. In this way, autophagy can be distinguished from heterophagy, where cells degrade extracellular material. De Duve’s “autophagy” likely described macroautophagy, which we will call autophagy, the focus of this review. Autophagy, which is highly conserved from yeast to humans, is a bulk degradation process involved in the clearance of long-lived proteins and organelles. During autophagy, phagophores [also called pre-autophagosomal structures (PAS)] elongate and fuse while engulfing a portion of cytoplasm within double-membraned vesicles, called autophagosomes. The autophagosomes first fuse with endosomes to form hybrid organelles called amphisomes that later fuse with acidic lysosomes where the entrapped cytosolic contents are degraded (Fig. 1). There are two other forms of autophagy, which we will not review, but will briefly mention. The first, chaperone-mediated autophagy (CMA), involves selective translocation of the cytosolic proteins that are marked by a pentapeptide motif with a consensus sequence similar to KFERQ across the lysosomal membrane. Cytosolic chaperones aid in the target recognition and unfolding, and a multimer comprising the lysosomal protein LAMP-2a (lysosomal-associated membrane protein-2a) subunits is thought to be rate-limiting for target translocation into lysosomes. The second is microautophagy, a poorly understood phenomenon in mammalian cells, where the cytosolic contents are engulfed by direct invagination of the lysosomal membranes into tubulovesicular structures.

Until the early 1990s, autophagy was predominantly studied using morphological and biochemical methods. In 1992, Ohsumi’s group demonstrated that autophagy occurred in Saccharomyces cerevisiae (baker’s yeast). Soon thereafter, Ohsumi’s lab and others, like Klionsky and Thumm, published data using this tractable organism to identify autophagy genes (Atg genes). The discovery of yeast autophagy genes was followed by the identification of mammalian orthologs with similar roles and provided a series of reagents for characterizing the molecular machinery of the system as well as its roles in normal physiology and disease (240).

Autophagy in mammalian systems occurs under basal conditions and can be stimulated by stresses like starvation, various pathologies, or by treatment with pharmacological agents like rapamycin. In addition to its roles in maintaining normal cellular homeostasis by liberating nutrients from macromolecules and by assisting the clearance of misfolded proteins and damaged organelles, autophagy is vital in a range of physiological and pathological situations, including during early embryonic development and neonatal starvation, for the degradation of disease-causing aggregate-prone proteins and in the clearance of pathogenic bacteria. Dysfunction in the autophagy pathway has been implicated in an increasing number of human diseases, from infectious diseases to cancer and neurodegeneration (341).

In this review, we aim to broadly outline the biology of mammalian autophagy and the key genes involved in the process. We will consider how autophagy is important for normal mammalian physiology and its roles in a range of disease. Finally, we will consider the possibility that autophagy upregulation may be beneficial for certain human diseases.

II. AUTOPHAGY MACHINERY

More than 30 different genes regulating autophagy have been identified in yeast, and many of these have
mammalian orthologs. In addition, many genes with multiple functions, including roles in autophagy that were not identified in the relatively specific yeast screens, have been identified. These genes can be grouped according to their functions at key stages of the autophagy pathway: initiation, elongation, maturation, and fusion with the lysosomes.

A. Initiation of Autophagosome Formation

The membrane source from which autophagosomes arise is still a matter of debate, and there may be multiple sources. It has been hypothesized that autophagosomes can either be generated de novo from preexisting intracellular precursor molecules, or could arise from other intracellular membrane structures like the endoplasmic reticulum (ER) (8). The latter hypothesis has recently been supported by more evidence suggesting that ER could contribute to autophagosome formation (168, 582). Autophagosomes have frequently been reported to form in the vicinity of ER. Furthermore, rough ER was found to surround both the outer and inner surfaces of the cup-shaped pre-autophagosomal structures. Accumulation of these ER-associated early autophagic structures was seen in cells that had a block in maturation of pre-autophagosomal structures. ER association with the outer surface was, however, not observed when the pre-autophagosomal structures matured into autophagosomes. Thus the ER may be one of the membrane sources contributing to the maturation of pre-autophagosomal structures (168, 582). It has recently been suggested that mitochondria may be a membrane source to autophagosomes during starvation (150a). Recently, we showed that plasma membrane is a key contributor for autophagosome precursors (415a). The formation of new autophagosomes requires the activity of the class III phosphatidylinositol 3-kinase (PI3K), Vps34. Phosphatidylinositol-3-phosphate (PI-3-P), the product of Vps34 activity, plays an essential role in the maturation of pre-autophagosomal structures (168, 582). It has recently been suggested that mitochondria can either be generated de novo from preexisting intracellular precursor molecules, or could arise from other intracellular membrane structures like the endoplasmic reticulum (ER) (8). The latter hypothesis has recently been supported by more evidence suggesting that ER could contribute to autophagosome formation (168, 582). Autophagosomes have frequently been reported to form in the vicinity of ER. Furthermore, rough ER was found to surround both the outer and inner surfaces of the cup-shaped pre-autophagosomal structures. Accumulation of these ER-associated early autophagic structures was seen in cells that had a block in maturation of pre-autophagosomal structures. ER association with the outer surface was, however, not observed when the pre-autophagosomal structures matured into autophagosomes. Thus the ER may be one of the membrane sources contributing to the maturation of pre-autophagosomal structures (168, 582). It has recently been suggested that mitochondria may be a membrane source to autophagosomes during starvation (150a). Recently, we showed that plasma membrane is a key contributor for autophagosome precursors (415a). The formation of new autophagosomes requires the activity of the class III phosphatidylinositol 3-kinase (PI3K), Vps34. Phosphatidylinositol-3-phosphate (PI-3-P), the product of Vps34 activity, plays an essential role in the early stages of the autophagy pathway. Recent studies have identified strong colocalization of early autophagosome markers in PI-3-P-enriched structures that were formed upon starvation (8). The precise role of PI-3-P is still unclear; however, this raises the importance of PI-3-P effector proteins in autophagosome formation. Indeed, the recently identified mammalian Atg18 homolog WIP1-1 binds to PI-3-P and is recruited to autophagosomal membranes, especially upon autophagy induction (401). The small GTPase Rab5, an early endocytic protein, interacts with and activates Vps34 (58, 483). Inhibition of Vps34 activity by 3-methyladenine (3-MA) or wortmannin leads to inhibition of autophagosome formation (26, 391, 472).

Vps34 is part of the autophagy-regulating macromolecular complex (PI3K complex) consisting of Beclin 1/Atg6, Atg14/barkor, and p150/Vps15 (Fig. 1) (189, 227, 500). The activity of Vps34 is enhanced by its interaction with Beclin 1 (123). Several Beclin 1 binding proteins have been identified, and disruption of their interaction with Beclin 1 affects autophagosome formation. The Beclin 1 binding partners that induce autophagy include ambra-1 (110), UVRAG (298), and bif-1 (506). On the other hand, the binding of the anti-apoptotic proteins Bcl-2 or Bcl-XL to Beclin 1 inhibits autophagy (389). Furthermore, the inositol 1,4,5-trisphosphate receptor (IP3 R) that can bind to Bcl-2 can also interact with Beclin 1. The IP3 R antagonist xestospongin B decreases this interaction and induces autophagy (552). The Beclin 1-Bcl-2 interaction was also diminished by xestospongin B, even though this drug does not affect the IP3 R-Bcl-2 interaction (67). Another component in the IP3 R complex, NAF-1 (nutrient-deprivation autophagy factor-1), has recently been identified and acts at the ER (45). NAF-1 interacts with Bcl-2 at the ER and stabilizes the Bcl-2-Beclin 1 interaction, while knockdown of NAF-1 decreases Bcl-2-Beclin 1 interaction and promotes autophagy (45). Activation of c-Jun NH2-terminal kinase-1 (JNK1) during starvation results in phosphorylation of Bcl-2 and Bcl-XL that releases their binding to Beclin 1, thus inducing autophagosome formation (502).

A second macromolecular complex implicated in the initiation step of autophagosome formation is the FIP200 (163)-ULK1/Atg1 complex (44, 46, 126, 178, 209). Atg13 binds ULK1 or its homolog ULK2 and mediates their interaction with FIP200. Under nutrient deprivation conditions, Atg13 and ULK1/2 are dephosphorylated (see section v for details), thereby activating ULK1/2, which phosphorylates FIP200 to induce autophagosome formation (46, 126, 178, 209).

B. Elongation

Elongation of phagophores probably requires membrane input from other organelles. Atg9 is one protein that may play this role. Atg9 is a transmembrane protein that cycles between the trans-Golgi network and endosomes, probably carrying membrane for expansion of phagophore (584). Other components of Atg9 sorting remain to be identified.

Two ubiquitin-like reactions are involved in the elongation of the pre-autophagosomal structures. In the first of the reactions, the ubiquitin-like protein Atg12 is covalently tagged to Atg5 (342). Atg12 is first activated by Atg7 (E1 ubiquitin activating enzyme-like) and then transferred to Atg10 (E2 ubiquitin conjugating enzyme-like). Atg12 is finally linked by its COOH-terminal glycine to an internal lysine (K130) residue of Atg5 (342). The Atg12-Atg5 then forms a conjugate with Atg16L1 (Atg12-Atg5-Atg16L1), resulting in an 800-kDa complex containing Atg12-Atg5-Atg16L1 tetramers that are linked via the coiled-coil domain of Atg16L1 (340). This complex is es-
sential for the elongation of the pre-autophagosomal membrane, but dissociates from fully formed autophagosomes.

The second ubiquitin-like reaction involves the protein microtubule-associated protein 1 light chain 3 (MAP1-LC3/LC3/Atg8). LC3 is synthesized as a precursor
form and is cleaved at its COOH terminus by the protease Atg4B (170, 516), resulting in the cytosolic isofrom LC3-I. LC3-I is conjugated to phosphatidylethanolamine (PE) in a reaction involving Atg7 (E1-like) and Atg3 (E2-like) to form LC3-II (211, 518). LC3-II is specifically targeted to the elongating autophagosome membrane and, unlike the Atg12-Atg5·Atg16L1 complex, remains on completed autophagosomes until fusion with the lysosomes, after which LC3-II on the cytoplasmic face of autolysosomes can be delipidated by Atg4 and recycled (516). LC3 is also found on the internal surface of autophagosomes, and this is degraded in the autolysosomes.) The relatively specific association of LC3-II with autophagosomes makes it an excellent marker for studying autophagy (241).

Cross-talk between the two ubiquitin-like systems has been reported. The Atg12-Atg5·Atg16L1 complex can function in an E3 ubiquitin-ligase-like manner to facilitate LC3-I conjugation to PE (157). The Atg16L1 complex is believed to bring LC3 to the site of lipidation for the final conjugation with PE, but the mechanism by which LC3 is targeted to specific membranes remains to be identified (121). Atg10 can interact with LC3 and facilitate LC3 conjugation to PE (358). Similarly, Atg3 coimmunoprecipitates with Atg12, and overexpression of Atg3 increases Atg5·Atg12 conjugation (517). LC3 can mediate membrane tethering and hemifusion, and these functions may be crucial for the expansion and/or assisting the final fusion of the double-membrane cups into fused vesicles (355). Consistent with this hypothesis, loss of Atg3 or a loss-of-function mutation in Atg4B leads to decreased closure of isolation membranes (120, 494).

C. Maturation and Fusion

Unlike in yeast cells where autophagosomes are formed at the single phagophore assembly site next to the vacuole, mammalian autophagosomes are formed randomly in the cytoplasm. Autophagosomes move bidirectionally along microtubules with a bias towards the microtubule organising center (MTOC), where the lysosomes are enriched. This transport towards MTOC requires the function of dynein motor proteins (233, 413, 566). Depolymerization of microtubules or inhibition of dynein-dependent transport results in inhibition of autophagy (245, 560). Autophagosomes first fuse with endosomes and then with lysosomes where the fate of the autophagosomes ends. The fusion step involves proteins such as ESCRT, SNAREs, Rab7, and the class C Vps proteins (7, 109, 147, 199, 282, 428). Mutation or loss of proteins important for formation of multivesicular bodies (MVBs) (e.g., ESCRT) leads to an inhibition of autophagosome maturation (109, 282, 428). UVRAG, a Beclin 1 interacting protein, is also involved in the maturation step by recruiting the fusion machinery on the autophagosomes. This function is independent of its interaction with Beclin 1 (299). UVRAG recruits the class C Vps proteins and via this interaction activates Rab7, thereby promoting fusion with late endosomes and lysosomes (299). A recently identified Beclin 1 interacting protein, Rubicon, also functions in the maturation of autophagosomes. Rubicon is thought to be a part of a distinct Beclin 1 complex containing hVps34, hVps15, and UVRAG that suppresses autophagosome maturation (329, 603). More work is required to exactly characterize the different Beclin 1 complexes and where in the autophagy pathway they act.

In addition to the fusion machinery, proper lysosomal function is also essential for fusion to be successful. Inhibition of the lysosomal H+·ATPase by chemicals like bafilomycin A1 inhibits the fusion of autophagosomes with endosomes/lysosomes (577). This might be because lysosomal acidification is required for normal fusion. However, the possible mechanisms responsible are unclear.

A recent study has identified an alternative autophagy pathway that seems to be activated when mouse cells lacking Atg5 or Atg7 were treated with stressors like etoposide. This Atg5/Atg7-independent autophagy requires certain other key autophagy proteins, like Ulk1 and Beclin 1, and the progression of this autophagy process occurs in a Rab9-dependent manner (362).
III. SELECTIVE AUTOPHagy

Autophagy has long been considered to be a nonselective bulk degradation pathway. However, in the last few years, several forms of selective autophagy have been identified which lead to degradation of specific organelles, proteins, and pathogens in yeast, flies, and mammals. Whether nonselective autophagy really occurs in a random manner is an interesting question. Recently, a study of the dynamics of 1,500 proteins revealed they were degraded in an ordered fashion during starvation, implying selectivity under conditions typically associated with nonselective autophagy (260). Cytosolic proteins were degraded rapidly, whereas proteins annotated to various complexes and organelles were degraded later at different time periods. Selective autophagy may be involved, possibly via positive selection for incorporation into autophagosomes [e.g., by posttranslational modifications like ubiquitination or acetylation (202, 238)] and/or negative selection (e.g., due to decreased accessibility of certain proteins/organelles for autophagic engulfment). However, some of these observations may also be unrelated to any selection and may be the consequence of multiple different pathways contributing to degradation of a given substrate; many proteins are degraded both by the ubiquitin-proteasome system (where substrates are “primed” for degradation by E3 ligases) and autophagy.

A variety of organelles are cleared by autophagy. Homeostatic levels of ER, peroxisomes, mitochondria, and ribosomes are maintained by ER-phagy, pexophagy, mitophagy, and ribophagy, respectively (20, 259, 432, 530). Lipid degradation by autophagy (macrolipophagy) and degradation of pathogens (xenophagy) have also been described as specific phenomena (289, 563). Mitochondria are a major source of reactive oxygen species (ROS) and are therefore especially prone to ROS damage. Maintaining a healthy population of mitochondria is essential for the well-being of cells. The major degradative pathway in mitochondrial turnover is mitophagy (230). Mitophagy has been directly visualized in hepatocytes from transgenic mice expressing green fluorescent protein (GFP) fused with LC3 (230, 343). GFP-LC3 formed small green dotted structures that became associated with mitochondria following starvation. These structures became crescent-like in shape and went on to completely sequester individual mitochondria.

Mitophagy can be induced by nutrient deprivation and photodamage. Both these stimuli can induce the mitochondrial permeability transition (MPT) (286). Unlike in yeast cells, a common feature of mammalian mitophagy is loss of membrane potential. During MPT, the permeability transition pore, a nonselective large-conductance inner mitochondrial membrane channel, opens, resulting in depolarization and mitochondrial outer membrane rupture. Cyclosporin A, an MPT blocker, can prevent mitophagy occurring in mitochondria undergoing MPT (230). In HeLa cells, inhibitor factor 1 (IF1), a nuclear DNA encoded protein that regulates F1F0-ATP synthase, is involved in mitochondrial clearance by autophagy (39). Mitophagy is also induced during normal developmental processes like reticulocyte maturation or development of sperm cells. Mitochondrial clearance in reticulocytes required the Bcl2-related protein Nix along with Ulk1 activity (267, 469). Nix has recently been shown to bind to LC3/GABARAP and thus may target mitochondria to autophagosomes (366, 468). During hypoxia, BNIP3, a protein whose expression is suppressed in many forms of cancer, is involved in mitochondrial clearance as a survival mechanism to control generation of ROS (13, 598).

One way that mitophagy may be beneficial would be if it could selectively enhance removal of dysfunctional mitochondria. This may occur via the protein Parkin, which is mutated in certain cases of autosomal recessive Parkinson’s disease. Parkin is selectively recruited to dysfunctional mitochondria with low membrane potential. After recruitment, Parkin facilitates the engulfment of dysfunctional mitochondria by autophagosomes and thus allows selective removal of damaged mitochondria (357). The relocation of Parkin to mitochondria relies on the expression of phosphatase and tensin homolog (PTEN)-induced putative kinase 1 (PINK1), another protein mutated in forms of autosomal recessive Parkinson’s disease. Overexpression of the wild-type, but not the mutant, PINK1 causes translocation of Parkin to mitochondria (554). Loss of PINK1 induces oxidative stress and subsequently leads to the clearance of depolarized mitochondrial by autophagy requiring beclin-1 (55, 76).

Another way that one may be able to mediate selective clearance of mitochondria appears to be related to the increased likelihood that a small mitochondrial could be engulfed by an autophagosome, compared with a larger mitochondrion. Mitochondria undergo multiple cycles of fusion and fission in a “kiss and run” pattern. Fission events often generate unlike daughter mitochondria: one daughter has increased membrane potential [delta psi(m)] and a high probability of subsequent fusion, while the other has decreased membrane potential and a reduced probability for a fusion event. This predicts the formation of a subpopulation of nonfusing mitochondria that also have reduced delta psi(m) and decreased levels of the mitochondrial fusion protein OPA1. Inhibition of fission decreases mitochondrial autophagy and results in the accumulation of oxidized mitochondrial proteins, reduced respiration, and impaired insulin secretion. These studies suggest a fusion-fission-related process that enables selective removal of damaged mitochondria by autophagy (540).
Peroxisome abundance is increased in cells in response to peroxisome proliferators, a process that is counterbalanced by degradation of peroxisomes by pexophagy (432). Evidence for the contribution of the autophagic machinery in peroxisomal degradation in mammals comes from conditional knockout mice for Atg7, an essential autophagy gene. In control hepatocytes, excess peroxisomes were surrounded by autophagosomes, suggesting a selective process. Atg7-deficient hepatocytes had impaired removal of excess peroxisomes, and these excess peroxisomes were not surrounded by autophagosomes (194). It is possible that one way that peroxisomes may be targeted to autophagosomes is via ubiquitination of cytosolic-facing proteins. Such proteins may be recognized by the ubiquitin-binding protein p62 that is targeted to autophagosomes by binding to Atg8 (231).

ER volume is increased in response to ER stress where an excess of unfolded proteins is sensed by the unfolded protein response (UPR). When the UPR becomes inactivated, the ER reestablishes its homeostatic volume by ER-phagy (20). Electron microscopic studies in yeast have revealed large vesicles, bound by double membranes, densely filled with stacked membrane cisternae and no other organelles, indicating a selective process (20). Autophagy may also play a role during the UPR by eliminating areas of ER that are enriched in potentially dangerous unfolded proteins. In mammalian cells, autophagy occurs in response to ER stress and ER protein aggregates (545), but whether or not this is a selective process is yet to be demonstrated.

Regulation of selective autophagy has been well studied in yeast where elegant genetic experiments have elucidated genes that are specifically involved in the different selective forms of autophagy, for example, Atg19 and Atg20 in ER-phagy, Uth1, Aup1, Atg32 in mitophagy, PEX3 and PEX14 in pexophagy, and Ubp3p/Bre5p in ribophagy (216, 259, 376, 545). Much less is known about the molecular targets and signaling pathways responsible for selective pexophagy and ER-phagy in mammals. Indeed, it is frequently very difficult to make a strong claim for selective autophagy without identifying candidate modulators that can influence autophagic degradation of the specific organelle/protein in question, without influencing autophagic clearance of other substrates.

Selective autophagy is not limited to degradation of organelles like mitochondria and the ER. Glycogen autophagy occurs in mammals under conditions of demand for the massive hepatic production of glucose, such as in the postnatal period. Glycogen autophagy appears to be a selective process, since autophagic vacuoles are predominantly distributed at the borders of glycogen stores and contain various quantities of glycogen (256). Glycogen autophagy is induced by glucagon, which is secreted after birth as a result of the normal postnatal hypoglycemia. Glucagon elevates cAMP levels resulting in the formation of hepatic autophagic vacuoles, activation of the glucose-hydrolyzing acid glucosidase, and degradation of glycogen to glucose (256).

IV. CROSS-TALK BETWEEN THE UBIQUITIN-PROTEASOME SYSTEM, CHAPERONE-MEDIATED AUTOPHAGY, AND AUTOPHagy

A. Ubiquitin-Proteasome System and Autophagy

Besides autophagy, the other major route for intracellular protein degradation is the ubiquitin-proteasome system (UPS). In contrast to macroautophagy, this process is highly specific. In mammalian cells, about a thousand E3 enzymes are responsible for the specific detection and binding of their substrates, which include misfolded proteins and short half-life regulatory proteins. In this process, the ubiquitously expressed, 76-amino acid protein ubiquitin is first activated by an E1 enzyme and subsequently transferred onto an E2 ubiquitin-conjugating enzyme. The interaction between an E2 enzyme (carrying the activated ubiquitin molecule) and an E3 enzyme (carrying the degradation substrate) leads to the transfer of the ubiquitin onto the target substrate. In this reaction, a covalent isopeptide bond is formed between the COOH-terminal glycine of the ubiquitin molecule and the lysine residues in the target protein (171). The ubiquitin molecule itself contains seven internal lysines at positions 6, 11, 27, 31, 33, 48, and 63. In a second cycle, the cascade of the tagged protein is destined for different pathways. A protein that carries a chain of at least four K48-linked ubiquitins can be recognized by the machineries that allow transfer to the proteasome. At least in some circumstances, this appears to be mediated by ubiquitin-binding proteins, like p97 (152).

The proteasome consists of a 20S central complex and two 19S lid complexes. The barrel-shaped central complex comprises four stacked rings. The two identical outer rings are each made up of seven α-subunits (termed α 1–7), and the two identical inner rings each consist of seven β-subunits (termed β 1–7). The proteolytic activity resides in the inner two β-rings. The two 19S lid complexes that bind to either side of the central complex restrict access to the proteolytic sites. Subunits of these lid complexes bind to ubiquitin chains on degradation substrates, release the ubiquitin chains, and allow the substrate to enter the central, proteolytic complex. Proteasomal degradation of target substrates results in short

Physiol Rev • VOL 90 • OCTOBER 2010 • www.prv.org
polypeptides that are released into the cytoplasm or the nucleus, which are then further degraded into amino acids by peptidases (171).

The UPS and autophagy have long been regarded as two separate cellular pathways with distinct functions. It was previously thought that autophagy predominantly degraded long half-life proteins and that the clearance of the short half-life proteins that were typical UPS substrates would not be affected by a compromise in autophagy. However, recent findings suggest that the two systems do “communicate,” at least under certain circumstances, and might even have compensatory functions in some cases. Indeed, short half-life proteins normally degraded by the UPS can be degraded by autophagy under certain conditions (117, 294), and long-lived proteins can also be degraded by the UPS (116). It is now clear that a number of proteins can be degraded by both autophagy and the proteasome (71, 81, 225, 559). Furthermore, mutations that interfere with the proteasomal degradation of a protein may increase the dependency of such proteins on autophagy for their degradation, as this will then become the default clearance route. This is especially true for mutated proteins with an increased aggregation tendency, as oligomeric and higher-order structures will become inaccessible to the narrow proteasome barrel.

Numerous studies in yeast and mammalian cells, including primary neurons, have reported that proteasome inhibition leads to the upregulation of autophagy (49, 88, 89, 193, 418). This cross-talk may be mediated by JNK1 activation following proteasome inhibition, which would be predicted to induce autophagy via Bcl-2 phosphorylation, thereby reducing its ability to bind and inhibit the function of Beclin 1 (337, 389, 562). In vivo data further support these findings: impairment of the proteasome leads to neurodegeneration in the fly eye. This phenotype is enhanced when essential autophagy genes are knocked down, and rescued by autophagy induction with rapamycin (380).

While proteasome inhibition may induce autophagy, the converse does not occur. Indeed, inhibition of autophagy leads to impaired degradation of substrates destined for the proteasome (255). This is dependent on the ubiquitin-binding protein p62, which is an autophagy substrate that accumulates in autophagy-deficient cells (25, 250). The resulting elevated levels of p62 appear to compromise the UPS by delaying the delivery of ubiquitinated substrates to the proteasome, by sequestering them away from other ubiquitin-binding proteins (e.g., p97). Therefore, prolonged inhibition of autophagy would also result in the reduced flux through UPS; this may be relevant to the interpretation of studies examining the effects of autophagy knockout in various tissues, since some of the effects may be secondary consequences of UPS compromise (e.g., p53 elevation and apoptosis) (306, 310).

An interesting coordinate regulation of autophagy and the UPS appears to occur during muscle atrophy. In this condition, both proteasomal and autophagic protein degradation are upregulated, and this upregulation is coordinated by the transcription factor FoxO3 (600). In this case, FoxO3 appears to mediate independent effects on autophagy and the UPS (600).

Recently, a growing amount of data have drawn attention to p62 and its possible role in connecting autophagy with the UPS. p62 is cleared by both the UPS and autophagy (250, 255) and is commonly detected in ubiquitin-containing protein aggregates associated with various neurodegenerative diseases (25, 91, 268–270, 352). p62 polymerizes via its NH2-terminal PB1 domain and binds to polyubiquitin chains via its COOH-terminal UBA domain. In vitro data reveal that p62 interacts with LC3, and it has been suggested that this may facilitate the autophagic clearance of ubiquitin-positive protein aggregates (25, 250, 381). The strongest support for this hypothesis comes from studies using an artificially ubiquitinated peroxisomal integral membrane protein, where it was demonstrated that a single ubiquitin molecule is sufficient as a degradation signal, in contrast to the tetra-ubiquitin chain that is necessary to signal for the degradation of proteasome clients (231). However, definitive data supporting this mechanism for the clearance of endogenous proteins are still awaited. p62 is unlikely to be necessary for the clearance of most autophagic substrates, as there does not appear to be a defect in bulk autophagy in p62 knockout mice (250). Another LC3 interactor with many functional similarities to p62 has recently been identified [NBR1 protein (neighbor of BRCA1 gene 1)], and it is possible that there may be redundancy for their putative roles in autophagy (236–238, 273). Also, HDAC6, which also binds ubiquitin and interacts directly with microtubules, is involved in autophagic degradation of mutant proteins during proteasomal inhibition (193, 380). A recent study reported that degradation of polyubiquitinated proteins by the proteasome or autophagy is mediated by BAG1 and BAG3, respectively. A switch from BAG1 to BAG3 in aged cells suggests that autophagy is used more extensively for the turnover of polyubiquitinated proteins (125). Although ubiquitinated proteins are autophagy substrates, more work is needed to understand the exact role of ubiquitination as a signal for selective autophagy.

B. ERAD and Autophagy

Most secreted and transmembrane proteins in eukaryotic cells are folded and modified in the ER before they are further transported towards their destination. Proteins that fail to fold properly in the ER initially receive aid by an intricate system of molecular chaperones. However, proteins that do not fold at this stage are ret-
rot translocated to the cytoplasm and targeted for proteasomal degradation via cytosolic polyubiquitination, a process called ER-associated degradation (ERAD).

When misfolded proteins are not exported efficiently to the cytoplasm and accumulate in the ER, the UPR is often induced. Three main pathways are activated to alleviate the accumulation of proteins in the ER. 1) Inositol-requiring protein-1 (IRE1) oligomerizes in the stressed ER and via X-box binding protein-1 (XBP1) activates the transcription of UPR genes, whose products are involved in lipid synthesis, or ERAD, or code for chaperones. 2) ER stress leads to the processing of activating transcription factor-6 (ATF6). The resulting ATF6 fragment translocates to the nucleus and activates UPR genes. 3) Protein kinase RNA (PKR)-like ER kinase (PERK) oligomerizes under ER stress and phosphorylates the α-subunit of eukaryotic translation initiation factor-2 (eIF2α), thus inhibiting translation, thereby decreasing the ER protein load. Additionally, PERK activates UPR gene transcription (423, 548).

Many studies have reported that ER stressors, like DTT, tunicamycin, or thapsigargin, or proteasome inhibitors induce the formation of autophagosomes (19, 89, 180, 257, 367, 433, 583). The activation of autophagy under ER stress may have a cytoprotective effect, as genetic or chemical inhibition of autophagy leads to a higher susceptibility towards cell death (19, 89, 367). The molecular pathway that is responsible for the induction of autophagy after ER stress is not entirely clear. Different data suggest an involvement of IRE1, signaling via JNK-1 but not XBP1, and independence of PERK1 and ATF6 (89, 367). The involvement of JNK-1 is particularly interesting, as phosphorylation of Beclin 1 by this kinase is essential for the induction of autophagy under starvation conditions (562). Another mechanism may involve Death-associated protein kinase 1 (DAPK1) activation by ER stress leading to Beclin 1 phosphorylation, which induces autophagy by reducing Beclin 1 binding to Bcl-xL (142, 592). On the other hand, Kouroku et al. (257) demonstrated that ER stress caused activation of autophagy via PERK1 and eIF2α. Calcium release due to ER stress has also been implicated in the pathway leading to autophagy induction (180, 433). However, some of these calcium studies were based on experiments using thapsigargin, which may have incorrectly interpreted that the increased numbers of autophagosomes occurring after thapsigargin treatment were the result of increased autophagosome synthesis. It appears that thapsigargin causes a calpain-dependent reduction in autophagosome synthesis along with a block in autophagosome-lysosome fusion. So, although this compound increases autophagosome numbers (due the block in autophagosome-lysosome fusion), its overall effects are to impede autophagic flux and the clearance of autophagic substrates (566).

C. CMA and Autophagy

In addition to the relationships with the UPS, (macro)autophagy is also modulated by the activity of CMA. Deletion of the lysosomal receptor LAMP-2a, a key component of CMA, induces macroautophagy (321, 322). Whereas macroautophagy is a relatively unspecific degradation pathway, CMA is highly specific. Thus it is somewhat surprising that autophagy-incompetent cells activate CMA (221), as this pathway requires a specific pentapeptide signal in its substrates. While only 30% of the cytoplasmic proteins carry the required signal (56), enhanced degradation of these proteins may be sufficient to reduce the overall load of proteins and thereby alleviate cell stress.

V. SIGNALING PATHWAYS REGULATING MAMMALIAN AUTOPHAGY

Several signaling pathways seem to regulate autophagy in mammalian cells. Similar to yeast, the classical pathway involves the serine/threonine kinase, mammalian target of rapamycin (mTOR). The induction of autophagy by mTOR inhibition under starvation conditions is a well-known phenomenon in mammalian cells (509). Various pathways and small molecules regulating autophagy via mTOR and mTOR-independent mechanisms, which have been identified in recent years, are described below.

A. mTOR Pathway

The mTOR pathway is the most studied pathway regulating mammalian autophagy (Fig. 2). mTOR [also known as rapamycin and FKBP12 target-1 (RAFT1), rapamycin target-1 (RAP1), FKBP12-rapamycin-associated protein (FRAP)] is an important signaling molecule that regulates diverse cellular functions, such as initiation of mRNA translation, cell growth and proliferation, ribosome biogenesis, transcription, cytoskeletal reorganization, long-term potentiation, and autophagy (445). mTOR is the mammalian ortholog of the yeast protein kinase TOR that negatively regulates autophagy (291). The mTOR pathway involves two functional complexes: a rapamycin-sensitive mTOR complex 1 (mTORC1), which regulates autophagy, consisting of the mTOR catalytic subunit, raptor (regulatory associated protein of mTOR), GβL (G protein β-subunit-like protein; also known as mLST8), and PRAS40 (proline-rich Akt substrate of 40 kDa); and mTOR complex 2 (mTORC2) consisting of mTOR, rictor (rapamycin-sensitive companion of mTOR), GβL, SIN1 (SAPK-interacting protein 1), and PROTOR (protein observed with rictor) that is not a direct autophagy regulator (145, 581).
Many diverse signals, such as growth factors, amino acids, glucose, energy status, and different forms of stress, regulate the raptor-mTOR (mTORC1) pathway (445). The activity of mTORC1 can be inhibited by rapamycin (sirolimus), a lipophilic macrolide antibiotic first isolated from *Streptomyces hygroscopicus* (145, 365). Rapamycin is a potent autophagy inducer in various cell lines from yeast to mammalian cells, including neurons (28, 365, 415, 416, 427). In mammalian cells, rapamycin forms a complex with the immunophilin FK506-binding protein of 12 kDa (FKBP12), which then recruits phosphoinositide-dependent kinase 1 (PDK1) and Akt to the cell membrane. Akt is then phosphorylated and activated, which in turn phosphorylates and inactivates tuberous sclerosis complex (TSC) 1/2, leading to activation of Rheb and consequently mTORC1. Akt can also be phosphorylated and activated by mTORC2. AMPK, a cellular energy sensor of changes in the intracellular ATP/AMP ratio, directly phosphorylates TSC2, thereby providing the priming phosphorylation for subsequent phosphorylation of TSC2 by glycosynthase kinase 3 (GSK3) to inhibit mTORC1 signaling. The mTOR pathway involves two functional complexes: a rapamycin-sensitive mTORC1 that regulates autophagy, consisting of the mTOR catalytic subunit, raptor, mLST8, and PRAS40; and mTORC2 comprising of mTOR, rictor, mLST8, SIN1, and PROTOR. Amino acids activate mTORC1 via Rag GTPases and suppress autophagy. The rate-limiting factor of amino acid signaling is L-glutamine, which is initially taken up by its high-affinity transporter SLC1A5, followed by its efflux by the SLC7A5/SLC3A2 bidirectional transporter in exchange for uptake of essential amino acids, which subsequently activates mTORC1. The ULK1-Atg13-FIP200 complex acts as an integrator of the autophagy signals downstream of mTORC1. Under nutrient-rich conditions, mTORC1 suppresses autophagy by interacting with this complex and mediating phosphorylation-dependent inhibition of Atg13 and ULK1. Under starvation conditions or rapamycin treatment, mTOR dissociates from the complex, resulting in dephosphorylation-dependent activation of ULK1 and ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself, which triggers autophagy. The mTORC1 pathway regulates cell growth mainly through 4E-BP1 and p70S6K. Phosphorylation-dependent activation of p70S6K can also inhibit IRS1, thereby exerting a feedback loop mechanism.
also known as PHAS-1) at Thr389/Thr421/Ser424 and Thr37/Thr46, respectively (242, 445, 464). Interestingly, a study by Scott et al. (471) showed that p70S6K is a positive regulator of autophagy. When TOR is inactivated during starvation-induced autophagy in the Drosophila fat body, p70S6K needs to be activated for some time to allow maximal autophagy, after which loss of p70S6K activity prevents excessive autophagy in TOR-inactive state, probably by inhibiting the PI3K pathway through a negative-feedback loop involving downregulation of the insulin receptor substrates (IRS) (243, 471). Furthermore, p70S6K activation appears to be necessary but not sufficient to induce autophagy, as it activates autophagy in TOR mutants but not in wild-type Drosophila (471). Whether these effects of p70S6K on autophagy are seen in mammals is unclear.

A recent study indicates that rapamycin does not inhibit mTORC1 completely, due to rapamycin-resistant functions of mTORC1. Instead, a selective ATP-competitive small molecule mTOR inhibitor, Torin1, was found to induce autophagy to a much greater extent than rapamycin (528). Although Torin1 inhibits both mTORC1 and mTORC2 directly, its effects on autophagy are mTORC2 independent but mTORC1 dependent due to suppression of rapamycin-resistant functions of mTORC1 that are required for inhibition of autophagy (528).

Other conditions, such as nutrient deprivation, also stabilize the raptor-mTOR complex, thereby inhibiting mTORC1 and inducing autophagy (228, 242, 332). Under conditions of limited extracellular amino acids, autophagy recycles intracellular constituents to provide an alternative source of amino acids (242). Conversely, diverse extracellular signals, such as amino acids and growth factors, activate mTORC1 and suppress autophagy (145, 242, 332). Recently, the rate-limiting factor that enables essential amino acids to inhibit mTORC1 has been identified as L-glutamine (360). The influx of L-glutamine by its high-affinity transporter SLC1A5 initially increases its intracellular concentration. The heterodimeric SLC7A5/SLC3A2 bidirectional transporter then uses L-glutamine as an efflux substrate in exchange for the cellular uptake of essential amino acids, which subsequently activates mTORC1 (360). However, the role of glutamine may be tissue specific, since this amino acid stimulates autophagy in intestinal cells (434). The Rag GTPases mediate amino acid signaling to mTORC1 activation by binding to raptor and further redistributing mTOR to a subcellular compartment containing its activator rheb (440). A recent report suggests that amino acids induce the translocation of mTORC1 to lysosomal membranes where the Rag GTPases reside. This recruitment of mTORC1 requires a trimeric Ragulator complex containing the proteins encoded by the MAPKSP1, ROBLD3, and c11orf59 genes. Thus, in the presence of amino acids, the Rag GTPases that are tethered to lysosomal surface by the Ragulator act as a docking site for mTORC1, which is then activated by Rheb (439).

The other mTOR complex, mTORC2, which phosphorylates (Ser-473) and activates Akt, is known to be rapamycin insensitive (195, 444). However, recent studies have shown that prolonged treatment with rapamycin inhibits mTORC2 activity and Akt signaling in certain mammalian cell types (446, 595). Rapamycin derivatives such as temsirolimus (CCI-779) and everolimus (RAD001) inhibit the activity of mTORC1, thereby reducing the phosphorylation of p70S6K and 4E-BP1 (595).

Many of the molecular mechanisms linking TOR signaling to the autophagic machinery have been dissected in yeast, where the Atg1 kinase and the Atg1-Atg13-Atg17 complex are thought to act downstream of TOR regulating autophagosome formation (212, 573). Recent studies have identified some of the molecular components downstream of mTORC1 in mammalian autophagy. Atg13 binds to the mammalian Atg1 homologs ULK1 or ULK2 and mediates the interaction of ULK1/2 with FIP200, thereby forming a ULK1/2-Atg13-FIP200 stable complex that signals to the autophagic machinery downstream of mTOR (126, 178, 209). Under nutrient-rich conditions, mTORC1 suppresses autophagy through direct interaction with this complex and mediates phosphorylation-dependent inhibition of the kinase activities of Atg13 and ULK1. Under starvation conditions or rapamycin treatment, mTOR dissociates from the complex, resulting in the inhibition of mTOR-mediated phosphorylation of Atg13 and ULK1. This leads to dephosphorylation-dependent activation of ULK1 (and ULK2) and ULK1-mediated phosphorylations of Atg13, FIP200, and ULK1 itself, which triggers autophagy (126, 178, 209). Therefore, the ULK1-Atg13-FIP200 complex acts as an integrator of the autophagy signals downstream of mTORC1. However, it is not clear yet how phosphorylation of these proteins regulates their activities.

B. PI3K Pathway

A major signaling cascade controlling mTORC1 is the PI3K pathway (Fig. 2) (242). The binding of growth factors or insulin to cell surface receptors activates the class IA PI3K, wherein its regulatory subunit mediates activation of its p110 catalytic subunit by the direct interaction with phosphorytrosine residues of the activated receptors or adaptor proteins. Activated PI3K then converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$), which in turn recruits pleckstrin homology (PH) domain proteins, such as the serine/threonine kinases phosphoinositide-dependent kinase 1 (PDK1) and Akt/ PKB, to the plasma membrane (40). Akt is activated by its phosphorylation on two different sites. The rictor-mTOR
complex phosphorylates Akt on Ser-473, which may facilitate the phosphorylation by PDK1 of the activation loop of Akt on Thr-308. Akt has been shown to modulate autophagic activity regulated by the PI3K pathway. Mutations causing Akt activation or inactivation cause suppression and induction of autophagy, respectively (80).

The different classes of PI3K have distinct effects on autophagy (334). Increases in the class I PI3K product, PIP₃, inhibit autophagy by inducing Akt activation (391), whereas hyperactivity of the phosphoinositide phosphatase PTEN (phosphatase and tensin homologue deleted from chromosome 10), a tumor suppressor, stimulates autophagy by inhibiting the PI3K pathway (6). In contrast, increasing the levels of the class III PI3K product, PI-3-P, stimulates autophagy (391). Autophagy can be downregulated by inhibitors of PI3K activity, such as 3-MA, wortmannin, and 2-(4-morpholinyl)-8-phenylchromone (LY294002) (26, 391, 472).

The phosphorylation-dependent Akt activation results in the phosphorylation of a host of other proteins, including the tumor suppressor proteins mutated in tuberous sclerosis which form a complex called the tuberous sclerosis complex (TSC), consisting of a heterodimer of TSC1 (hamartin) and TSC2 (tuberin). TSC1/2 affect cell growth and survival (40, 445). TSC1/2, an upstream integrator of various signals regulating the mTORC1 pathway, is the GTPase-activating protein (GAP) for the Ras family GTP-binding protein rhes, which directly binds and activates the raptor-mTOR complex (130, 315, 521). Several other kinases, including AMP-activated kinase (AMPK), ribosomal S6 kinase 1 (Rsk1), and extracellular signal regulated kinase 1/2 (ERK1/2), signal to mTORC1 by phosphorylating TSC2 and inhibiting the activity of the TSC1/2 heterodimer (445).

Apart from amino acids, mTORC1 can be a sensor of changes in the cellular energy state via AMPK (334). AMPK, which senses changes in the intracellular ATP/AMP ratio, directly phosphorylates TSC2, thereby providing the priming phosphorylation for subsequent phosphorylation of TSC2 by glycogen synthase kinase 3 (GSK-3) to inhibit mTOR signaling (186). We have recently shown that inhibition of GSK-3β suppresses autophagy by activating mTOR (449). While AMPK activation inhibits TOR and stimulates autophagy in yeast, the AMPK activator AICAR inhibits autophagy in mammalian cells, but this is probably not due to direct effects on AMPK (333). Recent evidence indicates that AMPK is required for mammalian autophagy, as inhibition of AMPK activity by compound C or a dominant negative construct strongly suppresses autophagy (333, 336). AMPK also mediates hypoxia-induced autophagy by downregulating mTOR (382). Both LKB1 and Ca⁺⁺/calmodulin-dependent protein kinase kinase-β (CaMKKβ) play important roles in the phosphorylation and activation of AMPK. In addition, AMPK can be activated by other upstream kinases, but the physiological significance of this is currently unclear.

C. Other Protein Kinases

Apart from mTOR and certain protein kinases like AMPK discussed above, several other kinases have been reported to regulate autophagy. Physiological or pharmacological stimulation of IKK (IκB kinase) induces autophagy via AMPK activation and mTOR inhibition, but in an NFκB-independent manner (68). Eukaryotic translation initiation factor 2α (eIF2α) kinases, which are serine/threonine kinases that regulate stress-induced translational control programs, are also involved in the regulation of stress-induced autophagy. The mammalian eIF2α kinase signaling pathway is essential for both virus- and starvation-induced autophagy, which is antagonized by the herpes simplex virus (HSV)-encoded neurovirulence gene product ICP34.5 (511). Eukaryotic elongation factor-2 (eEF-2) kinase, also referred to as Ca⁺⁺/calmodulin-dependent kinase III, also regulates autophagy via the mTOR pathway. Inhibition of eEF-2 kinase inhibits autophagy. The activity of eEF-2 kinase is enhanced in glioblastoma and other malignancies. Nutrient deprivation, which decreases the activity of S6 kinase, was shown to enhance the activity of eEF-2 kinase activity (569).

The nitrogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, which is commonly activated in cancers, regulates autophagy. Carcinogens such as lindane, which activates MAPK/ERK pathway, impair autophagy (62). However, starvation-induced autophagy activates the ERK1/2 MAPK pathway, and inhibition of the pathway by a MAPK kinase (MAPKK) MEK1/2 inhibitor (PD-98059) suppresses starvation-induced autophagy in human colon cancer HT-29 cell line (371). A recent study shows that sustained MEK/ERK activation results in complete disassembly of both mTORC1 and mTORC2, strongly enhancing Beclin 1 activity, resulting in cytodestruction, while moderately enhanced Beclin 1 activity, resulting in cytoprotection, is mediated via transient activation of MEK/ERK (556). Cogorno and colleagues (369) have shown that a cytoplasmic heterotrimeric Gₛ₃ protein regulates autophagy in a human colon cancer HT-29 cell line. Autophagy is minimal when the Gₛ₃ protein is in the GTP-bound form and becomes stimulated when GDP is bound to the Gₛ₃ protein (370). A similar function of Gₛ₃ protein in the regulation of the anti-autophagic activity of insulin in the liver has also been reported (138). Likewise, Go-interacting protein (GAIP), which is a regulator of G protein signaling (RGS) protein and activates the hydrolysis of GTP by the Gₛ₃ protein, increases autophagy (372). Activation of ERK1/2 stimulates autophagy by phosphorylating GAIP, which stimulates its GTPase activity towards the GTP-
bound conformation of the G_{i3} protein. Phosphorylation of GAIP by the ERK1/2 MAPK-dependent pathway is sensitive to amino acids, since the inhibition of autophagy by amino acids correlates with the inhibition of the ERK1/2 MAP kinases and a low level of GAIP phosphorylation (371). Amino acids interfere with ERK1/2-dependent autophagy by inhibiting the activation of the kinase Raf-1 (388).

Autophagy is also modulated by cell hydration, which is sensitive to a p38 MAPK inhibitor (SB203580) that prevented autophagy inhibition, implicating a role of p38 MAPK in autophagy (167, 555). Accumulation of mutant glial fibrillary acidic protein (GFAP) in Alexander disease induces autophagy by activating p38 MAPK and inhibiting mTOR signaling pathways (514). Recent studies show that the negative regulation of autophagy by p38α was due to a direct competition with mAtg9 for binding to p38-interacting protein (p38IP) (561).

Recently, the stress-activated signaling molecule, c-Jun NH\_2-terminal kinase 1 (JNK1), but not JNK2, was shown to be an important mediator of starvation-induced autophagy in mammalian cells. Starvation signal activates JNK1, which in turn phosphorylates Bcl-2 at multiple sites (Thr-69, Ser-70, and Ser-87) in the unstructured loop, thereby disrupting the Bcl-2-Beclin 1 complex and triggering autophagy (562).

D. mTOR-Independent Pathways

Apart from the classical mTOR pathway, we have recently described mTOR-independent pathways regulating autophagy in mammalian systems (Fig. 3) (451).

1. Phosphoinositol signaling pathway

The first evidence for the existence of mTOR-independent regulation of mammalian autophagy comes from
studies showing that autophagy is negatively regulated by intracellular inositol and inositol 1,4,5-trisphosphate (IP$_3$) levels (448). This pathway is stimulated by G protein-coupled receptor-mediated activation of phospholipase C (PLC), which hydrolyzes PIP$_2$ to form IP$_3$ and diacylglycerol (DAG) (22). IP$_3$ functions as a second messenger and binds to its receptors (IP$_3$R) on the ER, thereby releasing the stored ER Ca$^{2+}$ into the cytoplasm, which then elicits a range of cellular responses (23). IP$_3$ is degraded by a 5'-phosphatase and inositol polyphosphate 1-phosphatase (IPPase) to form inositol monophosphate (IP$_1$) (313). Inositol monophosphatase (IMPase) catalyzes the hydrolysis of IP$_1$ into free inositol, which is required for the phosphoinositol signaling (311).

We have previously shown that mood-stabilizing drugs, like lithium, carbamazepine, and valproic acid, all of which reduce intracellular inositol levels (568), induce autophagy (448). Lithium (Li$^+$) inhibits a group of Mg$^{2+}$-dependent phosphomonoesterase enzymes, IMPase and IPPase, by competing with the cofactor Mg$^{2+}$ (154, 185, 425). Its inhibitory effect, primarily on IMPase, prevents inositol recycling, leading to depletion of the cellular inositol pool and inhibition of the phosphoinositol cycle (21). A specific competitive IMPase inhibitor, L-690,330, mimics the autophagy-inducing effects of lithium (448). On the other hand, valproic acid reduces inositol levels by inhibiting myo-inositol-1-phosphate (MIP) synthase that catalyzes the rate-limiting step of inositol biosynthesis (475). Autophagy induction by the inositol-lowering agents are likely to be mediated at the level of, or downstream of, reduced IP$_3$, as they are abolished by pharmacological treatments that increase IP$_3$ levels, such as myo-inositol or an inhibitor of prolyl oligopeptidase (448). Furthermore, overexpression of cytosolic IP$_3$ kinase A, which lowers IP$_3$ levels by phosphorylating it to inositol 1,3,4,5-tetrakisphosphate (IP$_4$), induces autophagy (566). IP$_3$-regulated autophagy is mTOR independent, and rapamycin has no effects on IP$_3$ levels, implicating distinct regulation of mammalian autophagy by mTOR-dependent and mTOR-independent pathways (448). IP$_3$R, as mentioned earlier, has been identified as a new regulator of the Beclin 1 complex. Xestospongin B, an IP$_3$R antagonist, induces autophagy by disrupting the interaction of Beclin 1 and IP$_3$R/Bcl-2 complex (552).

2. cAMP-Epac-PLC-ε-IP$_3$ pathway

We have recently described various components of an mTOR-independent autophagy pathway in a screen with United States Food and Drug Administration-approved drugs and pharmacological probes, where the cAMP-Epac-PLC-ε-IP$_3$ and Ca$^{2+}$-calpain-G$\alpha_\alpha$ pathways link in a cyclic fashion (451, 566). These pathways have multiple targets for chemically or genetically inducing autophagy, independent of mTOR activity (Fig. 3).

An upstream signaling cascade influencing IP$_3$ levels is the cAMP-Epac-PLC-ε pathway. Autophagy is negatively regulated by elevated intracellular cAMP levels, which can be reduced by chemical agents to stimulate autophagy (365, 566). Pharmacological treatment with imidazoline-1 (I1R) receptor agonists, such as clonidine and rilmenidine, or the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine, induce autophagy by reducing cAMP levels (566). The two major targets of cAMP are Epac, which is a guanine nucleotide exchange factor, and protein kinase A (PKA) (254). The negative regulation of autophagy by cAMP was found to be mediated via Epac, and not through PKA. Activation of Epac by elevated cAMP levels activates Rap2B, a small G protein that subsequently activates PLC-ε (222, 566). Induction of autophagy by dominant-negative Rap2B can abolish the inhibitory effects of an Epac-specific cAMP analog, implicating that Rap2B is a downstream autophagy regulator of Epac (566). Furthermore, overexpression of PLC-ε, a Rap2B effector, which hydrolyzes PIP$_2$ to form IP$_3$, results in the accumulation of autophagy substrates like aggregate-prone intracytoplasmic proteins, mimicking what would be seen when autophagy is inhibited (566). Conversely, overexpression of cytosolic IP$_3$ kinase A, which catalyzes the phosphorylation of IP$_3$, generating IP$_4$, decreased the accumulation of such autophagy substrates. Thus the link from cAMP via Epac to PLC-ε culminating on the generation of IP$_3$ is consistent with our earlier findings, where pharmacological manipulations that result in elevated intracellular IP$_3$ levels were shown to negatively regulate autophagy (448).

3. Ca$^{2+}$-calpain-G$\alpha_\alpha$ pathway

Stimulation of autophagy by reduced intracellular IP$_3$ levels is likely due to inactivation of the signal for ER Ca$^{2+}$ release, since an increase in cytosolic IP$_3$ levels results in IP$_3$ binding to its receptors (IP$_3$R) on the ER to trigger depletion of the ER Ca$^{2+}$ store and increased intracytosolic Ca$^{2+}$ (21). Indeed, it has been recently shown that genetic knockdown or pharmacological inhibition of IP$_3$R induces autophagy (67). Moreover, elevated intracytosolic Ca$^{2+}$ levels negatively regulate autophagy, which was first reported by Seglen and co-workers (141). Chemical agents like thapsigargin (an ER Ca$^{2+}$/Mg$^{2+}$-ATPase inhibitor that releases Ca$^{2+}$ from ER stores) or ionomycin (Ca$^{2+}$ ionophore that releases Ca$^{2+}$ from intracellular stores), which increase intracytosolic levels of Ca$^{2+}$, inhibit autophagy (141). Data from our screen hits reveal that L-type Ca$^{2+}$ channel antagonists, such as verapamil, loperamide, amiodarone, nimodipine, and nitrendipine, which prevent the influx of extracellular Ca$^{2+}$ by inhibiting the L-type Ca$^{2+}$ channels on the plasma membrane and thereby decreasing intracytosolic Ca$^{2+}$ levels, induce autophagy. Conversely, the L-type Ca$^{2+}$ channel...
agonist (±)-BAY K 8644, which increases intracytosolic Ca^{2+}, inhibits autophagy (566). Some of the Ca^{2+} channel blockers found in our screen as autophagy inducers were also reported by Yuan and colleagues (599) in an independent screen. Furthermore, modulation of cytosolic Ca^{2+} levels by K_{ATP} channel openers, such as minoxidil, decrease whole cell L-type Ca^{2+} channel currents and induce autophagy (566).

Elevated intracytosolic Ca^{2+} activates a family of Ca^{2+}-dependent cysteine proteases called calpains, including the ubiquitously expressed calpain 1 (μ-calpain) and calpain 2 (m-calpain) (139). Calpain activation inhibits autophagy, whereas chemical inhibition of calpain by calpastatin and calpeptin, or genetic knockdown, induces autophagy (566). The effects of L-type Ca^{2+} channel activators can be abrogated by reducing calpain activity, suggesting that calpain is acting downstream of the autophagy-regulatory effects of free cytosolic Ca^{2+} (566). Calpain activation inhibits autophagy by cleaving the α-subunit of heterotrimeric G proteins (G_{α}α) (566), which results in constitutive activation of these proteins, leading to greater adenyl cyclase activation and subsequent cAMP production (455). Chemical or genetic inhibition of G_{α}α increases autophagy, which also abrogates calpain-induced autophagy impairment. Activation of G_{α}α with its natural ligand, pituitary adenyl cyclase-activating polypeptide (PACAP), or calpain activation, suppress autophagy, and this inhibitory effect is abolished by an adenyl cyclase inhibitor 2',5'-dideoxyadenosine that reduces cAMP levels (566). This creates a potential cyclic pathway where calpain regulates autophagy through G_{α}α that signals via the cAMP-Epac-PLC-ε-IP_{3} pathway, which modulates calpain activity by influencing Ca^{2+} levels (Fig. 3) (566). It is yet to be elucidated how this mTOR-independent cyclic pathway signals to the autophagic machinery, although this pathway may have several branching points that link to autophagosome formation. Other Ca^{2+}-mobilizing approaches (31, 128, 141) do not, however, exert the same effect on calpains and have been reported to induce autophagy. It is possible that cytosolic and organellar Ca^{2+} differentially regulate autophagy, and calpain activation depends on Ca^{2+} signals in a spatially restricted subcellular domain (181).

E. Other mTOR-Independent Autophagy Regulators

Several other mTOR-independent small molecule modulators of mammalian autophagy have been reported, whose targets are currently unknown (451, 452). We recently identified a number of small molecule autophagy regulators by a chemical screening approach (450). A primary screen in yeast with 50,729 compounds revealed various enhancers [small-molecule enhancers of rapamycin (SMERs)] and suppressors [small-molecule inhibitors of rapamycin (SMIRs)] of the cytostatic effects of rapamycin in yeast. A secondary screen in mammalian cells with the SMERs and SMIRs, tested independently without rapamycin, identified 3 SMERs and 13 SMIRs that induced or inhibited autophagy, respectively. Further screening of the structural analogs of these SMERs has identified 18 additional candidates that induce autophagy (450).

Trehalose is another effective mTOR-independent autophagy enhancer (447), which is a disaccharide and also functions as a "chemical chaperone" due to its ability to influence protein folding through direct protein-trehalose interactions (50).

1. Additive induction of autophagy by mTOR and mTOR-independent pathways

We have recently shown that autophagy can be upregulated additively by simultaneously using mTOR inhibitors and mTOR-independent autophagy enhancers (451). Although lithium induces mTOR-independent autophagy by inhibiting IMPase, another intracellular target of lithium, GSK-3β suppresses autophagy by activating mTOR (186, 448, 449). To counteract the autophagy-inhibitory effects of mTOR activation resulting from lithium treatment due to GSK-3β inhibition, the use of rapamycin in combination with lithium enables greater upregulation of autophagy by simultaneously inducing autophagy through mTOR inhibition and lowering IP_{3} levels (449).

Several other mTOR-independent small molecule autophagy enhancers have been shown to additively upregulate autophagy in combination with rapamycin, such as trehalose, SMERs, IMPase inhibitor L-690,330, and the calpain inhibitor calpastatin (447–452, 566). Such combination treatments facilitate greater upregulation of autophagy, compared with autophagy induction by each of the compounds alone.

F. Ceramides and Other Bioactive Sphingolipids Regulate Autophagy

Another class of molecules that regulate autophagy is the sphingolipids, a major constituent of biological membranes, some of which play an important role in cell signaling as second messengers. A large number of sphingolipid subspecies in the cells influence membrane structure, interactions with the extracellular matrix and neighboring cells, vesicular trafficking, and the formation of specialized structures such as phagosomes and autophagosomes (143, 460). Thus sphingolipids are emerging as major players in several aspects of cell physiology and in the pathology of many diseases including cancer and Alzheimer’s disease (143, 460). Many categories of sphingolipids have been shown to be abnormal in cancer (151), and these can coordinate signals from the external envi-
The metabolism of sphingolipid is a highly dynamic process generating second messengers, including ceramide, sphingosine, and sphingosine 1-phosphate (SIP) (160, 288, 327, 373, 495). Resting cells typically have very low levels of sphingolipid metabolites. Metabolism and signaling are commonly initiated by activation of different forms of sphingomyelinases, enzymes that cleave the membrane lipid sphingomyelin to ceramide and phosphocholine. Ceramide is the central molecule in sphingolipid metabolism, with roles in cell growth, cell death, proliferation, and stress response (160, 288, 327, 495). Ceramide is produced by de novo synthesis in the ER or by the hydrolysis of sphingomyelin by acid sphingomyelinases (localized in acidic compartments) and neutral sphingomyelinases (localized in the plasma membrane and mitochondria). Ceramide is engaged in the biosynthesis of glucosylceramide and of sphingomyelin (160, 288, 327, 338). Ceramide can also generate ceramide 1-phosphate, sphingosine, and SIP. Ceramide is deacylated to generate sphingosine, which is phosphorylated by sphingosine kinases (SphK) to produce SIP (495). Cells maintain a dynamic equilibrium in the levels of ceramide, sphingosine, and SIP. It is now generally accepted that ceramide, sphingosine, and SIP have opposing effects in cellular stress responses (160, 288, 327, 373, 495). While ceramide and sphingosine are often associated with cell growth arrest and the induction of cell death, SIP promotes cell proliferation and cell survival (309). Thus the dynamic balance between ceramide, sphingosine, and SIP is important in determining whether cells survive or die (73, 495).

In many cell types, increased ceramide leads to cell growth arrest and apoptosis (159, 247, 287, 327). Ceramide increases are often necessary for apoptosis (197, 307, 383), and certain anticancer drugs induce apoptosis via increasing de novo synthesis of ceramide (20, 133, 134). Sphingosine is not produced by de novo biosynthesis; it is only formed by deacylation of ceramide (495). Sphingosine is a protein kinase C inhibitor (158), and increased sphingosine levels also inhibit cell growth and induce apoptosis (72). In contrast to ceramide and sphingosine, SIP promotes cell growth and inhibits apoptosis (405, 495, 496). Many external stimuli, particularly growth and survival factors, activate SphK, leading to an increase in SIP levels and a concomitant decrease in ceramide levels. Enzymes that interconvert ceramide, sphingosine, and SIP regulate the antagonistic effects of these metabolites. Thus conversion of ceramide and sphingosine to SIP simultaneously removes proapoptotic signals and creates a survival signal, and vice versa. This led to the proposal of a “sphingolipid rheostat,” according to which the relative amount of these antagonistic metabolites are critical in determining cell fate (73). In agreement, it has been shown that increased SIP protects against ceramide-induced apoptosis, and depletion of SIP enhances ceramide-induced apoptosis (73, 74, 100, 570).

The existence of this sphingolipid rheostat and its essential involvement in regulating cell growth, proliferation, stress responses, and cell death pathways has been further extended to encompass autophagy. The addition of exogenous ceramide can induce autophagy (77), and the autophagy induced by agents such as the estrogen receptor antagonist tamoxifen results in an increase in the endogenous ceramide levels. Therefore, endogenous ceramide can act as a mediator in the modulation of autophagy by other agents (458). Ceramide treatment results in downregulation of nutrient transporters in mammalian cells, thus interfering with the uptake of essential nutrients like amino acids (144). This could explain why amino acid starvation and ceramide treatment trigger autophagy via the same molecular switches. Exogenously added ceramide (C2-Cer) and elevation of endogenous levels of ceramide with tamoxifen increase Beclin 1 (458), an upstream regulator of autophagy (219), whereas suppression of Beclin 1 expression blunts the autophagic response to ceramide in a glioblastoma cell line. It is possible that ceramide facilitates the interaction of PI3K with upstream regulators of autophagy (594). Class I PI3K and PKB/Akt are suppressors of autophagy, and ceramide is a well-known activator of the phosphoprotein phosphatase 2A (PP2A), which, in turn, inhibits Akt activation (458, 467, 606). A short-chain permeable analog of ceramide (C2-Cer) increases the transcription of the BH3-only protein BNIP3 in two different malignant glioma cell lines (77). BNIP3 has been shown to positively modulate autophagy during hypoxia by displacing Bcl-2 from the Bcl-2/Beclin 1 complex (597). Since BNIP3 is also proapoptotic, its induction could result in activation of both autophagy and cell death. Finally, it has been recently reported that ceramide can facilitate the dissociation of the Bcl-2/Beclin 1 complex by inducing the JNK1-mediated phosphorylation of Bcl-2, mimicking the effect observed during nutrient starvation (387, 389, 562). Thus, in addition to the PP2A-mediated effect on the Akt/PKB pathway, induction of autophagy by ceramide seems to rely also on a dual mechanism converging on the dissociation of the Bcl-2/Beclin 1 complex (387, 389, 562).

Another backbone lipid metabolite implicated in autophagy is SIP (276). Overexpression of SphK1 promotes autophagy, which is not inhibited by the ceramide neosynthesis (276). Different from the ceramide-mediated autophagy, SphK1-mediated autophagy is associated neither with an increase in Beclin 1 protein nor with an inhibition of Akt or the class I PI3K (276). However, mTOR is inhibited by SphK1 overexpression, suggesting that SIP can modulate autophagy by inhibiting mTOR independently of the class I PI3K arm. In addition, the suppression of SphK1 activity increases apoptotic cell death, whereas SphK1 protects cells from apoptosis dur-
ing nutrient starvation (276). Starvation stimulates both autophagy and endogenous SphK activity. Interestingly, the stimulation of SphK activity during nutrient deprivation has also been reported in yeast, suggesting a general role for SphK during starvation in eukaryotic cells (275). Together, these findings suggest that modulation of autophagy adds a further layer of complexity to the roles exerted by SphK1 (SIP) in cell survival (73).

To summarize, the control of autophagy by ceramide and SIP suggests a novel cell function for the sphingolipid rheostat (73, 307). Both the tumor suppressor ceramide and the tumor promoter SIP can trigger autophagy but with different outcomes on cell survival and death. In cancer cells, the survival role of SphK1 (SIP)-induced autophagy and the role of ceramide in cell death with autophagic features are compatible with their functions as tumor-promoting and tumor-suppressing molecules, respectively (373). However, further investigations on the interplay between sphingolipid metabolism and autophagy are required, since a better understanding of these interrelationships could lead to new strategies for controlling autophagy in muscular and neurodegeneration, infection, cancer, and other diseases.

G. Reactive Oxygen Species and Autophagy

Autophagy is also modulated by ROS, the highly reactive molecules that result from the incomplete reduction of oxygen (52). The major (although not exclusive) source of ROS under physiological conditions is the electron transport chain, where ~1–3% of the oxygen used by mitochondria is converted to ROS (11). Generally speaking, the cell is well insulated against increases in ROS by a number of protective systems. When production of ROS outstrips degradation, oxidative stress is said to occur. The generally deleterious effect of ROS on intracellular structures and their association with disease (for example, neurodegeneration) means they have traditionally been considered as harmful (410, 536). However, ROS also have physiological roles in the cell, notably in signaling (75). ROS can have a wide range of reactions with target enzymes that may influence their activity and can also exert effects via redox-regulated transcription factors (10). Examples of redox regulation of enzyme activity include the activation of nuclear factor-κB (NFκB) following the activation of NFκB inducing kinase by H₂O₂ (295). Evidence has recently begun to emerge suggesting that some species of ROS are important signaling molecules that regulate autophagic activity.

Starvation (a potent inducer of autophagy) has been reported to increase levels of ROS in a PI3K-dependent manner, and treatment with antioxidants ameliorates the ability of starvation to induce autophagy (462). One way in which ROS may be acting to regulate autophagy is by the modulation of the action of Atg4 on Atg8. The lipidation of Atg8/LC3 is essential for the successful induction of autophagy. Atg4 both primes Atg8 by cleavage of its COOH terminal and also delipidates LC3-II on the cytosolic surface of autolysosomes, thus allowing it to be recycled. Whilst the former reaction does not appear to be redox dependent, Atg4 is inactive and unable to cleave Atg8 from membranes when in its oxidized state (462). It is therefore possible that under oxidative conditions Atg4 is oxidized and inactive, which allows Atg8 to lipidate and thus initiate autophagy, whilst reduced Atg4 is active favoring Atg8 delipidation. The redox regulation of Atg4 appears to be mediated by the oxidation of a specific residue, Cys81 (462). Whilst Atg4 may be important, it may not be the only pathway involved. Other evidence suggests that increased levels of ROS (in particular hydrogen peroxide) may result in increased autophagic activity in human cancer cell lines and that this might in part be related to increased expression of Beclin 1 (54). A recent study has shown that in response to elevated levels of ROS, cytoplasmic ATM (ataxia-telangiectasia mutated) is rapidly activated, resulting in downstream activation of the TSC2 tumor suppressor via the LKB1/AMPK pathway. This leads to inhibition of the mTORC1 complex to induce autophagy, suggesting that multiple pathways might exist for mTORC1 regulation and autophagy in response to distinct stimuli (2).

Starvation may not be the only stimulus that results in both increased ROS and autophagy. Hypoxic conditions can also induce ROS generation (10). Drugs, including a mannose-binding lectin, a celecoxib derivative, methamphetamine, and pilocarpine-induced status epilepticus have all been shown to induce both autophagy and ROS (42, 127, 253, 302). Interestingly, when increases in ROS were abrogated in these studies using antioxidant drugs as diverse as N-acetylcysteine, catalase, glutathione, melatonin, and vitamin E, induction of autophagy was also inhibited.

The increased autophagic activity in response to ROS generation by starvation is clearly a survival pathway. Other evidence implicating autophagy as a defense against oxidative stress includes the ability of autophagy to specifically degrade damaged mitochondria (“mitophagy”), the degradation of oxidized proteins by both chaperone-mediated autophagy and macroautophagy, and increases in ROS leading to both apoptosis and autophagy where autophagy appears to be protecting against cell death (226, 285, 574, 580). Conversely, autophagy has been associated with autophagic (“type 2”) cell death in response to oxidative stress, for example, following focal cerebral ischemia, and in these circumstances inhibiting autophagy with drugs or knocking down genes essential for autophagy can decrease cell death (53, 90, 239, 564). In the context of apoptosis, inhibition autophagy has been shown to mediate an increase in ROS via the selective

Physiol Rev • VOL 90 • OCTOBER 2010 • www.prv.org
autophagic degradation of catalase (587). Thus, in certain conditions, autophagy upregulation may cause an increase in ROS, rather than being a result of it, and indeed, the increase in ROS following starvation may be at least partially autophagy dependent (462). In such circumstances, it is possible a positive-feedback loop may occur where autophagy is stimulated in the presence of increased ROS which in turn leads to the degradation of catalase and a further increases in ROS and autophagy (587).

Increasing interest in the relationship between ROS, apoptosis, and autophagy has led to some intriguing new hypotheses. These include the idea that mitochondria may provide the organizing sites for autophagosome biogenesis and may even be the source of the autophagosomal membrane (461). Improved understanding of the relationship between ROS and autophagy will help us to appreciate the interactions between these important cellular processes but may also have therapeutic applications. Examples include neurodegenerative diseases, where autophagy and oxidative stress are both important pathways (and have been used as therapeutic targets), or in cancer therapy where selective differences in the sensitivity of malignant cells to autophagy and ROS might be exploited in treatment (10, 457).

VI. AUTOPHAGY, METABOLISM, CELL DEATH, DEVELOPMENT, AND AGEING

A. Autophagy, Autophagic Cell Death, and Apoptosis

Autophagy is essential for maintaining homeostasis, which involves protein degradation for energy needs, and for the removal of damaged substrates for de novo biosynthesis. As a result, autophagy is important in promoting cell survival in different conditions, such as protein aggregate-induced stress, nutrient and growth factor deprivation, ER stress, and pathogen infection. Indeed, autophagy blockade sensitizes cells to a range of proapoptotic stimuli, particularly those acting on the intrinsic (mitochondrial-dependent) apoptosis pathway (30, 414). Some of these effects may be attributable to secondary effects of long-term autophagy blockade, like p62 accumulation slowing the delivery of ubiquitinated substrates to the proteasome leading to an accumulation of proteins like p53 (255). However, both genetic and chemical inducers of autophagy acting on mTOR-dependent and -independent pathways also protect against subsequent proapoptotic insults in cells and Drosophila, supporting the protective role of this pathway in many circumstances (414, 447). This protective effect is not simply a function of autophagy liberating fuels for cells, but appears to be related to decrease in the amount of mitochondria (or amount of mitochondria as a consequence of mitophagy). This, in turn, results in less release of toxic molecules like cytochrome c from mitochondria in response to proapoptotic insults (414).

1. Autophagic cell death: what does it mean?

While autophagy is generally considered to be a cytoprotective process, the occurrence of increased numbers of autophagosomes in dying cells has led to the concept of autophagic cell death (also sometimes referred to as type II cell death). In certain instances, like chloroquine myopathy (463), the toxicity is clearly unrelated to increased autophagic flux, as chloroquine is a weak base that prevents lysosomal acidification and ultimately reduces autophagosome-lysosome fusion. Thus, in some cases, increased numbers of autophagosomes in dying cells may be the consequence of decreased autophagic substrate clearance. In other cases, cell death may occur alongside (but not due to) autophagy, if common upstream pathways are activated. However, an attractive model would be that autophagic cell death (associated with increased autophagic flux) primarily occurs in development or during homeostasis processes, where redundant cells require elimination. However, this model is challenged by observations that Beclin 1-null Caenorhabditis elegans (505) and mice lacking Beclin 1 or Atg5 (407) exhibit increased cell death. Generally, the role of autophagy in mammalian cell death remains unclear, although some studies suggest autophagy may regulate cell death in normal cells under certain stress conditions.

2. Autophagy-induced cell death

It has been proposed that uncontrolled autophagy may lead to cell death, presumably due to excessive degradation of cellular constituents. This model is suggested by studies showing that Beclin 1 mutants that are unable to bind to Bcl-2 induce excessive autophagy and cell death, which can be inhibited with siRNA against Atg5 (389). In Drosophila melanogaster, overexpression of a consitutively active Atg1 mutant induces intensive autophagy and early degradation of salivary glands, which can be blocked by Atg12 elimination, but not by overexpression of the apoptosis inhibitor p35 (470). However, it is possible that some of these effects may be due to functions of these genes that are independent of autophagy. For example, Beclin 1 knockout mice show embryonic lethality, while Atg5 and Atg7-null mice (which are autophagy-null) are live born. Furthermore, Atg1 appears to modulate cell growth by inhibiting the activity of S6 kinase, which regulates cell growth, and some of its effects may be autophagy independent (283).

Some have suggested that autophagy may contribute to cell death, when chemical inhibitors of autophagy reduce toxicity. For instance, the autophagy inhibitor 3-MA...
inhibits cell death in tumor necrosis factor (TNF)-induced human T lymphoblastic leukemia cells (205), in nerve growth factor (NGF)-deprived sympathetic neurons (576), in chloroquine-induced cortical neurons (591), in low potassium-induced cerebellar granule cells (41), and in serum-deprived PC12 cells (541). However, in these autophagy inhibition experiments, it is unclear if the inhibitor is mediating autophagy-dependent rescue or whether the protection is mediated via some autophagy-independent target of the chemical.

It has also been suggested that autophagy can directly kill cells where the apoptosis machinery is absent. Cell death was blocked when Atg7 or Beclin 1 was knocked down by RNAs in mouse L929 cells treated with the pan-caspase inhibitor zVAD (586). Also, RNAi against Atg5 and Beclin 1 blocked cell death of bax \(^{-/-}\), bak \(^{-/-}\) murine embryonic fibroblasts (MEFs) induced by etoposide (481). However, it is important to note that the autophagic cell death occurs under conditions of apoptosis deficiency rather than under physiological conditions.

3. Cross-talk between autophagy and apoptosis

In general, the conclusion of autophagic cell death is largely based on nonphysiological conditions such as chemical inhibition, excessive autophagy, or apoptosis deficiency. Therefore, whether autophagic cell death exists physiologically is still unclear in many circumstances (261, 341). However, numerous data indicate areas of cross-talk between autophagy and apoptosis. The interplay between autophagy and apoptosis suggests that there may be coordination between both of these pathways that may be important throughout development and homeostasis. Death-associated protein kinase (DAPK) family members and DAPK-related protein kinase-1 (DRP-1) induce cell death depending on their kinase activity, but DAPK and DRP-1 also induce autophagy in apoptosis-resistant MEFs (184). The potent apoptosis inhibitor Bcl-2 inhibits starvation-induced autophagy by its direct interaction with Beclin 1. Interestingly, only ER-targeted Bcl-2 but not mitochondria-targeted Bcl-2 inhibits autophagy (389). The tumor suppressor p53 can trans-activate autophagy-inducing genes. In genotoxic stress conditions, autophagy can be induced in a p53-dependent manner (108). Most recently, Kroemer and colleagues (522) discovered that inactivation of p53 triggers autophagy, and this discovery led them to suggest that cytoplasmic p53 may act as a master repressor of autophagy. Atg5 is critical for autophagy at the stage of autophagosome precursor synthesis (213, 266). Yousefi et al. (585) showed that Atg5 is cleaved by calpains to form a 1–193 amino acid cleavage product. The death-inducing activity of this truncated form of Atg5 was observed in the absence of autophagy. Instead, the truncated form of Atg5 may inactivate the anti-apoptotic activity of Bcl-xL by displacing Bcl-xL-Bax complexes, thereby promoting Bax oligomerization. The caspase activation resulting from this process will further reduce autophagosome synthesis by cleaving Beclin-1 (308). Thus calpain activation may impact on autophagy via multiple mechanisms.

The common factors/components and the link between autophagy and apoptosis indicate the coregulation mechanism of both pathways may be important in diverse aspects such as development, tissue homeostasis, and disease occurrence.

B. Autophagy in Development

Autophagy is important during critical mammalian developmental stages in which nutrients are restricted. One such stage is preimplantation development of embryos (539). Autophagy is low in unfertilized oocyte and increases shortly after fertilization. Autophagy is transiently suppressed between the late one-cell and middle two-cell stages and then activated again after the late two-cell stage. Autophagosome formation continues to increase through the four- to eight-cell phase. The complete loss of autophagy in oocytes, as seen in Atg5 knock-out mice under the zonapellucida glycoprotein (Zp3), oocyte specific promoter, halted proper development of embryos before the blastocyst phase. Atg5-deficient oocytes fertilized by Atg5 null sperm failed to develop beyond the four- and eight-cell stages but could develop if fertilized by wild-type sperm. A lack of autophagy from the start of oogenesis does not seem to affect oocyte formation or fertilization. It is not known whether the main role of autophagy in preimplantation development is to provide nutrients to the growing embryo or to clear maternal proteins (538, 539). Other possible consequences of autophagy deficiency are also possible in this context, including secondary effects on the UPS, which may impact on the coordination of the levels of critical proteins required for regulating cell division. It is possible that some of these secondary consequences of autophagy compromise may contribute to the necessity for autophagy during T-cell development. Deletion of Atg5 or Atg7 in T cells was accompanied by a decrease in thymocyte and peripheral T-cell numbers and also resulted in a decrease in T-cell survival (497).

Sudden termination of the fetal nutrient supply from the mother presents a stressful situation for the newborn infant before it establishes breast-feeding. During this transition period, autophagy provides the necessary nutrients to the infant through increased turnover of proteins. This has been shown experimentally in a transgenic mouse model expressing GFP fused LC3 to visualize autophagosomes in vivo (343). In these mice, autophagy in various tissues is enhanced soon after birth, peaked \(-6\ h\) after birth, and declined back to basal levels within 24–48
To assess the specific role of autophagy, the effect of starvation during this critical period in Atg5 and Atg7 knockout mice was studied. While mice generated from animals with conditional deletion of Atg5 in oocytes and Atg5 null sperm cannot develop beyond the four- and eight-cell stages, conventional Atg5−/− mice generate maternally derived Atg5 proteins stored in oocytes are sufficient to rescue the autophagy-deficient phenotype of early embryogenesis (538, 539). These mice develop normally with only a slightly lower birth weight for the Atg7−/− mice, and a significant weight reduction in the Atg7−/− mice (251, 266). Atg5 and Atg7 knockout mice die within 1 day of birth (251, 266). Under forced starvation conditions, the survival time of the Atg5 knockout mice was nearly half (12–13 h) that of their Atg5−/− or wild-type littermates (21–24 h) (266). These experiments support the role of autophagy in normal developmental processes that occur in the context of diminished nutrient supply, lending further importance to the need for the functional turnover of amino acids. Autophagy can have differential roles during and after development. A cardiac-specific knockout of Atg5 during cardiogenesis resulted in cardiac dysfunction only after treatment with pressure load, while cardiac-specific knockout in adult mice developed cardiac hypertrophy and contractile dysfunction under normal conditions, suggesting that upregulation of autophagy may be an adaptive response in failing hearts, while basal autophagy in adult hearts may serve a homeostatic function (354).

Additional characterization of autophagy deficiency has been performed in Atg7 and Atg8 (autophagy-related 8a), Drosophila melanogaster mutants (208, 359). Autophagy is highly activated in larval tissue at the onset of metamorphosis. Although Atg7 mutant flies are morphologically normal, they are sensitive to stressful conditions and show a decreased level of cell death-induced autophagy during metamorphosis (208). Increased lethality and a decrease in GFP-LC3 levels in Atg7-deficient flies indicated that autophagy deficiency was deleterious during stress-inducing conditions such as starvation, oxidative stress, and metamorphosis (208). Furthermore, autophagy genes regulate dauer formation (a stage of reversible developmental arrest during unfavorable conditions) in C. elegans nematodes (335). Also, environmental conditions (like limited food or increased temperature) that stimulate dauer formation can also induce autophagy (335). Thus autophagy seems to play an important role in many developmental processes in eukaryotes.

**C. Erythropoiesis**

Erythropoiesis or erythrocyte/red blood cell (RBC) maturation is a specific cell developmental process requiring autophagy. During mammalian erythropoiesis, late-stage erythroblasts enucleate to become reticulocytes. The reticulocytes subsequently get rid of the internal organelles and are released into the circulation where they mature into biconcave erythrocytes.

Autophagy has been suggested to participate in reticulocyte maturation. Studies in embryonic erythroid cells show that during this maturation process, the number of mitochondria in the cells is reduced, while at the same time there is a substantial increase in the autophagy of mitochondria (508). Studies in both humans and rats have shown that autophagy participates in the degradation of mitochondria during erythroid maturation (172, 223). Furthermore, autophagosomes have been observed in erythrocytes of different mammalian species and in embryonic erythroid cells (176, 258, 486). Thus it is believed that autophagy plays a critical role in the terminal differentiation of red blood cells.

Recently, Nix, a Bcl-2 only member of the Bcl-2 family, has been implicated in the regulation of erythroid maturation via mitochondrial autophagy (441). Nix−/− mice exhibited anemia with reduced numbers of mature erythrocytes. The Nix−/− erythrocytes contained an abnormal subpopulation of cells that were devoid of ribosomes but retained their mitochondria (469). Nix−/− RBCs also showed increased autophagosomal structures; however, all Nix−/− RBCs containing autophagosomes also had many mitochondria outside the autophagosomes, suggesting a probable defect in the sequestration of mitochondria by autophagosomes (441).

**D. Autophagy and Ageing**

In addition to playing critical roles in development, autophagy has also been implicated in ageing, at least in model organisms. The intracellular accumulation of DNA, protein, lipids, and cellular organelles is one of the common features of all age-related changes (547), and this hints that one may be able to attenuate ageing by enhancing their clearance. This accumulation of damaged macromolecules during ageing occurs due to defective clearance and a general decline in the housekeeping mechanisms. It has been observed that the autophagic activity and lysosomal proteolytic activities decline with age, and this correlated with accumulation of damaged proteins and organelles, a characteristic feature of ageing (70, 92, 526, 553). Furthermore, many environmental modulators of ageing such as starvation, hyperthermia, and hypoxia also affect the autophagic pathway (16, 48, 382, 392, 547). Upregulation of rate-limiting components of the autophagic pathway prevents the age-dependent accumulation of damage in neurons and enhances longevity in Drosophila (485). On the contrary, inactivation of autophagy (by reducing activity of vps-34 (161), TOR/mTOR (161), unc-
The idea that autophagy may regulate lipid generation was from experiments in immature liver (22). Further links between autophagy and ageing are supported by the observations that distinct stress resistance and longevity signaling pathways, such as FoxO (600), p53 (522), SIRT1 (280, 437), and NFκB (435, 436), are also potent regulators of autophagic degradation. SIRT1, FoxO3, and p53 can clearly suppress NFκB signaling, which results in enhanced autophagic degradation accompanied by an increase in life span (435, 436). The role of the deacetylase SIRT1 in autophagy is supported by the discovery that many of the Atg proteins can be acetylated by p300. Knockdown of p300 reduces acetylation of Atg5, Atg7, Atg8, and Atg12 and induces autophagy (281).

E. Autophagy and Lipid Metabolism

Mammals survive starvation by activating proteolysis to produce an amino acid pool that is used, in part, for energy metabolism through glyconeogenesis (266), while esterified fatty acids are mobilized to skeletal muscle and other tissues of the body for metabolism through β-oxidation (593). The ability to synthesize sterol esters or triacylglycerols and store these neutral lipids in cytoplasmic lipid droplets is a universal property of eukaryotes (319). When energy is required, intracellular signaling pathways can trigger the hydrolysis of triglycerides to fatty acids in lipid droplets by the process of lipolysis, which is mediated by lipase enzymes (97). Lipolysis and autophagy are known to share similarities in regulation and function. Recently, Singh and co-workers (487) demonstrated that autophagy could mediate fat mobilization and breakdown in liver cell cultures and mouse liver hepatocytes. During fasting, this can be mediated via mTOR inhibition. In a process that the authors coined as macrolipophagy, portions of lipid droplets become trapped inside the double-membrane-bound autophagosome vesicles, after which they are transported to lysosomes and are degraded to fatty acids.

Autophagy may also be involved in lipid droplet formation, as their formation is impaired in hepatocytes with Atg7 deficiency (480). This raises a possible paradoxical situation where autophagy is involved in both lipid droplet synthesis and formation. However, these two studies were done in mice of different ages. The study showing that autophagy regulates lipid droplet catabolism was performed in mature liver (4-mo-old mice), while much of the support for the role of autophagy in lipid droplet generation was from experiments in immature liver (22 days old). The idea that autophagy may regulate lipid droplet formation during development has echoes in a subsequent study that showed that knockdown of Atg7 in adipocytes resulted in lean mice with decreased white adipose mass, which had features of brown adipocytes. This was associated with decreased levels of stored lipid in these cells (487). Thus it appears that autophagy deficiency may have different net effects on lipid droplet formation and degradation in different cell types, and this may also vary at different stages of development.

VII. AUTOPHAGY AND IMMUNITY

Autophagy functions in diverse aspects of immunity. Autophagy can target pathogens that reside in the cytosol or within phagosomes for lysosomal degradation and can participate in the effective elimination of viruses, bacteria, and parasites. In addition, autophagy plays an important role delivering antigens to the MHC class II molecules. Autophagy may also contribute to several independent stages of antigen presentation, including the uptake of antigens from the cytosol or from phagosomes (Fig. 4).

A. Autophagy, Antigen Processing, and Presentation

Antigen processing and presentation need to be delicately regulated, since underactivity of the system may cause predisposition to infectious diseases, while hyperactivity will predispose to autoimmune disease. Recent data suggest a key role for autophagy in this system for normal immunity, for control of infectious agents, and also in autoimmune diseases.

The basis of T-cell immunity is the recognition of antigenic peptides presented on the MHC class I and class II molecules by specific T-cell receptors, CD8+ T cells, and CD4+ cells, respectively. The MHC class I and II molecules are specialized in the antigens they acquire and present: the class I molecules are known to report on intracellular events, including viral infections as well as bacterial or cellular transformations, whereas the MHC class II molecules are known to sample the extracellular milieu (34, 201). Biosynthesis of MHC class I molecules can be summarized in six steps: 1) acquisition of the antigenic peptide, 2) tagging of the peptide for destruction, 3) proteolysis, 4) peptide delivery into the ER; 5) peptide binding to MHC class I molecules, and 6) display of peptide-MHC class I complexes on the cell surface of antigen-presenting cells (APCs), like macrophages and dendritic cells. In the case of the MHC class II molecules, antigen presentation adopts the endocytic pathway to sample antigens. The extracellular antigens are taken up into a membrane-bound compartment known as the phagosome. Following a series of modifications, the phagosome ultimately fuses with lysosomes forming...
“phagolysosomes” where the contents of the phagosomes, including degraded proteins, can interact with the MHC class II molecules. The peptides are loaded onto the peptide-binding groove of the MHC class II molecules, which later interact with the CD4$^+$ T cells (201).

Antigen-presenting cells like macrophages and dendritic cells, which present MHC class II molecules, engage in macroautophagy and signaling inputs from cytokines can modulate this process (164, 466). In macrophages, signaling inputs, such as TLR ligands, including lipopolysaccharide (LPS), TLR7 ligands, imiquimod, and siRNA, also induce autophagy and enhance mycobacterial colocalization with autophagosomes resulting in their elimination (84, 575). TLR ligands are also known to facilitate Beclin 1 recruitment to the phagosome followed by Atg5- and Atg7-dependent recruitment of LC3 (84, 443, 575). When these autophagy markers are not recruited to phagosomes, then 

S. cerevisiae

is not eliminated. Collectively, these data implicate TLR signaling in autophagy induction (278). In addition to the stimulation of autophagy by TLR ligands, there is also a feedback mechanism with autophagy stimulating TLR signaling. The known case for this involves delivery of viral replication intermediates to TLR7 in endosomes of dendritic cells, which present MHC class II molecules, engage in macroautophagy and signaling inputs from cytokines can modulate this process (164, 466). In macrophages, signaling inputs, such as TLR ligands, including lipopolysaccharide (LPS), TLR7 ligands, imiquimod, and siRNA, also induce autophagy and enhance mycobacterial colocalization with autophagosomes resulting in their elimination (84, 575). TLR ligands are also known to facilitate Beclin 1 recruitment to the phagosome followed by Atg5- and Atg7-dependent recruitment of LC3 (84, 443, 575). When these autophagy markers are not recruited to phagosomes, then 

*S. cerevisiae*

is not eliminated. Collectively, these data implicate TLR signaling in autophagy induction (278).

Phagosome maturation is a key step for antigen acquisition and is restricted to APCs. Autophagosomal proteins participate in the maturation of phagosomes. In the presence of TLR signaling, LC3 and Beclin 1 were found to translocate to the phagosomal membrane during the early stages of phagocytosis. However, these were not the double-membrane structures found in autophagy, and therefore, the phenomenon may be different from conventional autophagy (443). More recently, proteomics analyses of phagosomes isolated from the cultured 

Drosophila

cells identified Atg9, an autophagosomal marker, as a phagosomal protein (132). This association of the autophagosomal protein with phagosomes for antigen presentation may be very transient (499). Antigen processing and presentation and T-cell selection in the thymus may also be affected by autophagy, as the autophagosomal pathway intersects with the endosomal pathway.

One of the prime examples of a role for autophagy in bacterial antigen presentation pertains to 

*Mycobacterium tuberculosis*

. It has been known that immunity against tuberculosis is mediated by macrophages and dendritic cells (112), but 

Mycobacteria

have evolved mechanisms to evade these cellular defenses, using strategies like preventing the fusion of the infected phagosomes with lysosomes that can kill the bacteria and produce peptides for antigen presentation (421). To ensure that the antituberculosis vaccines are not made ineffective by the hijacking of the cellular defenses by the pathogen itself, studies have been undertaken to improve vaccine efficacy (198). The findings revealed that autophagy induction in

![FIG. 4. Immune signals contributing to autophagy and immunological mechanisms regulated by autophagy. Antigen-presenting cells like macrophages and dendritic cells, which present MHC class II molecules, engage in autophagy and signaling inputs from cytokines can modulate this process. In macrophages, signaling inputs, such as TLR ligands, also induce autophagy and enhance mycobacterial colocalization with autophagosomes resulting in their elimination. PKR, an interferon-inducible protein kinase known to repress host and viral protein synthesis and viral replication, has also been shown to regulate autophagy. Interferon (IFN)-γ upregulates autophagy as a mechanism for the elimination of intracellular organisms such as *Mycobacterium tuberculosis* and *Listeria monocytogenes*.](http://physrev.physiology.org/)

Physiol Rev • VOL 90 • OCTOBER 2010 • www.prv.org

Downloaded from http://physrev.physiology.org/ by 10.220.32.246 on October 19, 2017
APCs corresponds well with improved immune response. Thus the performance of BCG vaccine can be significantly enhanced when it is combined with enhanced autophagy-based antigen presentation.

Although MHC class I molecules traditionally sample intracellular antigens, autophagy can deliver endogenous antigens to the MHC class II pathway, as seen in the example of MHC class II presentation of the EBNA1 viral antigen to the CD4+ T cells (465). Inhibiting autophagy, using 3-MA or Atg12 knockdown, blocks the presentation of the antigen to the CD4+ T-cells. Furthermore, blocking lysosomal acidification and autophagosome maturation results in EBNA1 accumulation in autophagosomes. These studies show that cytosolic antigens can be degraded by autophagy and delivered as peptides to the MHC class II pathway (379). In addition to viral antigens, autophagy also facilitates MHC class II-restricted antigen presentation of certain self, tumor, and model antigens (32, 95). In a recent study, peptides recovered in a complex with MHC class II originated from cytosolic or nuclear proteins, and their proportion increases upon starvation-induced autophagy (85, 93, 351). Thus MHC class II, although set up to scan the extracellular milieu, is also used for intracellular proteins.

All of the above examples of the role for autophagy in antigen presentation involve macroautophagy. However, CMA also plays a role in MHC class II-restricted presentation. For example, LAMP-2a together with heat shock cognate protein 70 can transport a cytoplasmic antigen to MHC class II molecules and contribute to antigen presentation (604).

Lastly, autophagy is also known to play a role in class I MHC processing and presentation. Autophagy is thought to be involved in clearing ubiquitinated cytoplasmic protein aggregates (341). Dendritic cells show the presence of aggresome-like structures (dendritic cell aggresome-like induced structures, DALIS) containing polyubiquitylated proteins, and peptides derived from DALIS can be presented by MHC class I molecules. Such aggresome-like structures are also seen in response to stress in many immune and nonimmune cells and are cleared by autophagy-dependent mechanisms (162, 251, 396). Given that the autophagosomal membrane may have some contribution from the ER, the MHC class I machinery may be present in the autophagosome. These data suggest a role for autophagy in MHC class I presentation. However, treatment with autophagy inhibitors, like wortmannin or 3-MA, failed to affect MHC class I-restricted presentation of an endogenous antigen by EBV-transformed B cells. In addition, colocalization between MHC class I molecules and GFP-labeled LC3 was not observed in epithelial cells (361, 379, 466).

In keeping with the functional pleiotropy of autophagy, these data suggest a new link between autophagy and the adaptive immune response.

### B. Autophagy and Infectious Diseases

Autophagy functions as an important defense mechanism against infections by sequestering and degrading pathogens in autolysosomes. This process has been termed xenophagy and differs from the classical autophagosome in that it appears to be selectively directed to the pathogen (289, 465).

Autophagic degradation has been proposed as part of the innate immune response against a number of microbial pathogens that invade eukaryotic cells. Group A Streptococcus is internalized into autophagosome-like structures and degraded in an Atg5-dependent manner (353). Stimulation of autophagy by starvation or treatment with rapamycin results in an increase in Mycobacterium tuberculosis clearance, which suggests that this process acts as part of the immune response against this pathogen. Furthermore interferon-γ (IFN-γ), a cytokine associated with protective immunity against mycobacteria (112), causes an increase in autophagy (146). More recently, the identification of small molecules that had been shown to enhance the growth-inhibitory effects of rapamycin in S. cerevisiae and therefore induce autophagy in an mTOR-independent manner, resulted in a reduction in the number of viable intracellular mycobacteria in macrophages (111). A number of other pathogens, like Toxoplasma gondii, Listeria monocytogenes, Salmonella enterica, or Rickettsia conorii, have been found to be targeted by autophagy (reviewed in Refs. 289, 290, 465).

The ubiquitin system plays a major role in recognition of certain cytosolic bacteria in mammalian cells, where ubiquitin is found to accumulate around the surface of the bacteria. It remains to be clarified whether ubiquitin conjugates to the bacterial protein itself, or whether ubiquitinated host proteins are targeted to the bacterial surface (390, 546). How the ubiquitin serves as a signal for bacterial degradation was not known until the recent discovery of the adaptor protein NDP52 (nuclear dot protein 52kDa) that recognizes ubiquitinated Salmonella enterica and also recruits LC3, thereby possibly targeting the bacteria for degradation by autophagy. Depletion of NDP52 resulted in hyperproliferation of bacteria in HeLa cells and enterocytes and increased the number of cells with ubiquitin-coated bacteria, suggesting that NDP52 might be one of the adaptors that targets ubiquitinated bacteria for degradation (529).

In contrast to bacterial pathogens, only a few viruses have been reported to be degraded by autophagy. The first evidence of autophagy as a host defense mechanism against viruses was the observation of herpes simplex virus (HSV) and human cytomegalovirus (HCMV) in autophagosomes (492). The discovery of the protective effect of the anti-apoptotic gene Bcl-2 in Sindbis encephalitis virus in mice permitted the identification of Beclin 1 as an antiviral host defense protein, which was later

---

*Mammalian Autophagy in Physiology and Pathophysiology 1050*
demonstrated to be essential for autophagosome formation (301). Autophagy has recently been shown to play a direct antiviral role against vesicular somatitis virus (VSV) in *Drosophila*, which required the P3K/Akt signaling pathway (477). In plants, tobacco mosaic virus (TMV) replication is also limited by autophagy through Beclin 1 (304).

Innate and adaptive immune control by autophagy has subjected pathogens to an extreme evolutionary pressure, favoring the appearance of mechanisms to evade this response and subvert it for their own benefit. These mechanisms target autophagy at different levels: inhibiting the autophagic machinery and, therefore, avoiding its killing function, or disrupting autophagosome degradation, resulting in the accumulation of these membrane structures, which has been exploited by some pathogens to favor their replication.

Inhibition of autophagy has been reported in the case of some bacterial pathogens, such as *Shigella flexneri* (368) or *M. tuberculosis* (146). Once *M. tuberculosis* infects host phagocytic cells, it interferes with phagosomal-lysosome fusion by decreasing phagosomal acidification and by producing mycobacterial lipids that mimic phosphatidylinositol-3-phosphatase (PI-3-P)-dependent trafficking pathways in the macrophage (86, 550). On the other hand, a number of pathogens use autophagy for their own replication, including *Brucella abortus, Legionella pneumophila, Porphyromonas gingivalis*, and *Coxiella burnetii*. Instead of being processed by the endosomal pathway, these pathogens divert their trafficking to autophagosome-like vacuoles. In this way, they not only avoid their degradation but increase the availability of short peptides that they use as carbon and energy sources (96, 148, 398, 503).

Viruses have also developed strategies for evading host defense autophagy, as well as for subverting it to facilitate their own replication. One of the best characterized examples is the HSV type 1 (HSV-1) neurovirulence protein ICP34.5. HSV-1 is the main cause of sporadic viral encephalitis in adults, and deletion of ICP34.5 attenuates its neurovirulence (57). This protein targets autophagy by two different mechanisms. The first of these mechanisms inhibits signaling pathways that regulate autophagy, while the second acts directly on the autophagic machinery. HSV-1 is degraded in autophagosomes due to PKR (double-stranded RNA-dependent protein kinase)-mediated induction of autophagy (512). PKR is an interferon-inducible protein kinase known to repress host and viral protein synthesis and viral replication, through phosphorylation and activation of the translation initiation factor eIF2α (reviewed in Ref. 131), which has also been shown to regulate autophagy (511). The virulence factor ICP34.5 blocks this effect by recruiting protein phosphatase 1α (PP1α), which counteracts PKR-dependent eIF2α phosphorylation (511). On the other hand, ICP34.5 also binds directly to Beclin 1, and this interaction antagonizes autophagosome formation. Beclin 1-mediated inhibition of autophagy is necessary for HSV-1 neurovirulence and requires PKR (377). ICP34.5 interacts with Beclin 1 and PP1α through different domains, which underscores that its neurovirulence and ability to inhibit autophagy occur by two independent mechanisms. Although Tallozcy et al. (512) showed that this factor antagonizes PKR-dependent degradation of HSV-1 through autophagy, both in murine embryonic fibroblasts (MEFs) and in mouse neurons (512), this finding has been controversial. When HSV-1 replication was studied in *ATG5*−/− MEFs, it showed the same replication limitation as in wild-type MEFs, while *pkr*−/− MEFs did not show such defects. This suggests that, although the virulence factor ICP34.5 antagonizes autophagy, HSV-1 replication in cultured cells is not limited by autophagy (3). In addition, in infections with other members of the herpesvirus family, the Kaposi sarcoma-associated virus (KSHV), Beclin 1 is downregulated due to the expression of viral Bcl2 proteins, which interact with Beclin 1 even more effectively than their cellular counterparts (389, 562).

Autophagosome formation is also inhibited during infection by HCMV, a herpesvirus that is an opportunistic pathogen and the major cause of congenital infections leading to birth defects. This virus has developed different strategies to avoid the autophagic response. During HCMV infection, autophagy is inhibited by an mTOR-dependent mechanism, as this signaling pathway is activated and infected cells cannot respond to the induction of autophagy by rapamycin. In addition, infected cells are resistant to the mTOR-independent inducer of autophagy lithium chloride (47).

Instead of antagonizing autophagy, infection by poliovirus, rhinovirus, and many other RNA viruses induces the accumulation in the cytoplasm of double-membrane vesicles that display hallmarks of autophagosomes (196, 400). Expression of the poliovirus proteins 2BC and 3A induces LC3 and LAMP-1 colocalization in these vesicles. In addition, stimulation of autophagy increases viral yield, while its pharmacological inhibition, or the absence of autophagic genes, inhibits viral replication. RNA viruses require cellular membranes for assembling their replication complex, so they induce the formation of autophagosome-like structures to serve as membrane scaffolds for their replication (196). It has also been suggested that these double-membrane structures allow the release of these cytoplasmic viruses by a nonlytic mechanism (235).

Recently, a number of examples of viruses that enhance autophagic flux resulting in the promotion of viral replication are emerging, although the mechanisms still need to be identified. Autophagosome formation is induced upon Dengue-virus 2 (DV2) infection, and this autophagy induction increases virus replication yields (284). Hepatitis C virus (HCV) suppresses autophagosome-lyso-
some fusion, and this process is dependent on the UPR. Inhibition of this fusion promotes autophagosome accumulation and increases virus replication (489, 490).

A different autophagy-dependent mechanism that viruses can use for their own benefit has been described for the human immunodeficiency virus 1 (HIV-1). This virus induces apoptosis in infected and uninfected CD4⁺ T cells due to the interaction of the envelope protein of HIV-1 with the chemokine receptor CXCR4 on these lymphocytes. It has been reported that this interaction leads to an increase in Beclin 1 levels in CD4⁺ T cells, which results in the induction of cell death with autophagic features (106; reviewed in Ref. 105). However, this process is not straightforward, as others have reported that HIV-1 decreases LC3 and Beclin 1 levels in infected cells and that disruption of autophagy allows host defense escape and promotes its replication (605).

Evasion of autophagy might have implications in other noninfectious diseases associated with viral infection as has been proposed for Alzheimer’s disease (AD). HSV-1 infection in combination with genetic determinants has been suggested to contribute to AD etiology. Although the mechanisms used by this virus are unknown, it has been hypothesized that the autophagy inhibition mediated by HSV-1 could also result in decreased degradation of the components of the amyloid plaques and consequently favor AD progression (191). Consequently, the identification of strategies that pathogen proteins use to impair host autophagy may not only be relevant for developing effective treatments against infection but may also have possible benefits in other contexts.

C. Autophagy and Crohn’s Disease

Autophagy, autoimmunity, and infectious agents intersect in Crohn’s disease (CD), a chronic debilitating inflammatory condition of the intestine that causes diarrhea, malabsorption, fistulation, and intestinal obstruction. The prevalence in Western populations is 100–250 per 100,000 with peak incidence in early adult life (346, 426). Symptoms include abdominal pain, diarrhea, anorexia, and malnutrition. The pathophysiology of CD is poorly understood. It is generally accepted that in genetically susceptible individuals, commensal intestinal bacteria trigger an aggressive mucosal adaptive immune response, leading to relapsing intestinal inflammation and tissue damage (453). Smoking is known to predispose, and several causative infectious agents have been postulated, including Mycobacteria paratuberculosis, Listeria monocytogenes, Klebsiella pneumoniae, and various strains of adherent/invasive Escherichia coli, although the evidence for each of these organisms is inconclusive (99). The mainstay of treatment for CD is immune suppression (corticosteroids, azathioprine, methotrexate, and anti-tumor necrosis factor antibody therapy). However, the majority of CD patients require major abdominal surgery over the course of their illness due to treatment failure or disease complications that include bowel obstruction due to fibrotic stenosis and intra-abdominal or perianal abscess due to intestinal fistulation and perforation (64).

Autophagy was first associated with CD pathogenesis in 2007, when genetic studies reported robust associations between variants in two autophagy genes, Atg16L1 and IRGM, and Crohn’s disease risk (385, 419). Functional studies have since implicated the disease-associated variant in Atg16L1 with impaired autophagic clearance of intracellular bacteria, abnormal intestinal Paneth cell function, and increased production of the proinflammatory cytokine IL-1B (37, 264). IRGM regulates autophagic clearance of invasive bacteria (488).

Atg16L comprises a coiled-coil domain that interacts with Atg5 and a WD repeat domain that is postulated to interact with other proteins (340). The strongest CD association at the Atg16L1 locus is with a nonsynonymous single nucleotide polymorphism (SNP) that codes for a polar threonine to alanine switch (termed the T300A variant) near to the WD repeat interaction domain of the protein. Knockdown of native Atg16L and reconstitution with the risk-associated T300A variant in HeLa has no effect on Atg16L binding to ATG5 or on basal autophagy, compared with replacement with the wild-type variant. However, the T300A variant is associated with impaired autophagosomal capture of Salmonella typhimurium when cells are incubated with live bacteria (264). Mice deficient in the coiled coil domain of Atg6L1 (Atg16L1 ΔCCD) die shortly after birth, and embryonic fibroblasts are null for autophagy. Fetal hepatic macrophages derived from Atg16L1 ΔCCD mice express high levels of the proinflammatory cytokine IL-1B, when incubated with LPS or live E. coli. Lethally irradiated wild-type mice reconstituted with fetal hepatic cells from Atg16L1 ΔCCD mice develop a severe colitis when fed dextran sulfate and have markedly elevated serum IL-1β levels (431). In a separate study, a gene trap vector was used to generate viable mice that express Atg16L at 30% of wild-type levels. Cell lysates from these Atg16L hypomorphic mice have impaired basal autophagy, and examination of the intestine reveals abnormal Paneth cell morphology (37). Paneth cells are specialized intestinal epithelial cells that secrete granules containing α-defensins, secretory PLA₂, and lysozyme (399), and the distribution of these granules is disordered in the Atg16L hypomorphic mice. Paneth cells are found in greatest abundance in the terminal ileum, the most common site of CD inflammation, and analysis of terminal ileal resection specimens shows the same abnormal Paneth cell morphology in patients homozygous for the T300A variant as is seen with the Atg16L hypomorphic mice, compared with patients homozygous for the wild-type variant who have...
normal Paneth cell morphology (37). Interestingly, CD risk-associated variants in nucleotide-binding and oligomerization domain (NOD)2 are also associated with abnormal Paneth cell function, in particular with reduced production of α-defensins (217). Two members of the NOD-like receptor family, NOD1 and NOD2, recognize bacterial peptidoglycan to trigger immune responses. Stimulation of NOD1 or NOD2 has been shown to induce autophagy and could thus control infection (60, 535). Activation of NOD2 by muramyl dipeptide, a bacterial ligand, can induce autophagy in monocyte-derived dendritic cells and requires its downstream mediator RIPK-2 (receptor-interacting serine-threonine kinase-2). Stimulation with muramyl dipeptide results in redistribution of MHC class II molecules to the surface and also redistributes LC3 to HLA-DM (intracellular protein involved in peptide presentation by MHC class II)-containing compartments. This redistribution was inhibited in dendritic cells with CD-associated variants in NOD2 or Atg16L1 which were incapable of autophagy induction (60). A related study by Travassos et al. (535) showed that NOD1-deficient MEFs or the CD-associated NOD2 mutant affected bacterial infection (535). This was attributed to defective autophagy in these cells, as they show that NOD1 and NOD2 interacted with Atg16L1 and recruited Atg16L1 to plasma membrane at the site of bacterial infection, which was affected in the NOD2 variant associated with CD (535).

The association with CD at the IRGM locus was initially reported at a cluster of intronic SNPs spanning the 5’ end of the gene. Subsequent resequencing of this region has failed to find any CD-associated polymorphisms in coding regions (385). However, examination of copy number variation has identified a 20-kb deletion polymorphism immediately upstream of IRGM, which is in perfect linkage disequilibrium with the most strongly associated risk allele of the index studies. Comparison with the chimpanzee genome suggests that this deletion polymorphism is ancestral, and the more recent deletion polymorphism is associated with CD risk. IRGM encodes a 178-amino acid protein belonging to the p47 immunity-related guanosine triphosphatase family. The murine homolog LRG-47 is induced by IFN-γ and upregulates autophagy as a mechanism for the elimination of intracellular organisms such as M. tuberculosis and Listeria monocytogenes (59, 107). Human IRGM lacks the IFN-γ response element, but functional studies in human cell lines suggest that IRGM can also regulate autophagic clearance of intracellular pathogens: HeLa cells infected with Salmonella typhimurium encapsulate the bacteria inside autophagosomes, and this is almost completely abolished when cells are transfected with siRNA against IRGM (330). Knock-down of IRGM in U937 cells is associated with reduced clearance of M. tuberculosis (488). Conversely, overexpression of IRGM in HeLa enhances autophagic capture of Salmonella typhimurium (330).

Overall, the recent genetic evidence and functional data suggest that autophagy of intracellular pathogens is defective in patients with CD, leading to persistence of the organisms within intestinal cells and recruitment of the adaptive immune response. In view of the limitations of treatments currently available for CD, pharmacological upregulation of autophagy may offer a novel therapeutic strategy (323).

D. Autophagy and Parasites

The interplay between autophagy and parasitic infection is complex, as this process in the host may be relevant to parasitic survival and autophagy may also play important roles in the pathogens themselves. In certain contexts, autophagy is protective for the host. This includes Toxoplasma gondii, a parasite that causes central nervous system infections in immunocompromised hosts. Toxoplasma gondii elimination from macrophages depends on autophagy. However, in astrocytes, this parasite is degraded from the cytoplasm. It is possible that autophagy is involved in the removal of partially degraded parasitic material in astrocytes and that this enables trafficking of such material to the endolysosomal system to enable MHC class II antigen presentation (155).

Some of the cellular defense against T. gondii may involve autophagy proteins via autophagy-independent mechanisms. Host Atg5 is essential for in vivo resistance to this pathogen, as it is required for IFN-γ-induced damage to the T. gondii parasitophorous vacuole membrane and subsequent parasite clearance. This Atg5-dependent process may be independent of autophagy but still requires Atg5, which appears to enhance parasite clearance via recruitment of a GTPase to the vacuole membrane (601).

In certain cells, at least, T. gondii induces mTOR-independent autophagy. It has been proposed that this may be a way that this parasite exploits the host cell machinery to liberate nutrients for the parasite, under limiting conditions (558).

Autophagy may allow entry of certain parasites into the host cells. This is exemplified by Trypanosoma cruzi, the organism that causes sleeping sickness. In cells infected with this parasite, the vacuole that surrounds the organism is decorated with antibodies to LC3, and autolysosomes were seen to be recruited to parasite entry sites. The induction of autophagy in cells prior to parasite exposure enhanced parasite invasion, while the converse was seen with autophagy inhibition, suggesting that autophagy may facilitate infection of this agent (422). A similar phenomenon has also been reported for Leishmanai amazonensis in mouse macrophages (but the effects were mouse strain specific) (397).

Many of the parasites themselves have Atg gene orthologs. Entamoeba histolytica and its reptilian relation...
Entamoeba invadens have the Atg8 conjugation system (but no Atg5–12 system). There is increased Atg8 ortholog conjugation in this parasite during logarithmic growth and encystations, and PI-3-K inhibitors reduce their growth, suggesting that “autophagy” may be relevant to the survival of this organism (although it was not clear the extent to which this system was crucial for protein catabolism) (394). It is interesting to note that a similar situation has been reported for T. cruzi (5) and that such an Atg8 system is also seen in Acanthamoeba and is highly expressed during encystation (348). Furthermore, the Atg8 ortholog is associated with vacuolar structures and knockdown of the Acanthamoeba Atg8 gene reduced “autophagosome” formation and encystation (348). While the Atg8 vesicles formation in this organism had similarities with autophagy and the vesicles did colocalize with Lyso-tracker red, it is still not clear if these are acting like conventional autophagosomes and mediating significant levels of protein degradation.

The multitude of possible roles of autophagy in parasite growth, invasion, and clearance and the possibility that there may be autophagic-like processes that are important for the parasite itself, raises potential therapeutic strategies for such processes based on autophagy modulation, but such strategies may require a good understanding of the process in both host and parasite.

VIII. DISEASES ASSOCIATED WITH DEFECTIVE AUTOPHAGOSOME SUBSTRATE DEGRADATION

A. Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are a group of at least 40 neurodegenerative or myopathic diseases that are characterized by the accumulation of undegraded lysosomal substrates within the lysosomal lumen (124). LSDs are monogenic and generally autosomal recessive diseases caused by mutations that lead to complete or partial dysfunction of lysosomal proteins (typically, but not always, lysosomal hydrolases). It has been estimated that lysosomes contain 50–60 different hydrolases that are active within the acidic pH of the lysosomal lumen. In addition to a number of hydrolases, multiple integral membrane proteins have been identified, most with no known function (124). Despite the large differences in the pathology of these diseases, all result in the accumulation of undegraded cargo both within the lysosomal lumen and outside lysosomes. As it is clear that this accumulation affects numerous other pathways, it is not known which species and pathways contribute most significantly to the progression of these diseases. The earlier view of these diseases was that the toxicity that causes neurodegeneration in these conditions is primarily driven by the specific substrate that accumulates when its hydrolase is deficient.

Recent data suggest that LSDs manifest a more general impairment of autophagic protein degradation due to a defect in autophagosome clearance, in addition to the specific substrate of the deficient hydrolase. This was demonstrated using mouse models of multiple sulfatase deficiency (MSD) and mucopolysaccharidosis type IIIA (MPS-III A) (474). MSD, an aggressive neurodegenerative disorder that results in death, is caused by a mutation in the sulfatase modifying factor 1 (SUMF1) gene which encodes the formylglycine-generating enzyme (FGE) (63). In addition to an accumulation of lysosomes and undegraded lysosomal substrates, MSD mice accumulated autophagosomes. Elevated LC3-II levels detected by immunoblotting and LC3-positive vesicles in neuronal tissue confirmed an accumulation of autophagosomes. Analysis of cell lines derived from MSD mice and their wild-type littermates showed a decrease in the colocalization of the lysosomal marker LAMP-1 and LC3 in MSD mouse embryonic fibroblasts (MEFs). This indicates that the accumulation of autophagosomes results from failed fusion of autophagosomes with lysosomes. The mutation of SUMF1 in MSD mice prevents both the degradation of lysosomal cargo and the fusion of autophagosomes with lysosomes. The accumulation of the autophagy substrates within MSD MEFs further supports a deficiency in autophagy as a secondary disease mechanism in LSDs. Brain tissue of MSD mice showed an accumulation of ubiquitinataggregates. Similar phenomena have been described in mucopolysaccharidosis type III (474).

Certain other LSDs have been described characterized by defective autophagy. X-linked myopathy with excessive autophagy (XMEA) is a childhood-onset disease caused by hypomorphing alleles of the VMA21 gene (411). VMA21 encodes a chaperone protein important in the assembly of the V-ATPase, a proton pump complex responsible for the acidification of lysosomes (411). VMA21 deficiency results in raised lysosomal pH, which reduces the degradative capacity of lysosomes and blocks autophagy (411). In mucolipidosis type IV, there is a mutation in a TRP (transient receptor potential) channel on the lysosome, which causes overacidification of the lysosomes which is associated with decreased degradation of lysosomal contents (549).

Danon disease is caused by mutations in the LAMP-2 gene that lead to a LAMP-2 deficiency (363). Patient muscle biopsies show the accumulation of autophagosomes and glycogen granules within myofibers (79, 363). LAMP-2 deficiency also impairs phagosomal maturation in mouse neutrophils (15). LAMP-2 is required for the successful maturation of phagosomes and autophagosomes (24, 104, 182). LAMP-2 deficiency impairs the dynein-mediated transport of lysosomes to regions of the cell where fusion with autophagosomes occurs (24, 104). Pompe disease is...
caused by mutations in α-glucosidase, a lysosomal enzyme that hydrolyzes glycogen to glucose, thus resulting in abnormal accumulation of glycogen in lysosomes (409). It is thought that the decreased metabolism of glycogen to glucose results in an energy crisis within the cell and upregulates autophagy (408). In accordance with observations from other lysosomal storage disorders (474), the subsequent accumulating autophagosomes are unable to fuse with lysosomes that contain an excess of undegraded substrates (408).

B. Autophagy Deficiency: A Secondary Disease Mechanism in Neurodegeneration

LSDs manifest impaired autophagic flux due to lysosomal pathology, and at least in some cases this appears to be due to failure of fusion of autophagosomes and lysosomes, but not failure of trafficking of autophagosomes to lysosomes (200). Another group of diseases is associated with defects in the vesicular trafficking processes that enable autophagosomes to get to the vicinity of lysosomes, a step that is required for efficient autophagic flux.

Motor neuron disease refers to a group of sporadic and familial diseases characterized by the degeneration of motor neurons. A number of mutations affecting microtubule transport have been implicated in the development of motor neuron diseases (MND) in mouse models (150, 234, 274) and humans (403). Disruption of retrograde axonal transport of cargo by overexpression or depletion of dynein complex components in transgenic mice results in the progressive degeneration of motor neurons and the formation of inclusions (150, 234, 274, 403). The pathology and symptomatic progression of the disease in these transgenic models mimic those seen in some MND patients (e.g., with dynactin mutations). As dynein activity is crucial for appropriate microtubule-based delivery of autophagosomes (which are formed randomly in the cytoplasm) to lysosomes clustered at the microtubule-organizing center, mutations in the dynein machinery impair autophagosome clearance. Interestingly, the presence of aggregates in MND models supports a deficiency in autophagy as a secondary disease mechanism in these disorders. Indeed, an increase in autophagosome number and LC3-II levels can be observed in mice with dynein mutations (272, 413). Further research will be needed to clarify the relative importance of autophagy dysfunction in MND, particularly in forms not due to primary mutations of the dynein machinery, but where axonal transport deficiencies have been reported.

Another group of diseases that manifest impaired autophagic flux are due to mutations in the endosomal sorting complexes required for transport (ESCRT) complex machinery, which is critical for degradation of various integral cell membrane proteins. The turnover and recycling of integral membrane proteins from the plasma membrane is carried out largely by different functions of the endocytic pathway. Simple sorting by endosomes recycles proteins, returning them to the plasma membrane. A more intricate degradative system utilizes the multivesicular body (MVB), a specialized vesicular structure generated by invagination of the endosomal membrane to form luminal vesicles. The fusion of MVBs with lysosomes releases the vesicles into the acidic lumen of the lysosomes within which hydrolases degrade the vesicles and their cargo. The sorting of integral membrane proteins into the MVB pathway is dependent on monoubiquitination of the protein, a signal that designates the protein for incorporation into the luminal vesicles of MVBs (173, 316, 420, 424, 478). The fidelity of this process from protein sorting to endosomal-lysosomal fusion is maintained by the sequential interaction of four complexes termed the ESCRT complexes. The specific interactions of these complexes are necessary for the formation of the MVB and proper progression of endosomal-lysosomal fusion (reviewed in Ref. 567). Each of the ESCRTs is recruited to their particular function through multiple interactions with proteins, membranes, endosomes, and other ESCRTs. Early cargo sorting is carried out by the interactions of ESCRT-0, -I, -II with ubiquitinated cargo. ESCRT-III is required for disassembly of the ESCRT complex and recruitment of deubiquitinating enzymes (DUBs) to remove ubiquitin from the cargo prior to degradation. The final actions of ESCRT-III concentrate cargo in late endosomes.

In addition to known roles in HIV budding (349) and tumor suppression (344, 527, 542), the ESCRT machinery has been implicated recently in neurodegenerative disorders, such as frontotemporal dementia linked to chromosome 3 (FTD3) (491) and amyotrophic lateral sclerosis (ALS) (345, 386). A point mutation in the ESCRT-III subunit CHMP2B that alters a splice-site within the gene was found to be associated with a rare familial autosomal dominant form of FTD3 (491). Additionally, two different point mutations in CHMP2B have also been associated with a non-SOD1 form of ALS (345, 386). Despite the association of specific ESCRT machinery mutations with neurodegenerative disorders, the mechanism of disease remains largely unknown.

Expression of a deletion mutant of CHMP2B, synonymous to the splice mutant, increases neuronal and dendritic loss through an apoptosis-independent pathway (282). In cell and fly models, expression of this deletion mutant increased LC3-II levels, caused an accumulation of autophagosomes, and decreased the formation of MVBs. Experimental characterization of CHMP2B and a mutant form of SKD1 (356), first shown with the yeast homolog Vps4 (484) that prevents dissociation of the ESCRT-III complex prior to the next round of endocytic cargo sorting, indicates that the proper dissociation of the ESCRT-III complex is critical to both autophagosome maturation and proper fusion of autophagosomes with...
lysosomes. ESCRT dysfunction has been shown to cause an accumulation of autophagosomes in cortical neurons and flies (282).

The effect of ESCRT dysfunction on autophagy has been studied by the expression of mutants or the knockdown of genes in all three complexes and associated proteins (associated proteins: SKD1/Vps4 (356, 484) and fab1 (428); ESCRT-III: CHMP2B (109, 282), mSnf7–2/Vps32 (282, 428), and Vps24 (109); ESCRT-II: Vps25 (428) and Vps22 (109); ESCRT-I: Vps28 (428), Tsg101/Vps23 (109)). Data accumulated from studies of loss of function of various ESCRT genes in a wide range of cell types confirm that these defects result in deficient maturation of autophagosomes or in their inability to fuse with lysosomes and endosomes. Autophagosomes accumulate without degradation of their cargo leading to neurodegeneration in many cases. Additionally, many of these tissues display an increase in polyubiquitinated inclusions. It remains unclear whether this deficiency in autophagy is a direct effect of the disruption of the ESCRT complex on autophagosomes or indirect via endosomes and lysosomes. Overall, recent research suggests that the deficiency in autophagy caused by ESCRT dysfunction contributes to the neurodegeneration in FTD3 and ALS as a secondary disease mechanism.

IX. AUTOPHAGY AND AGGREGATE-PRONE PROTEINS

A. Autophagy Induction for Various Neurodegenerative Diseases

One of the areas where there is much support for autophagy induction as a therapeutic strategy is in a range of adult-onset neurodegenerative diseases. One of the common pathological features of most adult-onset human neurodegenerative diseases is the formation of intracytoplasmic aggregates within neurons and other cell types. For example, this is seen in AD (with tau accumulation), in tauopathies caused by tau mutations, in Parkinson’s disease (where α-synuclein forms Lewy bodies), and in polyglutamine expansion diseases, like Huntington’s disease, where the mutant protein is the primary constituent of the aggregates. Extensive data suggest that these aggregate-prone proteins mediate toxicity primarily via gain-of-function mechanisms associated with their propensity to aggregate (183).

In general, these intracytoplasmic aggregate-prone proteins are good autophagy substrates (415, 416). Intraneuronal proteins will be inaccessible to autophagy unless they have a significant intracytosolic residence time - for instance if they shuttle between the nucleus and cytoplasm) (192). This autophagy dependence has been demonstrated with both wild-type and mutant forms of tau (18), mutant forms of α-synuclein that cause familial Parkinson’s disease (559), and polyglutamine-expanded proteins causing Huntington’s disease, Kennedy’s disease, and the most common spinocerebellar ataxia (spinocerebellar ataxia type III) (18, 347, 415, 416). When autophagy is blocked, the clearance of the SDS-soluble forms of these proteins is retarded, leading to increased aggregation (as aggregation is a concentration-dependent phenomenon), and increased toxicity. Importantly, when autophagy is induced, the clearance of these proteins is increased and toxicity is reduced. This has been demonstrated in cell models, Drosophila, zebrafish, and mouse models for mutant huntingtin with both mTOR-dependent and mTOR-independent autophagy-inducing agents, and in Drosophila for other proteins, including tau, and polyglutamine-expanded androgen receptor in Kennedy’s disease (18, 415, 416, 447, 448, 566). This raises the possibility that autophagy induction may represent a therapeutic strategy for these diseases by enhancing the removal of the toxic aggregate-prone proteins.

Interestingly, the nonaggregate-prone species of many of these proteins (e.g., huntingtin and α-synuclein) show a much lower dependency on autophagy for their clearance, compared with their mutant counterparts (447, 559). This may be because the aggregate-prone species become inaccessible to the proteasome’s narrow entrance when they form oligomers, making autophagy a default clearance pathway. In contrast, the wild-type forms can be rapidly cleared by the proteasome. However, it is also possible that additional posttranslational modifications to target proteins may also enhance selectivity for autophagic degradation [e.g., acetylation (202), ubiquitination (238)]. This may have additional benefits in certain neurodegenerative diseases, allowing preferential clearance of the mutant protein without affecting wild-type protein levels.

B. Alzheimer’s Disease

While autophagy upregulation appears to be beneficial in animal models of neurodegenerative diseases like Huntington’s disease and tauopathies, it is important to understand if this strategy may be beneficial for the most common late-onset neurodegenerative disease, AD. AD comprises 50–70% of all dementias. Although many of the genetic and environmental factors determining AD risk are still largely unknown, it is thought that the extracellular amyloid beta (Aβ) deposits play a causative role in the development of the disease, along with the intraneuronal tau aggregates. Autophagosomes and autophagolysosomes accumulate in the brains of patients suffering from AD and in a mouse model of AD (364). Some researchers believe that autophagy is upregulated in the early progression of the disease before deposition of Aβ plaques, due to an increase in the ratio of
LC3-II to LC3-I by western blot, an increase in LC3 immunofluorescence in AD brains compared with control, and an increase in autophagic vesicles by electron microscopy. Additionally, Aβ production, measured by secretion of Aβ into the medium in cell models, is increased under conditions that induce autophagy and decreased when autophagy is blocked. It is postulated that the autophagosome may be the main site of Aβ processing, as Aβ processing enzymes and Aβ colocalize with autophagosomes (588, 589).

Conversely, it is thought that later in the progression of AD, autophagy is actually inhibited, possibly due to the accumulation of Aβ in lysosomes (28). The accumulation of this protein inhibits further lysosomal degradation of autophagic contents after the fusion of autophagosomes with lysosomes. Over time this leads to the accumulation of unfused autophagosomes and a block in autophagic flux, which may contribute to neurodegeneration. It is not completely clear if the accumulation of autophagosomes results mainly from the upregulation of autophagy, the inability of autophagosomes to mature via successful degradation by lysosomes, or some combination of the two. Despite these complexities, there have been suggestions that autophagy upregulation may be beneficial in AD. Beclin 1 appears to be a major player in autophagy deficiency in AD (395), since its protein and mRNA levels are lower in both human and mouse models of AD. This relationship seems to be inversely correlated with both progression of AD and age, as younger human patients and younger mouse models did not have lower levels of the protein. This decrease in Beclin 1 levels is specific to the neuronal populations affected in AD pathology, furthering the correlation between Beclin 1 and AD. Overexpression of Beclin 1 in AD mice models reduced intracellular accumulation of Aβ and extracellular deposition of Aβ plaques, while Aβ deposition and neurodegeneration were exacerbated by reduced Beclin 1 expression, thus providing support for its causal role in disease. These data raise the possibility that autophagy upregulation may be beneficial in AD by decreasing the levels of the two aggregate-prone proteins that characterize its pathology: tau and Aβ.

C. Autophagy and α1-Antitrypsin Deficiency

In addition to regulating clearance of intracytoplasmic aggregate-prone proteins, autophagy may also be relevant to diseases caused by proteins that aggregate in the ER, like α1-antitrypsin (A1AT) deficiency (103). A1AT is a serine protease inhibitor mainly synthesized by the liver. A1AT deficiency is an inherited autosomal codominant disorder, and it is the most common genetic cause of liver disease in children. It also causes chronic liver injury and hepatocellular carcinoma in adults (103).

Approximately 95% of all A1AT-deficient individuals are homozygous for the mutant Z allele of A1AT (A1ATZ), caused by a single point mutation at position 342 in the A1AT gene encoding the substitution of lysine for glutamate (Glu342Lys), thus producing the mutant protein PiZ (203). This mutation alters the folding pathway of the protein and makes it prone to polymerization within the rough ER of hepatocytes (305). Between 80 and 90% of this mutant protein is retained in the ER (4), and this retention is suggested as the basis for the pathogenesis of hepatic disease by a gain-of-toxic function mechanism. Studies using mice transgenic for human A1ATZ support this toxic gain-of-function mechanism for the liver disease in A1AT deficiency (43). These suggest that the pathways implicated in the disposal of the PiZ must play a role in the protection of the hepatocyte.

Studies in both mammalian and yeast systems have demonstrated that the proteasome participates in degradation of PiZ (524, 565). However, this pathway is not sufficient to allow the clearance of all the mutant protein. Autophagy is the other pathway implicated in degradation of PiZ. Autophagic vesicles have been observed in fibroblast cell lines expressing PiZ, in the liver cells of mice transgenic for human A1ATZ and in the hepatocytes of patients with A1AT deficiency (525). In cell line models, PiZ degradation was partially abrogated by chemical inhibitors of autophagy (525). Experiments in murine embryonic fibroblasts from Atg5-null mice showed a progressive accumulation of PiZ, which was detected in autophagosomes when autophagosome-lysosome fusion was inhibited by coexpression of a dominant-negative Rab protein (214). With the use of yeast models, it has been described that the absence of Atg6 or Atg14 reduced the clearance of PiZ (262).

Recently, it has been described that a mutant subunit of fibrinogen, which aggregates in ER and causes liver damage, in a rare form of inherited hypofibrinogenemia also depends on autophagy for disposal (263). These observations provide even more powerful evidence for the importance of autophagy in the response to the accumulation of aggregate-prone proteins in the ER that can cause liver damage.

X. OTHER DISEASES ASSOCIATED WITH CHANGES IN AUTOPHAGY

There are many other human diseases that have been associated with changes in autophagy, including muscle diseases, cardiomyopathies, stroke, pancreatitis, and cancer. While the causal role of autophagy in these conditions is often unclear or controversial, it is useful to review the current status of the literature, particularly given the importance of these conditions.
A. Autophagy in Muscle Atrophy, Myopathy, and Cardiac Disease

Skeletal and cardiac muscle are highly plastic tissues, capable of remodelling and adapting due to physiological stimuli and disease. There is accumulating evidence that autophagy is crucial for normal muscle function and may contribute to muscle remodelling. Furthermore, aberrant autophagy may cause myofiber dysfunction and myopathy.

Skeletal muscle acts as a large store of protein that can be catabolized during nutrient deprivation or disease to provide free amino acids that can be used for protein synthesis in vital organs (i.e., heart, brain, lungs), or for the generation of energy via hepatic gluconeogenesis. Muscle atrophy can occur in specific muscles in response to inactivity or denervation, or as a result of fasting or diseases such as cancer, AIDS, sepsis, diabetes, and renal failure (215). It was initially thought that the UPS was primarily responsible for the degradation of muscle proteins during atrophy (277). Indeed, activity of the UPS is elevated during atrophy due to the increased transcription of a set of atrophy-promoting genes termed atrogenes (27, 140). The atrogenes comprise several proteasome subunits and two muscle specific E3 ligases, atrogin-1 (MAFbx) and MuRF1 (27, 140). MAFbx and MuRF1 knockout mice are indistinguishable from wild-type mice (normal weight and muscle morphology) but have reduced levels of skeletal muscle atrophy in response to fasting and denervation (314, 600). Expression of constitutively active FoxO3 induces autophagy in skeletal muscle (314, 600), and FoxO3 binds directly to the promoter region of the LC3 gene in muscle from fasted mice (314). Thus the FoxO3 transcription factor can coordinately regulate the activity of both autophagy and the UPS in muscle cells in response to atrophy-inducing signals (Fig. 5). The IGF-I signaling pathway is able to regulate autophagy in skeletal muscle both via mTOR and FoxO3 (Fig. 5).

Bnip3 is thought to play a major role in mediating the effect of FoxO3 on autophagy, as Bnip3 knockdown can markedly reduce the induction of autophagy caused by expression of constitutively active FoxO3 in mouse skeletal muscle (314). In addition, Bnip3 expression is sufficient alone to induce high levels of autophagy in skeletal muscle (156, 314, 534) (The role of Bnip3 in autophagy in muscle will be discussed below.) Thus both Bnip3 and FoxO3 are candidate therapeutic targets for the treatment of muscle-wasting disorders.

Valosin-containing protein (VCP) belongs to a AAA+ ATPase family of proteins, mutations in which result in inclusion body myopathy (IBM) and Paget’s disease of the bone as well as frontotemporal dementia (IBMPFD). Muscles of patients have degenerating fibers, rimmed vacuoles (RV), and sarcoplasmic inclusions containing ubiquitin. A recent study has shown that inhibition of VCP activity results in impaired autophagosome-lysosome fusion leading to accumulation of autophagosomes. Expression of an IBMPFD VCP mutant showed similar results, with an accumulation of nondegradative autophagosomes (207).

The role of autophagy in cardiac disease is controversial, and the induction of autophagy can be protective or detrimental, depending on the disease state being studied and the experimental paradigm. The induction of autophagy in the heart may be protective by removing damaged proteins and organelles, and by the recycling of proteins, glycogen, and fatty acids, thus providing energy for the myofiber during starvation/energy deprivation. However, autophagic cell death has been implicated in cardiac disease (102, 244, 429, 482).

The importance of autophagy in maintaining normal cardiac function and in cardiac myofiber homeostasis was highlighted by studies showing dysfunction and disease in autophagy-deficient hearts (266, 354, 513). Mice deficient in Atg5 die postnatally with reduced levels of free amino acids and energy depletion in the heart, suggesting that autophagy is important for energy homeostasis during...
neonatal starvation (266). The inducible knock-out of Atg5 in the hearts of adult mice causes cardiac hypertrophy, left ventricular dilation and contractile dysfunction, accompanied by increased levels of ubiquitinated proteins and aggregated mitochondria (354). In addition, LAMP-2-deficient mice have compromised autophagy and show increased postnatal lethality with surviving mice developing a cardiomyopathy that mimics human Danon disease (a disorder caused by mutations in LAMP-2, discussed earlier) (513).

Cardiac muscle is a highly active tissue that is constantly under stress and hence has a rapid turnover of proteins. Levels of ubiquitinated proteins are elevated in hearts that show features of cardiomyopathy and failure (557) and in pressure-loaded hearts (520), and the induction of autophagy may be important in reducing this. Desmin-related cardiomyopathy (DRCM) is caused by mutations in the small heat shock protein αβ-crystallin (551) and is characterized by the formation of desmin and αβ-crystallin containing aggregates in cardiac tissue (438). Expression of mutant αβ-crystallin induces high levels of autophagy in the cardiomyocytes of DRCM transgenic mice (519). Mice overexpressing mutant αβ-crystallin (DRCM mice) were crossed with hemizygous Beclin 1 knockout mice. Inhibition of autophagy (by reducing Beclin 1 levels) dramatically accelerated myopathy and heart failure in DRCM mice, suggesting that the induction of autophagy in DRCM hearts is a protective response (519).

In addition to degrading damaged proteins, autophagy is also important for removing damaged organelles, particularly mitochondria (230, 414). Damaged mitochondria are central to the intrinsic pathway of apoptosis by releasing cytochrome c and apoptosis inducing factor (AIF) that activate downstream executioner caspases. Mitochondria also cause cellular damage by producing ROS. Removal of mitochondria may be particularly important during ischemia/reperfusion injury when oxygen starvation and subsequent reoxygenation leads to the uncoupling of oxidative phosphorylation, opening of the mitochondrial permeability transition pore, and subsequent swelling and damage to the mitochondria (153). Ultrastructural studies have shown the presence of mitochondria in the autophagosomes of lamb fetal heart cultures after hypoxia (476), and ischemia has been shown to upregulate autophagy in the heart (82, 102, 578, 579).

Induction of autophagy during ischemia is likely to be induced, in part, by AMPK. AMPK is the cell’s energysensing kinase and is activated by AMP, a sensitive indicator of the ADP/ATP ratio in the cell. Glucose depriv-
tion-induced autophagy is inhibited by dominant-negative AMPK in cultured cardiomyocytes, and ischemia-induced autophagy is reduced in hearts from mice overexpressing dominant-negative AMPK (328). In most cells, active AMPK can phosphorylate TSC2 leading to inhibition of mTOR and induction of autophagy (187). However, studies of ischemic tissue showed an induction of autophagy and active AMPK, associated with phosphorylation of eukaryotic elongation factor-2 (eEF-2), but not inhibition of mTOR (177, 328). Another mechanism mediating ischemia-induced autophagy is Bnip3. Bnip3 is a Bcl2-related, BH3-only protein that is induced by hypoxia in cardiomyocytes (265) and in ischemic regions of tumors (375). Dominant-negative Bnip3 blocks autophagy in cardiomyocytes during ischemia/reperfusion injury (156), and knocking out Bnip3 blocks autophagy in tumor cells (534). Bnip3 may induce autophagy by titrating Bcl2 and/or Bcl-xL from Beclin 1 (534), or by binding Rheb and inhibiting its GTPase activity thus inhibiting mTOR (297). ROS may also mediate ischemia-induced autophagy, since uncoupled mitochondria produce elevated levels of ROS which induce autophagy (462). Autophagy may also be induced by ER stress and the UPR, which is activated in ischemia/reperfusion injury and failing hearts (137).

Autophagy may be protective in ischemia. Prolonged cardiac ischemia causes a larger myocardial infarction in AMPK dominant-negative mice (with reduced levels of autophagy), compared with wild-type mice (328), and rapamycin pretreatment is protective against ischemia/reperfusion injury in cultured mouse cardiomyocytes (224). In contrast, the induction of autophagy during reperfusion may be detrimental to the cardiomyocyte (328, 543). During reperfusion, AMPK is no longer activated, but increases in Beclin 1 are observed (328). The induction of autophagy during reperfusion was reduced in hemizygous Beclin 1 knock-out mice, and this was associated with decreased cardiac infarction and cardiomyocyte death (328). In accordance, inhibiting autophagy by treatment with 3-MA protects cultured cardiomyocytes (543). Therefore, autophagy is induced by different intracellular signaling pathways in ischemia compared with reperfusion and has very different effects on cardiomyocyte survival in these contexts (Fig. 6).

The heart undergoes compensatory hypertrophy in response to hypertension or myocardial infarction (hemodynamic stress), which may lead to heart failure. Autophagy is inhibited during cardiac hypertrophy (78, 393), and induction of autophagy by treatment with rapamycin can prevent hypertrophy caused by aortic banding (149) and thyroid hormone treatment (271). Rapamycin was even able to reduce established cardiac hypertrophy and improve cardiac function in pressure-overloaded rats (331). Rapamycin most likely exerts its effects on cardiac myocyte size by both inhibiting mTOR-dependent protein synthesis and by activating protein degradation (autophagy).

Despite inhibiting cardiac hypertrophy, autophagy may, however, enhance the transition from hypertrophy to cardiac failure. Pressure-load-induced hypertrophy upregulated autophagy, but cardiac remodelling and heart failure were reduced in Beclin 1 hemizygous knockout mice that have reduced autophagy (607). Conversely, Beclin 1 overexpression induced autophagy and accentuated cardiac remodelling (607). The conflicting results of these studies may be due to the signaling pathways used to activate autophagy.

\[ \beta \text{-Adrenergic stimulation can also induce cardiac hypertrophy and heart failure (571) and inhibits autophagy in cardiomyofibers (393). Treatment with the } \beta \text{-adrenergic agonist isoproterenol caused cardiac hypertrophy in Atg5 knockout but not wild-type mice after only 7 days (354). This suggests that autophagy can protect against cardiac disease induced by adrenergic stimulation.} \]

B. Autophagy and Pancreatitis

The pancreas performs several duties critical to optimal digestive and metabolic function, including secretory enzymes (e.g., trypsin) via the pancreatic duct into the duodenum to combine with bile produced by the liver to aid digestion. Under normal physiological conditions, these powerful enzymes are synthesized as inactive precursor proenzymes (e.g., the trypsin precursor trypsinogen) that are stored safely in membranous zymogen granules in the pancreas (374). In acute pancreatitis, inappropriate and premature intrapancreatic activation of trypsin from trypsinogen leads to protease-mediated lytic forces being unleashed on the cellular structure of the pancreas itself rather than the intended target of intraluminal digestive tract contents (175).

When pancreatic acinar cells are exposed to alcohol, pancreatitis is mediated by fatty acid methyl esters, which are nonoxidative products of alcohol and fatty acids. These fatty acid methyl esters cause the release of Ca\(^{2+}\) from internal stores via IP\(_3\) receptor activation, and the Ca\(^{2+}\) results in necrosis due to the intracellular trypsin
activation (135). It appears that the abnormal trypsin activation in acute pancreatitis depends on endocytic vacuole formation and trypsinogen activation in that compartment (479).

Nearly three decades ago, electron microscopy studies noted an increased number of autophagosomes in patients suffering from acute necrotizing pancreatitis (169). Recent studies have shown that this increase in autophagosome number is due to defective autophagosome-lysosome fusion (114, 115). In experimental models of pancreatitis, this may be caused by a reduction in the lysosomal protein LAMP-2, which is required for normal autophagosome-lysosome fusion (114, 115). Another mechanism that may contribute to impaired autophagosome-lysosome fusion may be via IP3-mediated emptying of Ca2+ stores, since thapsigargin, a drug which empties these stores, also blocks autophagosome-lysosome fusion and causes a net increase in autophagosome numbers (566). The situation in pancreatitis may be exacerbated by impaired processing of the lysosomal proteases cathepsins B and L, which would further reduce lysosomal function (479). It has been suggested that the retarded autophagy in pancreatitis may cause an imbalance in the activities of the cathepsins B and L deficiencies, which may contribute to this disease, since cathepsin L degrades trypsinogen and trypsin, while cathepsin B converts trypsinogen to trypsin (479). Thus it has been proposed that the deleterious effects of cathepsin B in this context may outweigh the beneficial effects of cathepsin L, when the activities of both enzymes are reduced in pancreatitis.

Another possible link between pancreatitis and autophagy may be via the protein vacuolar membrane protein 1 (VMP1), which is induced during acute pancreatitis. VMP1 is a transmembrane protein able to induce autophagy even when cells are nutrient replete, while its expression is triggered by traditional precipitants of autophagy, including starvation, as well as the mTOR inhibitor rapamycin. Conversely, VMP1 small interfering RNA (siRNA) prevents autophagosome formation even in the presence of rapamycin or starvation (114). VMP1 also colocalizes with LC3 and works in addition with Beclin 1. While these VMP1 findings suggest a possible link between increased autophagy and pancreatitis, they do not address whether autophagy is protective or deleterious in this condition. However, these VMP1 studies are consistent with conclusions that pancreatitis may require autophagosome synthesis. Atg5 knockout in acinar cells protects mice from experimental pancreatitis, and this is associated with a significant reduction in trypsinogen activation (166, 317). Thus it is possible that the combined effects of increased autophagosome synthesis and decreased autophagosome-lysosome fusion may create a large pool of autophagosomes, which help to cause this disease.

C. Autophagy and Stroke

Stroke is defined as a neurological deficit of cerebrovascular cause that persists beyond 24 h. Deficits following cerebrovascular events which last less than 24 h are arbitrarily defined as “transient ischaemic attacks” or TIAs. Cerebral vascular accidents are most commonly occlusive in nature (accounting for some 80% of cases), with hemorrhagic stroke accounting for the remaining 20% (94). For this reason, most “stroke” research has concentrated on ischemia/reperfusion models of disease, and for the remainder of this review, we use the word stroke to describe only ischemia/reperfusion injury. Stroke is a major focus of biomedical research, because it is both common and results in significant mortality and morbidity; it is the second most common cause of death worldwide and a major cause of disability (94). The relationship between autophagy and ischemic stroke is complex, with evidence for both protective and deleterious roles for autophagy in this condition.

It has long been thought that a possible protective strategy for severe ischemia may be to expose tissues to previous, less severe ischemic insults and thus activate protective cellular responses prior to a larger insult, so-called ischemic preconditioning or IP (303). This technique has been found to be effective in limiting the damage caused by subsequent larger ischemic insults, and the mechanisms for this protective phenomenon have been investigated. One of many events that occur during ischemic preconditioning is the upregulation of autophagy. The mechanisms for the autophagy induction are not completely clear, but it is known that ischemic preconditioning results in an increase in ROS levels (which can induce autophagy) and that these seem to be important for the success of the technique, as ROS scavengers seem to lessen the effectiveness of IP (303). Similarly, pharmacological inhibitors of autophagy, such as 3-MA, have been shown to ameliorate the protective effects of IP in cell and animal models of ischemic stroke (303, 384).

Whilst it is possible autophagy may have a role to play in the protection seen with ischemic preconditioning, it may have a very different effect once a major episode of ischemia/reperfusion has occurred. Beclin 1 and autophagy are upregulated in rodents following ischemia/reperfusion injury, and the observation of high levels of cell death and autophagy in the absence of caspase activation has led to the suggestion that autophagy may be an important cell death pathway in these circumstances (1, 412). Introduction of beclin 1 RNAi via a lentiviral vector simultaneously with induction of ischemia in rats led to decreased infarct volume and neurological deficit, whilst Atg7-deficient neonatal mice exhibit almost complete protection from neuronal death following hypoxic/ischemic injury (246, 602). Note that experiments in adult Atg7-deficient mice are difficult, as Atg7 deficiency itself results
in neurodegeneration (249). Similarly, pharmacological autophagy inhibitors such as 3-MA significantly decrease infarct size (by nearly 50%), even when given more than 4 h after the onset of ischemia. Pan-caspase inhibitors had no such effect (404).

Although the study of autophagy in the context of cerebral ischemia is in a relatively early stage of development, compared with some other areas of autophagy research, the current experimental evidence, as summarized above, provides some exciting and testable hypotheses that may be of significant clinical relevance. It appears that autophagy is an essential part of the protective effect of IP. If this is indeed the case, then it may be that upregulating autophagy prior to a severe ischemic insult may be protective. This is nontrivial, as individuals at high risk of subsequent ischemic events (for example, those who have suffered a TIA) can be identified and could be targeted for treatment with autophagy upregulating drugs. Early evidence from in vitro experiments of ischemia reperfusion in other organs suggests this approach may have promise (224). Equally, once the infarct has occurred, then autophagic defenses may be overwhelmed, and current evidence suggests autophagy may play a significant role in neuronal death. The finding that autophagy inhibitors limit damage, even when given hours after the ischemic event, raises the tantalizing possibility that autophagy-inhibiting drugs may be effective in limiting the damage following acute stroke when administered in a timeframe achievable within clinical practice.

D. Autophagy and Cancer

The role of autophagy in cancer is complex and highly debated (Fig. 7). On one hand, autophagy, as a housekeeping process capable of preventing accumulation of toxic cellular waste, some of which may be carcinogenic, can act as a tumor suppressor. On the other hand, the ability of autophagy to support cell survival in conditions of hypoxia and nutrient deprivation may assist the survival of tumors, as these need to develop strategies to survive in sites where there is poor vasculature or reduced nutrient delivery to the core of a lesion. We will discuss both of these potential roles for autophagy in cancer.

Much circumstantial support exists for the concept that autophagy may act as a tumor-suppressor pathway. Many of the signaling pathways leading to tumorigenesis (e.g., upstream regulators in the mTOR signaling) overlap with those regulating autophagy. Furthermore, a number of autophagy genes such as Beclin 1, ATG5, ATG4c, and Bif-1 also have properties of tumor suppressors in mice (318, 406, 506, 585). Thus, for example, ATG4 knockout mice treated with a carcinogen have higher frequency of tumorigenesis compared with wild-type controls (318). Another argument in support of autophagy being protective against cancer is that the products of several tumor suppressor genes, such as DRAM, PTEN, DAPK, TSC1, and TSC2, positively regulate autophagy (6, 65, 108, 184). The same could also be true, at least in part, for the most frequently mutated in human cancers gene p53 (65, 108), though its role in autophagy remains controversial (see below). p53 can transactivate genes that induce autophagy, like DRAM and sestrin 1 and 2 (35, 65). Conversely, oncogenes such as class I PI3K, Akt/PKB, and Bcl-2, among others, inhibit autophagy (6, 389). Needless to say, the cellular functions of all these proteins are not limited to the regulation of autophagy, and therefore, this argument remains rather circumstantial. Autophagic activity is also reduced upon oncogenesis in murine pancreatic cancer models (417, 533). Thus, in general, there is a positive correlation between molecules that induce autophagy and tumor suppression, and between molecules that inhibit autophagy and tumor progression. As the cellular functions of these proteins are not limited to the regulation of autophagy, the case for their link with cancer is confounded by this caveat.

The strongest support to date for the role of autophagy as an oncosuppressor pathway probably comes from the observations that autophagic genes like Beclin 1, UVRAG, and Bif-1 are mutated in human cancers, which presumably leads to the impairment of autophagic activity in mutant cells (61, 188, 232, 298, 300, 590). The list may potentially be extended in the future to other autophagy genes, including ATG7 and ATG8 that are part of the loci frequently deleted in various types of tumors (12, 14, 51, 101, 339, 502). The high penetrance of such mutations in human cancers (Beclin 1, for example, is monoallelically deleted in 40–75% of sporadic human breast, ovarian, and prostate cancers; Refs. 129, 430, 515) suggests that impairment of autophagy might be an important
step of tumorigenesis. Indeed, hemizygous inactivation of Beclin 1 in mice causes increased cancer in these animals (590), while Beclin 1 overexpression suppresses tumor growth (300). However, the interpretation that these effects are due to autophagy needs to consider the caveat that Beclin 1 may have autophagy-independent functions. Indeed, Beclin 1 null mice exhibit embryonic lethality, while ATG5 or ATG7 null mice are born normal, if bred from heterozygous parents (251, 266, 590).

Several potential mechanisms can account for tumor suppression by autophagy. Induction of autophagy may lead to so-termed type II apoptosis-independent cell death. Type II cell death has initially been suggested on the basis of experiments with apoptosis-inhibited L929 cells, where chemical inhibition or knockdown of autophagy proteins suppressed cell death (586). It has also been reported that apoptosis-impaired MEFs treated with apoptosis inducers die of autophagy-dependent cell death (481). These initial observations were followed by numerous subsequent studies presenting similar findings both in vitro and in vivo (261, 312). In agreement with this, radiation treatment as well as many potential anticancer drugs (including rapamycin, tamoxifen, arsenic trioxide, histone deacetylase inhibitors, etc.) are known to induce autophagy, possibly leading to autophagic cell death (36, 218, 378, 456, 510). However, the sole existence of cell death by autophagy has recently become a subject of intense criticism and urgently requires further clarifications, as discussed above (261, 292, 293, 459).

Recently, White and colleagues (324) proposed two alternative explanations for a role of autophagy as a tumor suppressor independent of its pro-death or pro-survival effects. First, autophagy can prevent DNA damage, centrosome abnormalities, aneuploidy, and chromosomal defects especially in metabolically stressed cells (220, 326). This is likely to be due to the role of autophagy in the removal of damaged proteins and organelles, primarily mitochondria, thereby preventing increases of ROS and hence further damage following genomic instability (206). A recent study by White’s group has shown that tumor formation occurs in autophagy-deficient cells as a consequence of p62 accumulation, which enhances DNA damage by increasing the levels of ROS (325). Thus impaired autophagy can promote genomic instability leading to oncogenic activation and tumor progression. Second, autophagy may also achieve its oncoprotective role by preventing necrosis (83); impaired autophagy in metabolically stressed tumor cells that cannot die by apoptosis can enable death by necrosis, which is often associated with a chronic wound-healing response that is linked to tumor growth (83).

Another mechanism whereby autophagy may be protective against cancer is because it enables efficient cross-presentation of antigens (296). This process is important for the induction of adaptive immunity against cancer cells (and infectious agents).

While the idea of autophagy as a tumor suppressor process became well established over the past three decades and has been embraced by the majority of the scientific community, an equally impressive (if not more extensive) literature exists supporting the role of autophagy in the promotion of cancer. The simplest possible reason for this might be that autophagy contributes to tumor survival by allowing cells to sustain themselves under conditions of nutrient deprivation, and tumor cells may partially rely on autophagy for energy production. Indeed, it has been found that despite an overall reduction of autophagic activity in various malignancies, autophagosomes remain present, especially in the most metabolically stressed regions of the tumor (370, 402, 454). Metabolomic profiling of colon and gastric cancer tissues revealed very low glucose and high lactate and glycolytic intermediate concentrations, suggesting enhanced glycolysis and the Warburg effect. The clear accumulation of all amino acids except glutamine in the tumors was compatible with enhanced autophagic degradation of proteins and active glutamine breakdown for energy production, i.e., glutaminolysis (174). In this regard, it is interesting that Beclin 1, unlike many other tumor suppressor genes, has never been found to be biallelically mutated in malignant cells, suggesting certain dependence of tumors on autophagy (406, 590). In agreement with this, complete removal of Beclin 1 or Atg5 from cancer cells slows their proliferation and facilitates apoptotic cell death, suggesting a protective role for autophagy against apoptosis (30, 83, 179, 220). Similarly, pharmacological inhibition of autophagy in colorectal tumors causes nutrient deprivation-induced cell death, highlighting an essential role of autophagy for the survival of cancer cells (454). Another argument in favor of autophagy as a pro-tumorogenic process comes from the recent discovery that the reduction of p53 levels in many tumors can induce autophagy (522). This finding is rather unexpected taking into account the earlier evidence in favor of p53 as a positive regulator of autophagy (65, 108). Although the specific molecular mechanism of the negative regulatory effect of p53 on autophagy awaits further elucidation, this function of p53 seems to be independent of its transcriptional activity and is mediated by cytoplasmic, not nuclear, p53 (350, 522). It has been speculated that upregulation of autophagy in p53 mutant cells could potentially have the advantage for cancerous cells, as autophagy may increase their resistance to apoptosis, possibly due to removal of pro-apoptotic mitochondria (350, 427, 522). These and other findings have suggested that autophagy upregulation after chemo- and radiotherapy may be an attempt for self-preservation by malignant cells (36, 190, 218, 252, 378, 456, 510).
Our current knowledge on the relationship between autophagy and cancer contains much apparently contradictory data, and sometimes, opposing interpretations of the same experimental evidence exist in scientific literature. This reflects an extreme diversity of various pathologies united under the common name cancer, varied experimental techniques used by the investigators, and different stages of the disease being studied. Taking into account the importance of the problem, untangling these contradictions should be prioritized. A common theme, however, is beginning to emerge in the field. It is based firmly on the appreciation of autophagy as a tumor-suppressing process protecting the normal cell from the insults caused by the accumulation of unfolded, dysfunctional, aggregated proteins, increased levels of ROS, and DNA damage (see Fig. 7). Thus functional autophagy is essential in preventing tumor initiation, and therefore, autophagy upregulation could potentially be exploited in prophylactic treatments. The prosurvival effects of autophagy could, however, also be exploited by existing tumors by allowing the transformed tissue to survive in conditions of hypoxia and undernourishment. Thus reducing autophagy may be of benefit in existing tumors. This has led to pharmacologically favorable inhibitors of autophagy, like chloroquine and its derivatives, being tested in clinical trials as sensitizers for radio- and chemotherapy in several malignancies. The initial results of chloroquine treatment of patients diagnosed with glioblastoma multiforme confirmed some improvement of midterm survival and called for large-scale trial studies (493). Caution, however, is called for in interpreting the results of these and other clinical trials as all the drugs, either inhibitors or inducers of autophagy, tested to date have pleiotropic effects in humans, and it remains to be demonstrated whether the effect on autophagy is causative or merely an epiphenomenon.

**E. Autophagy and Type 2 Diabetes**

Type 2 diabetes is characterized by a high blood glucose accompanied by insulin resistance and relative insulin deficiency. It typically presents in adults but can be seen in children. At a pathological level, it is associated with the loss of pancreatic beta-cell function and beta-cell loss. Recent studies have reported increased numbers of autophagosomes in beta cells from type 2 diabetics (320). This has led to a suggestion that autophagy may be contributing to the disease.

The interpretation of how autophagy modulates this disease is not straightforward. Complete loss of autophagy in beta cells leads to their degeneration and impaired insulin secretion (98, 122, 210). Interestingly, a high-fat diet (which predisposes to type 2 diabetes) increases autophagy in beta cells in control mice and causes a dramatic loss of glucose tolerance in beta cell autophagy-deficient mice. These data suggest that autophagy allows these cells to respond appropriately to high-fat diets.

On the other hand, autophagy may contribute to type 2 cell death in type 2 diabetes (118). The pancreatic duodenal homeobox (Pdx1) gene encodes a transcription factor that regulates beta-cell homeostasis and survival. Complete loss of this gene causes pancreatic agenesis while hemizygous loss leads to beta-cell death and diabetes. Pdx1 may be an important player leading to beta-cell death in patients exposed to chronic high levels of glucose (reviewed in Ref. 119). In mice with hemizygous loss of Pdx1, there is increased autophagy. When Pdx expression was reduced in mouse insulinoma cells using RNAi, there was also increased autophagy, which preceded cell death. Blocking autophagy alleviated the starvation-induced cell death in this in vitro model. These findings were then extended to an in vivo study where Beclin 1+/− mice were crossed with the Pdx1+/− mice. Double hemizygous mice (with decreased Pdx and Beclin 1) had improved beta-cell function and beta-cell mass compared with Pdx1−/− mice, suggesting that autophagy may be contributing to the toxicity in this model (118).

The apparent discrepancies between these studies may reflect a number of reconcilable issues. First, complete autophagy loss may not be relevant to diabetes and may reflect an extreme nonphysiological situation. Second, extrapolating from the Pdx1+/− to human type 2 diabetes, in general, may be unreliable; the Pdx1 deficiency may only represent a minor subgroup.

**XI. CONCLUDING COMMENTS**

Recent studies have shown the relevance of autophagy to many areas of normal human physiology and to some of the important diseases. It is likely that more diseases with autophagy associations will be discovered in the future. This is an exciting evolving area, which is enriched by the possibilities that basic cell biology is so closely tied up with understanding of disease pathogenesis and possible therapeutic strategies.

Yet, there are many areas of the basic biology of autophagy that are not understood. For instance, it is still unclear what the membrane sources are for autophagosomes, or how many of the signaling pathways regulating autophagy impact on its machinery. Only by understanding the fundamental cell biology of this process will we be able to maximize our understanding of its roles in disease, and the potential of autophagy modulation for the alleviation of human suffering.

**ACKNOWLEDGMENTS**

Address for reprint requests and other correspondence: D. C. Rubinsztein, Dept. of Medical Genetics, Univ. of Cam-
bridge, Cambridge Institute for Medical Research, Addenbrooke’s Hospital, Hills Road, Cambridge CB2 0XY, UK (e-mail: dcr1000@hernes.cam.ac.uk).

GRANTS

We are grateful for funding from a Wellcome Trust Senior Fellowship in Clinical Science (to D. C. Rubinsztein), a Medical Research Council Programme Grant (to D. C. Rubinsztein), Action Medical Research and The Rosetrees Trust (Research Training Fellowship to B. R. Underwood), the Sackler Trust, funding from the National Institute for Health Research Biomedical Research Centre at Addenbrooke’s Hospital, and The Daphne Jackson Trust and the Isaac Newton Trust (to F. H. Siddiqi and D. C. Rubinsztein).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

REFERENCES

6. Acker AT, Walker SA, Magazu M, Chandra P, Roderick HL. Regulation of autophagy by reac-


21. Berridge MJ. Inositol trisphosphate and diacylglycerol: two inter-

25. Bjorkoy G, Lamark T, Brech A, Outzen H, Perander M, Over-


Physiol Rev  Vol 90 • October 2010 • www.prv.org

http://physrev.physiology.org/ by 10.220.32.246 on October 19, 2017 www.prv.org


261. LaMonte BH, Wallace KE, Holloway BA, Shelly SS, Ascano J, Tokito M, Van Winkle T, Howland DS, Holzbaur EL. Disruption...


Nezis IP, Simonsen A, Sagona AP, Finley K, Gauner S, Contamine D, Rusten TE, Stenmark H, Brech A. Ref(2) P, the Dro sophila melanogaster homologue of mammalian p62, is required
403. Pizarro-Cerdà J, Moreno E, Gorvel JP. Invasion and intracellu-

404. Porter EM, Bevins CL, Ghosh D, Ganz T. The multifaceted

405. Prentice E, Jerome WG, Yoshimori T, Mizushima N, Denison
MR. Coronavirus replication complex formation utilizes compo-

406. Proikas-Cezanne T, Ruckerbauer S, Stierhof YD, Berg C, Nor-
dheim A. Human WIPI-1 puncta-formation: a novel assay to assess

7-bladed WIPI protein family, is aberrantly expressed in human
cancer and is linked to starvation-induced autophagy. Oncogene 23:

408. Puls I, Jannakuty C, LaMonte BH, Holzbaur EL, Tokito M, 
Mann E, Fleoter MK, Bidus K, Dryna D, Oh SJ, Brown RH Jr, 

409. Puyal J, Vasin L, Mottier V, Clarke PG. Postischemic treatment
of neonatal cerebral ischemia should target autophagy. Ann Neural

410. Pyne S, Pyne NJ. Sphingosine 1-phosphate signalling in mamma-

411. Qu X, Yu J, Bhagat G, Furuya N, Bisboosh D, Troxel A, 
Rosen J, eskellinen EL, Mizushima N, Ohsumi Y, Cattoretigi T, 

412. Qu X, Zou Z, Sun Q, Lubyl-Phekl P, Cheng P, Hogan RN, 
Glipin C, Levine B. Autophagy gene-dependent clearance of apop-

413. Rakic N, Hill V, Shea L, Takikita S, Baum R, Mizushima N, 
Rahlon E, Plotz P. Suppression of autophagy in skeletal muscle
uncoversthe accumulation of ubiquitinated proteins and their po-
tential role in muscle damage in Pompe disease. Hum Mol Genet

414. Raben S, Robinson BH. Mitochondria, oxygen free radicals, dis-

JJ, Israelien N, Naranian T, Paroutis P, Guo R, Ren ZP, 
Mahuran DJ, Kissel JT, Kalimo H, Levy N, 

416. Raha S, Robinson BH. The multifaceted 
structures.


418. Rohde KH, Abramovitch RB, Russell DG. Mycobacterium tu-

419. Rocca A, Lamaze C, Subtil A, Dautry-Varsat A. Involvement of

420. Rohde KH, Abramovitch RB, Russell DG. Mycobacterium tu-
berculosis invasion of macrophages: linking bacterial gene expres-

421. Rogers WW, Arboit ML, Vazquez CL, Colombo M. The au-
phagic pathway is a key component in the lysosomal dependent entry of Trypanosoma cruzi into the host cell. Autophagy 5: 6–18, 2009.


425. Rubin GP, Hungin AP, Kelly PJ, Ling J. Inflammatory bowel disease: epidemiology and management in an English general prac-

426. Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. Poten-
tial therapeutic applications of autophagy. Nat Rev Drug Discov

427. Rusten TE, Vaccari T, Lindmo K, Rodahl LM, Neizis IP, Sem-
Jacobson C, Wendler F, Vincent JP, Brech A, Bilder D, Sten-
mark H. ESCLtts and Fasl regulate distinct steps of autophagy. 

Kawasaki M, Tsuchiya K, Nishigaki K, Ishikawa S, Minatoguchi S, Kato K, 
Fujihara W. Cardiomyopathy with prominent autophagic degener-
ation, accompanied by an elevated plasma brain natriuretic peptide level despite the lack of overt heart failure. Intern Med 43:


430. Saitoh T, Fujita N, Jang MH, Uematsu S, Yang BG, Satoh T, 

431. Sakai Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: autophag-

432. Sakaki Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: autophag-

433. Sakaki Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: autophag-

434. Sakaki Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: autophag-

435. Sakaki Y, Oku M, van der Klei IJ, Kiel JA. Pexophagy: autophag-

436. Salminen A, Kaarniranta K. NF-kappaB signaling in the aging

437. Salminen A, Kaarniranta K. Regulation of the aging process by

438. Salminen A, Kaarniranta K. NF-kappaB signaling in the aging

439. Salminen A, Kaarniranta K. Regulation of the aging process by

440. Salminen A, Kaarniranta K. Regulation of the aging process by
MAMMALIAN AUTOPHagy IN PHYSIOLOGY AND PATHOPhYSIOLOGY


