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REVIEW ARTICLE

Autophagy: Regulation and role in disease

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Abstract

Autophagy, a lysosomal process involved in the maintenance of cellular homeostasis, is responsible for the turnover of long-lived proteins and organelles that are either damaged or functionally redundant. The process is tightly controlled by the insulin-amino acid-mammalian target of the rapamycin-dependent signal-transduction pathway. Research in the last decade has indicated not only that autophagy provides cells with oxidizable substrate when nutrients become scarce but also that it can provide protection against aging and a number of pathologies such as cancer, neurodegeneration, cardiac disease, diabetes, and infections.

Keywords: *Aging; lysosomes; amino acids; Atg proteins; ATP; cancer; ceramide; mitochondria; rapamycin; reactive oxygen species; signaling*

Abbreviations and Glossary: 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMBRA1, activating molecule in Beclin1-regulated autophagy; AMPK, AMP-activated protein kinase; *ATG*, AuTophaGy related; BAD, Bcl-xL/Bcl-2-associated death promoter; Bak, Bcl-2 homologous antagonist/killer; Barkor, Beclin 1-associated autophagy-related key regulator; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; Bcl-xL, basal cell lymphoma-extra large; Beclin, 1, coiled-coil, myosin-like Bcl-2 interacting protein; BH3, Bcl-2 homology domain; Bif-1, Bax interacting factor 1; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3; Cln3, ceroid-lipofuscinosis, neuronal 3; $\Delta\psi_{mit}$, mitochondrial membrane potential; DAPK, death-associated protein kinase; eEF2, eukaryotic elongation factor 2; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; Erk, extracellular signal-regulated kinase; FIP200, focal adhesion kinase (FAK) family interacting protein of 200 kDa; FoxO, Forkhead box O; GCN2, general control non-derepressible-2; HE, hepatic encephalopathy; HIF, hypoxia inducible factor; HIV, human immunodeficiency virus; HMGCoA, 3-hydroxy-3-methyl-glutaryl-CoA; hVps34, homolog of yeast Vps34; IGF1, insulin-like growth factor; Ire1, inositol-requiring enzyme 1; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; Lamp-2, lyosomal-associated membrane protein 2; LC3, microtubule-associated protein 1 light chain 3, the mammalian homolog of yeast Atg8; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblasts; MHC, II, major histocompatibility complex class II; mTOR, mammalian target of rapamycin; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; *NPC1*, Niemann-Pick type C disease 1; p38, p38 mitogen-activated protein kinase; *PARK2*, Parkinson disease 2, Parkin; PDK1, phosphoinositide-dependent kinase-1; PE, phosphatidylethanolamine; PERK, PKR-like ER kinase; Pex, peroxisomal import protein; PI3K, phosphatidylinositol 3-kinase; PINK1, PTEN-induced kinase 1; PKB, protein kinase B; PKR, double-stranded RNA-dependent protein kinase; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-biphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PTEN, phosphatase and tensin homolog deleted on chromosome 10; Rag, Ras-related small GTP-binding protein; raptor, regulatory associated protein of mTOR; Rheb, Ras homolog enriched in brain; rictor, rapamycin-insensitive companion of mTOR; ROS, reactive oxygen species; S6, ribosomal protein S6; S6K, 70 kDa S6 kinase; sirtuin, silent mating type information regulation 2 homolog; SOD1, Cu/Zn superoxide dismutase; SQSTM1, sequestosome 1; Tap42, type 2A-associated protein of 42 kDa; TNFα, tumor necrosis factor α; TORC1, target of rapamycin complex 1; TORC2, target of rapamycin complex 2; TSC, tuberous sclerosis complex; ULK, UNC-51-like kinase; *UVRAG*, UV radiation resistance-associated gene; Vps, vacuolar protein sorting.

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Introduction

In order to maintain cellular homeostasis it is important not only that cellular components (*e.g.* proteins, organelles) are synthesized and assembled when needed but also that these components are degraded when they are either damaged or superfluous. When the degradation process does not occur properly the cell may either die or turn into a cancer cell in which growth proceeds unrestrained.

Whereas the ubiquitin-proteasome pathway is involved in the degradation of short-lived proteins,[1,2] the autophago-lysosomal pathway of protein degradation is responsible for the degradation of long-lived proteins and for the elimination of either damaged or functionally redundant organelles. Another lysosomal protein degradation system, chaperone-mediated autophagy, takes care of removing cytosolic proteins carrying a lysosomal targeting motif: these are recognized by a specific chaperone in the cytosol that delivers them to the lysosomes where they are internalized and degraded. This process has been described elsewhere.[3]

In the past, most attention has been devoted to the ubiquitin-proteasome-mediated catabolic pathway. However, the explosion of research on (macro)autophagy in the last decade highlights the importance of this process in cellular homeostasis.[4–6]

Formation of autophagosomes

During autophagy, which occurs in all eukaryotic cells, part of the cytoplasm containing the material to be degraded becomes surrounded by a double membrane (isolation membrane), which forms an autophagosome.[7] The autophagosome moves along microtubules in a dynein-dependent fashion and fuses with endocytic compartments (to form an amphisome[8]) and lysosomes in a manner that is dependent on the small GTPase Rab7 and the lysosomal membrane protein Lamp-2.[9–12] In this process, the outer autophagosomal membrane fuses with the lysosomal membrane, and the inner autophagosomal membrane vesicle is released into the lysosomal interior. This vesicle, with its sequestered macromolecular material, is then degraded, and the products return to the cytosol via specific permeases for reutilization in metabolism (Figures 1 and 2).[7]

The formation of autophagosomes, consisting of nucleation, membrane expansion, and vesicle closure, is the rate-limiting step in autophagy. This step requires 18 different *ATG* (AuTophaGy-related) genes that have been identified by genetic screens for autophagy mutants in yeast; many of these genes have mammalian counterparts. These have been reviewed in great detail elsewhere[7,13–17] and will be discussed here only briefly.

Central to autophagosome induction and formation in yeast are the Atg1-Atg13-Atg17 complex;

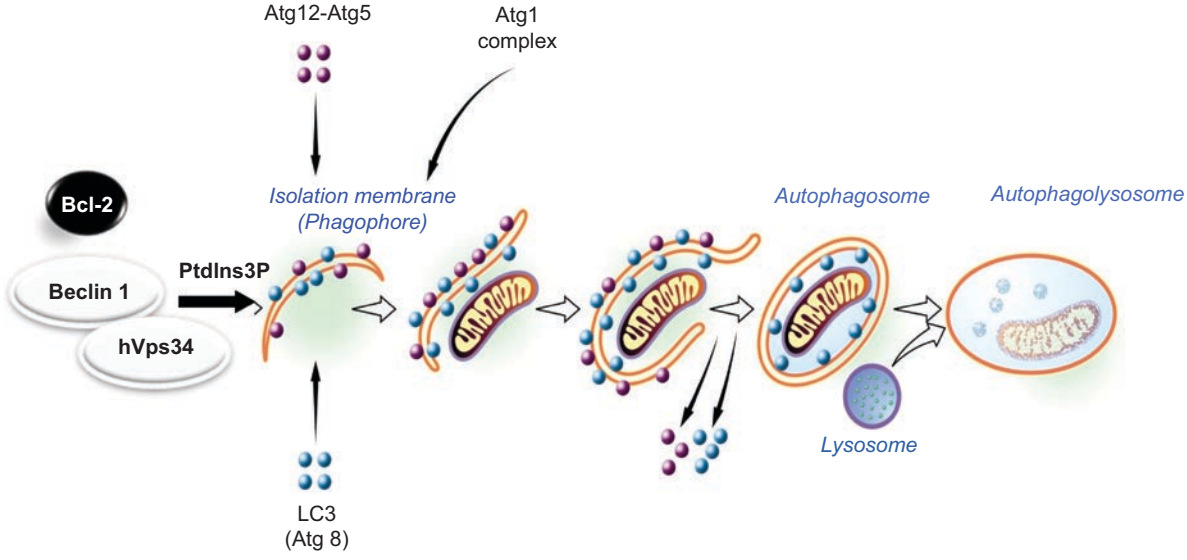

Figure 1. The autophagic process. Essential for the initiation of autophagosome formation is the association of Beclin 1 (Atg6) with the class III phosphatidylinositol 3-kinase hVps34 (homolog of yeast Vps34) before PtsIns(3)P is produced. For this to occur, Beclin 1 must first dissociate from its inhibitory complex with Bcl-2. Only the two-ubiquitin-like conjugation systems (Atg12 and Atg8/LC3) and the Atg 1 complex are represented in the figure. The fusion of the autophagosome with endosomes is not represented. For further details about the Atg machinery, see the text.

the phosphatidylinositol 3-kinase (producing PtdIns(3)P [phosphatidylinositol 3-phosphate]) complex (consisting of the class III phosphatidylinositol 3-kinase Vps34 [Vps, vacuolar protein sorting] and its regulatory protein kinase Vps15, and of Atg6 and Atg14); and the Atg5-Atg12-Atg16 multimere complex, which is needed in the process leading to the conjugation of Atg8 (LC3-I or microtubule-associated protein 1 light chain 3 in mammalian cells) with phosphatidylethanolamine (PE) to form Atg8-PE (LC3-II in mammalian cells). The deconjugation of Atg8-PE is catalyzed by the protease Atg4. Both Atg5-Atg12 and Atg8-PE are produced by two unique, ubiquitin-like conjugation systems: the formation of Atg5-Atg12 requires Atg7 (homologous to the E1 ubiquitin-activating enzyme) and Atg10 (homologous to the E2 ubiquitin-activating enzyme), whereas conjugation of PE with Atg8 (a ubiquitin-like protein) requires Atg7, Atg3 (another E2-like enzyme), and the Atg5-Atg12-Atg16 complex (E3-like).[13,18,19] Atg8-PE behaves like a membrane protein and is present on autophagosome membranes. Interestingly, *in vitro*, Atg8-PE mediates tethering between adjacent membranes and stimulates membrane hemifusion; it is thought that this may mimic the expansion of the autophagosomal membrane during autophagosome formation.[20]

Once the autophagosome has formed, Atg5-Atg12 is lost, but Atg8-PE (LC3-II) remains on the autophagosomal membranes; the inner membrane LC3-II is degraded by lysosomal enzymes after the fusion of autophagosomes with lysosomes, and the outer membrane LC3-II is deconjugated by Atg4 and returns to the cytosol. LC3-II is therefore a useful marker of autophagosomes but not of autophagic flux.[21–23] However, LC3-II may also be present on protein aggregates that are formed in a manner that is independent of autophagy.[23–25]

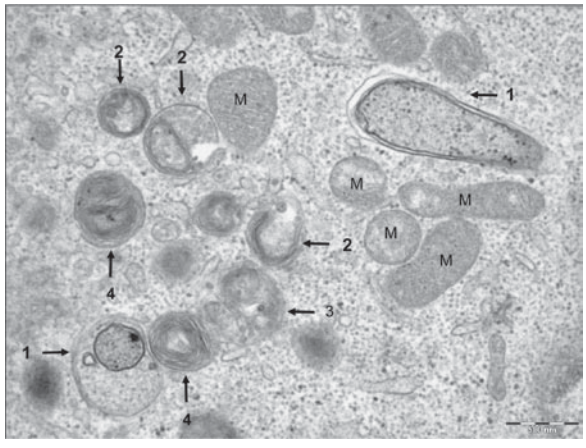

Figure 2. Autophagic organelles visualized by transmission electron microscopy in starved human fibroblasts. (1) and (2) Autophagosomes surrounded by a double-membrane with undigested cytoplasmic material inside. (3) An amphisome resulting from the fusion of an autophagosome with an endosome. This organelle has acquired acidic properties and degradative capacities. (4) Autolysosomes filled with undigested lipids. (M) Mitochondria. The figure is used by courtesy of Gérard Pierron (CNRS, Villejuif, France).

The source of the membrane used for autophagosome formation has been the subject of considerable debate.[12,16,26] Seglen and coworkers proposed that the sequestering membrane is a unique organelle with distinct morphological properties and coined the term "phagophore" for the membrane cisternae that form the new autophagosome;[8] the site where the autophagosome is formed is now designated the "phagophore assembly site" (PAS). Recent evidence indicates that the lipids for autophagosome formation may be derived from the endoplasmic reticulum (ER),[27] as had long been suspected.[28,29] Analysis of the dynamics of several PtdIns(3)P-binding proteins in mammalian cell lines has revealed that during starvation-induced autophagy, autophagosomes are formed in a PtdIns(3)P-enriched compartment that is dynamically connected to the ER. PtdIns(3)P presumably defines the site of autophagosome formation (*i.e.* the phagophore), as it is formed before LC3-II is present in the phagophore. Evidence has also been obtained that PtdIns(3)P may be required to join the ends of the membrane of the autophagosome in *statu nascendi* (termed the "omegasome" because of its shape) to form a vesicle.[27,30] Data obtained with yeast have also revealed the presence of PtdIns(3)P on the isolation membrane. PtdIns(3)P levels are highly enriched on the inner concave surface of the isolation membrane and near the elongating tips; its function is probably to recruit some Atg proteins to the membrane, to generate the negative curve of the inner membranes, and to maintain the edges of the isolation membranes.[31–33]

Regulation of autophagy

When the cellular nutrient supply is insufficient, autophagy becomes activated as a defense mechanism by which amino acids and other intracellular nutrients are recycled for cell survival[34,35] and to repress apoptosis.[36] For example, in newborn mammals, when the maternal supply is suddenly interrupted immediately after birth, starvation-induced autophagy in various tissues provides the necessary oxidizable substrates, as has been beautifully demonstrated in Atg5-deficient mice.[37] Another example is that of the adult mammalian muscle and liver which, in starvation, degrade proteins by autophagy in order to produce amino acids for the hepatic synthesis of

glucose, which is needed as substrate for energy production in brain and erythrocytes.[38]

Amino acids are classical (feed-back) inhibitors of autophagy, and they carry out this function by inhibiting the formation of autophagosomes[39] by a mechanism, the details of which are still obscure (see below). It must be stressed that ammonia, one of the major products of amino acid catabolism, can directly inhibit lysosomal function by causing an increase in intralysosomal pH because of its acidotropic properties and, in this way, can interfere with the autophagic pathway.[40,41] Autophagy has long been known to be under hormonal control: the process is inhibited by insulin and promoted by glucagon.[39,42]

Even under nutrient-rich conditions, some constitutive autophagy is still required for cellular quality control, *i.e.* to remove damaged intracellular structures and organelles, as indicated by experiments with Atg5 and Atg7-deficient mice.[43–46] How the autophagic system is able to recognize these aberrant structures will be discussed later.

Signaling pathways

Autophagy is regulated by several signal-transduction pathways (Figure 3). The most important is the insulin-growth factor-amino acid-mTOR (mammalian target of rapamycin) pathway: its inhibition activates autophagy in all eukaryotes.[47] Other mechanisms, such as the regulation of autophagy by the Erk1,2- and p38-related (Erk, extracellular signal-regulated kinase; p38, p38 mitogen-activated protein kinase) signaling pathways, do not operate in all cell types.[47] There is also controversy about whether these signaling pathways stimulate[48–50] or inhibit[51,52] autophagy. Likewise, uncertainty exists with regard to the role of the trimeric protein Gαi3 in regulating autophagy.[53,54] These differences are probably cell-type dependent.

Insulin, amino acids, and mTOR-mediated signaling

Because the insulin-amino acid-mTOR signaling pathway is the major mechanism by which autophagic activity is controlled, we will briefly describe some of its properties here. For more detailed discussion of the various components in this pathway, see Refs. [55–60].

The first part of the insulin-signaling pathway, upstream of mTOR, involves the insulin receptor, IRS1 and IRS2 (IRS, insulin receptor substrate), class I phosphatidylinositol 3-kinase (PI3K), producing PtdIns(3,4,5)P_3 [phosphatidylinositol 3,4,5-triphosphate] and PtdIns(3,4)P_2 [phosphatidylinositol 3,4-biphosphate]), phosphoinositide-dependent kinase-1 (PDK1) and protein kinase B (PKB) (Figure 3). This part of the pathway is involved in regulating muscle and adipocyte glucose transport.[61]

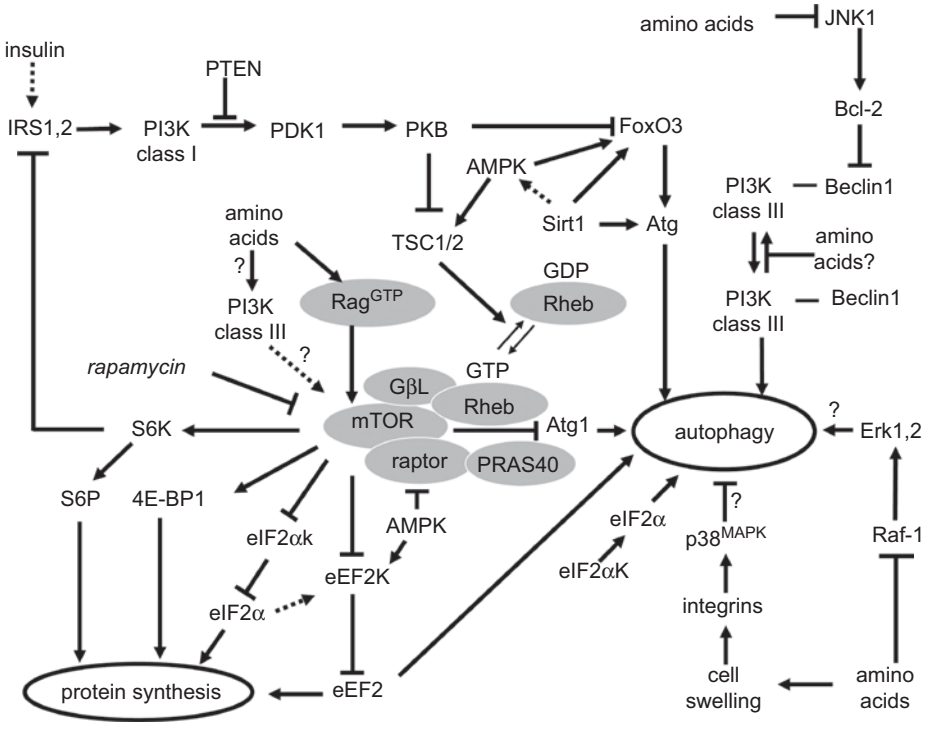

Figure 3. Amino acid-activated signaling and the regulation of autophagy. For the purpose of clarity the mTORC2 complex (containing mTOR and rictor) is not shown. Phosphorylated eIF2alpha and eEF2 stimulate autophagy; the dephosphorylated forms stimulate protein synthesis.

The second part of the insulin-signaling pathway, downstream of mTOR, involves components such as 4E-BP1 (eukaryotic translation initiation factor 4E binding protein 1), S6K (70 kDa S6 kinase), ribosomal protein S6, eIF2α (eukaryotic initiation factor 2α)-kinase, and eEF-2 kinase, proteins that are involved in regulating protein synthesis. The activity of mTOR is inhibited by the heterodimer TSC1/TSC2 (TSC, tuberous sclerosis complex): it acts as a GTPase-activating protein complex for the small G-protein Rheb (Ras homolog enriched in brain), which, in its GTP-form, binds to and activates mTOR. Protein kinase B phosphorylates TSC2, which results in inactivation of the TSC1/TSC2 complex and activation of mTOR.

mTOR is present in a complex, TORC1 (target of rapamycin complex 1), with raptor (regulatory associated protein of mTOR, a protein that acts as a scaffold for mTOR-mediated phosphorylation of mTOR substrates), the protein GβL, and the inhibitory protein PRAS40. In this form, mTOR activity is inhibited by rapamycin. However, when mTOR is present in a second complex, TORC2 (target of rapamycin complex 2), formed with the protein rictor (rapamycin-insensitive companion of mTOR), its kinase activity is rapamycin-insensitive and serves to stimulate protein kinase B phosphorylation.

An important aspect of the regulation of TORC1 activity is that upstream signaling by insulin alone does not activate mTOR unless low concentrations of amino acids are also present.[56,62–67] It is noteworthy that high concentrations of amino acids alone can activate mTOR downstream targets in a rapamycin-sensitive manner and do not require the presence of insulin.[62–64] Amino acids do not stimulate class I PI3K (phosphatidylinositol 3-kinase) or protein kinase B,[56,65–68] which implies that their mechanism of action is different from that of insulin (see also below). Inhibitors of class I PI3K interfere with amino acid-dependent signaling in the absence of insulin,[69] which suggests that basal activity of PI3K is sufficient for mTOR activation under these conditions.[70] Alternatively, these compounds may inhibit mTOR directly.[67] Because amino acids are still required for mTOR activation in TSC-null cells,[71,72] their site of action must be located downstream of TSC.

A finding of great importance for our understanding of the regulation of autophagy were the discoveries that activation of mTOR-mediated signaling by amino acids not only stimulates protein synthesis but also simultaneously inhibits autophagy and that rapamycin stimulates autophagy in the presence of amino acids.[62,63] Stimulation of autophagy by rapamycin has been found not only in mammalian cells but also in yeast,[73,74] *Drosophila*,[34] *Caenorhabditis elegans*,[75] trypanosomes,[76] and plants,[77,78] which demonstrates that the mechanism of autophagy control is evolutionarily widely conserved. Today it is generally accepted that rapamycin and related compounds are possible tools, in addition to amino acid starvation, for stimulating autophagy.[4,79–81] The recent development of ATP-competitive inhibitors of mTOR will also be useful in this regard.[81]

Whereas in hepatocytes, in the presence of low concentrations of amino acids, insulin stimulates mTOR signaling and simultaneously inhibits autophagy, glucagon has the opposite effects, *i.e.* it inhibits signaling and stimulates autophagy.[63] This is consistent with the physiological role of these hormones in controlling hepatic autophagy *in vivo*. The fact that opposite types of regulation of protein synthesis and of autophagic protein degradation occur via the same signaling pathway is considered to be efficient from the point of view of metabolic regulation.[63,82]

The molecular target of TOR in the autophagic machinery is probably the Atg1-Atg13-Atg17 complex, as experiments with yeast have indicated: inactivation of TOR by either starvation or rapamycin treatment is accompanied by dephosphorylation of Atg1 and Atg13 and increased binding of Atg1 to Atg13 and Atg17, resulting in increased Atg1 protein kinase activity. Under nutrient-rich conditions, Atg13 becomes hyperphosphorylated in a TOR-dependent manner, which reduces the association between Atg1 and Atg13; this, in turn, results in decreased Atg1 activity.[83,84] Activation of Atg1 kinase and of autophagy in yeast requires inhibition of Tap42-controlled protein phosphatase 2A (Tap42, type 2A-associated protein of 42 kDa), which suggests that Atg1 is not the direct target of protein phosphatase 2A.[85] In *Drosophila*, overexpression of the *Atg1* gene induces autophagy, and Atg1 protein kinase activity is required for this effect.[86] The homologs of Atg1 in mammalian cells are the ULK1 and ULK2 (ULK, UNC-51-like kinase) proteins, the protein kinase activity of which is also essential for autophagy.[87] Recent findings with mammalian cells have shown that mTORC1 is incorporated into a complex with ULK1, Atg13, and FIP200 (focal adhesion kinase [FAK] family interacting protein of 200 kDa), the mammalian functional homolog of Atg17, in a nutrient-dependent manner and that mTOR directly phosphorylates ULK1 and Atg13.[88,89]

Unexpectedly, and in contrast to the effect of rapamycin, interruption of amino acid signaling by the PI3K inhibitors wortmannin and LY294002 has been shown to inhibit rather than stimulate autophagy.[38,69] The reason is that these inhibitors inhibit not only class I PI3K but also class III PI3K, and the product of the latter lipid kinase, PtdIns(3)P, is essential for autophagy, as was demonstrated in experiments with

HT-29 cells, a human colon cancer cell line.[90] In contrast, the products of class I PI3K, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, have been found to inhibit autophagy, and overexpression of PTEN (phosphatase and tensin homolog deleted on chromosome 10), which degrades these lipids to PtdIns(4)P and PtdIns(4,5)P_2, respectively, stimulates autophagy.[91] 3-Methyladenine, the classical inhibitor of autophagy,[92] proved to be a PI3K inhibitor.[69,90] These observations were the first to show that phosphatidylinositolphospholipids are involved in regulating autophagy, and, as discussed above (see section, **Formation of autophagosomes**), we now know that PtdIns(3)P fulfills an essential role in autophagosome formation.

The fact that Beclin 1 (coiled-coil, myosin-like Bcl-2 interacting protein), the mammalian homolog of Atg6, binds to, and is an essential activator of, class III PI3K (see section, **Formation of autophagosomes**) is important for the regulation of autophagy in mammalian cells. However, Beclin 1, a BH3 (Bcl-2 homology domain) protein, can also associate with the anti-apoptotic proteins Bcl-2/Bcl-xL (Bcl-2, B-cell lymphoma 2; Bcl-xL, basal cell lymphoma-extra large), which contain a BH3-binding groove; this means that it must first dissociate from this inhibitory complex before it can activate class III PI3K and stimulate autophagy (Figures 1 and 4).[93] This may occur by JNK1-mediated (JNK, c-Jun N-terminal kinase) phosphorylation of Bcl-xL or, alternatively, by the displacement of Beclin 1 from its complex with Bcl-2 by other BH3-containing proteins, such as the pro-apoptotic proteins BAD (Bcl-xL/Bcl-2-associated death promoter) and BNIP3 (Bcl-2/adenovirus E1B 19kDa-interacting protein 3) (Figure 4). A recent report indicates that dissociation of Beclin 1 from its complex with Bcl-2 may also occur by phosphorylation of Beclin 1 through the action of the tumor suppressor DAPK (death-associated protein kinase), a Ca^{++}-calmodulin-activated protein kinase, the expression of which is lost in many malignancies.[94]

Apart from their ability to inhibit autophagy by activating mTOR, amino acids may also inhibit autophagy by causing a decrease in Beclin 1-associated class III PI3K activity,[95] perhaps because they promote the Beclin 1-Bcl-2 assocation.[96] The Beclin 1-PI3K class III complex also contains the proteins UVRAG (UV radiation resistance-associated gene), Ambra1 (activating molecule in Beclin1-regulated autophagy), Bif-1 (Bax interacting factor 1), and Atg14/Barkor (Beclin 1-associated autophagy-related key regulator) as additional protein components, which are indispensable for proper activation of class III PI3K and thus for the stimulation of autophagy.[93,97,98] Bcl-2 may also inhibit autophagy by another mechanism, *i.e.* by binding to the ER where it affects cellular Ca^{++} homeostasis,[99,100] because Ca^{++} is required for autophagy.[101]

Autophagy regulation and energy

Although mTOR is activated in the presence of amino acids, it can be inhibited by the activation of AMPK (AMP-activated protein kinase) when energy falls short (Figure 3).[102–106] In line with its function to stimulate catabolism,[107] AMPK is essential for

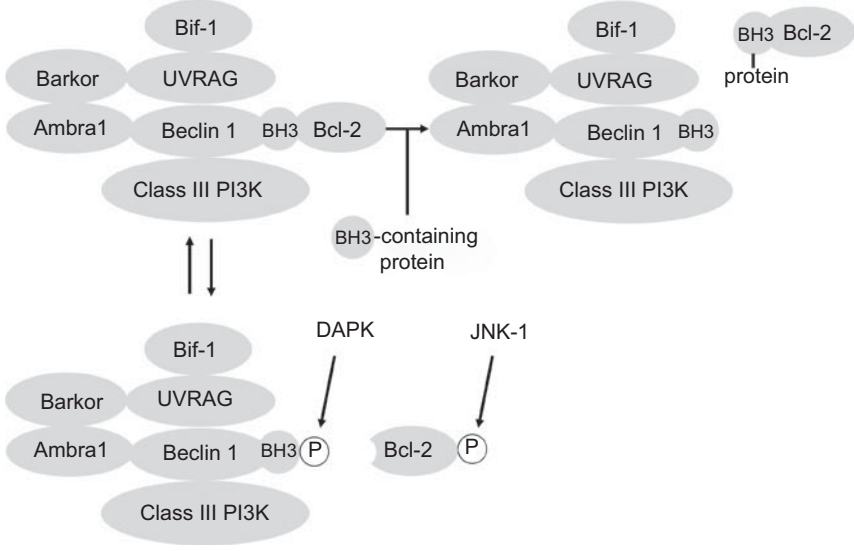

Figure 4. Regulation of the activity of the class III PI3K complex in autophagosome formation. Class III PI3K becomes active in association with Beclin 1. For this to happen, Beclin 1 must first dissociate from its inhibitory complex with Bcl-2. This may occur by JNK1-mediated phosphorylation of Bcl-2, by DAPK-mediated phosphorylation of Beclin 1, or by competition with other BH3-containing proteins (*e.g.* BAD or BNIP3); in the latter case these BH3-containing proteins form a complex with Bcl-2.

autophagy.[99,108,109] The p53 tumor suppressor activates AMPK, inhibits mTOR, and stimulates autophagy, particularly of mitochondria.[110] AMPK also underlies the stimulation of autophagy in cardiac[111] and cerebral[112] ischemia.

Progress in elucidating the effect of AMPK on autophagy has been hampered for several years because of the use of AICAR (5-aminoimidazole-4-carboxamide ribonucleoside), the classical activator of AMPK, which was found to inhibit autophagy[113]; this compound has many side effects,[108,114] including the inhibition of the binding of class III PI3K to Beclin 1.[115]

It has been suggested that, apart from its ability to inhibit mTOR, AMPK may also activate autophagy in an mTOR-independent manner by phosphorylating and activating eEF2 (eukaryotic elongation factor-2) kinase,[116] also known to be involved in autophagy (Figure 3).[117,118] Two mechanisms presumably contribute to the inhibition of mTOR by AMPK: the first mechanism proceeds via AMPK-mediated phosphorylation of TSC2[119] and the second via phosphorylation of raptor.[120]

Interestingly, not only does AMPK inhibit mTOR-dependent signaling, but also, conversely, inactivation of mTOR by amino acid depletion can result in the activation of AMPK, as observed in pancreatic β-cells[121] and in muscle[122] but not in isolated hepatocytes.[104] Likewise, the deletion of S6K [located downstream of mTOR (Figure 3)] in muscle cells stimulates AMPK activity and inhibits cell growth, which is restored when AMPK is blocked.[123] Opposite changes in mTOR and AMPK activity have also been observed in the liver and mammary gland of rats and in mammary carcinomas of rats subjected to dietary restriction.[124] These findings strongly suggest that mTOR signaling and AMPK can reciprocally inhibit one another.

Feedback interaction in the mTOR pathway and autophagy

Overactivation of mTOR signaling results in feedback inhibition of the insulin-signaling pathway by virtue of S6K-dependent phosphorylation of IRS1, which reduces the activity of class I PI3K (Figure 3).[125–128] This feedback may be part of a homeostatic mechanism that prevents the overactivation of mTOR by amino acids. It has been proposed that the overactivation of mTOR contributes to insulin resistance in obesity-linked diabetes. Although autophagy was not measured in these studies, this feedback mechanism could perhaps act as a safety mechanism to avoid the complete inhibition of autophagy because, even in the presence of excess nutrients, some autophagy is always needed to fulfill its house-keeping function, *i.e.* removal of redundant and/or damaged intracellular structures.

Does S6K play a role in autophagy?

Although TOR activation inhibits autophagy, paradoxically, S6K has been shown to be required for autophagy in the *Drosophila* fat body.[34] This would suggest that the same protein kinase is required for protein synthesis, an anabolic process, and for autophagy, a catabolic process—which does not seem to be logical. Furthermore, a requirement for S6K would be inconsistent with the stimulation of autophagy by rapamycin or amino acid depletion, both of which lead to S6K inactivation (see section, **Insulin, amino acids, and mTOR-mediated signaling)**. In an attempt to get round this dilemma, it was proposed that under nutrient-rich conditions the feedback of signaling upstream of mTOR by S6K results in decreased activity of class I PI3K, which would, in turn lead to decreased PtdIns(3,4,5)P_3 levels and thereby deinhibit autophagy.[129] This would be useful for preserving the house-keeping function of basal autophagy even under nutrient-rich conditions (see preceding paragraph). Conversely, when nutrients become scarce, the inactivation of TOR caused by a fall in amino acid concentration accelerates autophagy, provided sufficient S6K is still present. It has been suggested that in response to long-term starvation, S6K activity may become so low that class I PI3K is once again activated and restrains autophagy in order to prevent autophagic cell death.[34]

The proposal that S6K is essential for autophagy is, however, also difficult to reconcile with the observation that overexpression of Atg1 stimulates autophagy, inhibits cell growth, and, at the same time, inhibits the activity of TOR and S6K in both *Drosophila* and mammalian cells; knockdown of ATG1 has the opposite effects.[86,130] This suggests the existence of a self-reinforcing feedback loop, whereby increased Atg1 levels lead to the down-regulation of TOR activity, resulting in further activation Atg1.[86,130]

Other observations, at least in mammalian cells, are also at variance with a requirement of S6K for autophagy. Thus, S6K deletion in muscle cells does not affect autophagy, at least as measured by LC3-II accumulation.[131] Sch9 was recently identified as the yeast ortholog of mammalian S6K1.[132] In yeast, in a nutrient-rich medium, inactivating Sch9 triggers autophagy, in an Atg1-dependent manner, without inactivating TORC1.[133] This would be consistent with a negative impact of S6K on autophagy. Finally, deletion of *sch9* in yeast results in life-span extension.[134] Because autophagy is of vital importance for

longevity, this also argues against a possible role for S6K in the autophagic process (see below).

FoxO proteins

An important development in elucidating autophagy regulation was the finding that the expression of several autophagy genes is increased by the transcription factor FoxO3 (FoxO, class O of forkhead box transcription factors), the activity of which is inhibited by protein kinase B-mediated phosphorylation[135–137] and activated by AMPK (Figure 3).[138] In addition to its ability to inhibit Atg1, recent data have shown that mTOR can also inhibit the expression of autophagy genes by down-regulation of the tumor suppressor p73.[139] Controlling autophagy by FoxO3, acting in concert with the mTOR-signaling pathway, is therefore ideally suited to the role of autophagy as a survival pathway when nutrients become scarce. The fact that autophagy is controlled at the level of protein kinase B and mTOR and also at the level of the Beclin 1-PI3K class III association (see above) explains why rapamycin does not always stimulate autophagy to the same extent; the relative importance of these regulatory mechanisms are probably cell type-dependent.[41] The regulation of autophagy genes by FoxO3 can be considered as part of a general fasting response: the FoxO proteins inhibit progression of the cell cycle, suppress the expression of genes involved in glycolysis and lipogenesis, and stimulate the expression of the genes controlling gluconeogenesis and fatty acid oxidation.[140,141] In addition, FoxO proteins are known to induce the expression of genes that provide protection against oxidative stress, *e.g.* superoxide dismutase and catalase, and therefore play an important role in combating aging and disease (see also below).[141]

Recent evidence indicates that mTOR not only regulates protein synthesis and autophagy but also is required for lipogenesis.[142,143] Thus, the entire insulin-amino acid-mTOR pathway appears to coordinate the fluxes through the major metabolic pathways (Figure 5).

Mechanisms of amino acid signaling

The mechanism responsible for the activation of mTOR signaling by amino acids is still unknown. In most cell types, leucine, but not valine or isoleucine, is potent in stimulating mTOR signaling[65] and in inhibiting autophagy.[38] This rules out the possibility that the plasma membrane leucine transporter could be the amino acid receptor, as has been proposed.[56] The metabolism of leucine is not required,[144] and non-metabolizable analogues of leucine can mimic the effect of leucine on mTOR signaling.[145,146]

It has been suggested that the plasma membrane contains a leucine-specific receptor protein that controls autophagy independently of mTOR.[147] According to this view, leucine inhibits autophagy and stimulates mTOR signaling by different mechanisms. Presumably, however, the amino acid receptor is intra- rather than extracellular.[148–150] The amino acid transporters do play a role in controlling the intracellular concentration of leucine. For example, the level of leucine may be determined by the Na^+-concentration-dependent uptake of glutamine and the exchange of extracellular leucine for intracellular glutamine.[151,152] Indeed, this combination of amino acids is particularly effective in stimulating mTOR signaling, at least in hepatocytes,[153] Jurkat cells,[154] β-cells,[155] and HeLa cells,[152] and also in inhibiting autophagy (see section below on **Hypothesis: Could glutamate dehydrogenase be the amino acid sensor?**).

Various potential mechanisms of amino acid sensing have been discussed recently,[56,67,68,156] but none has received firm experimental support.

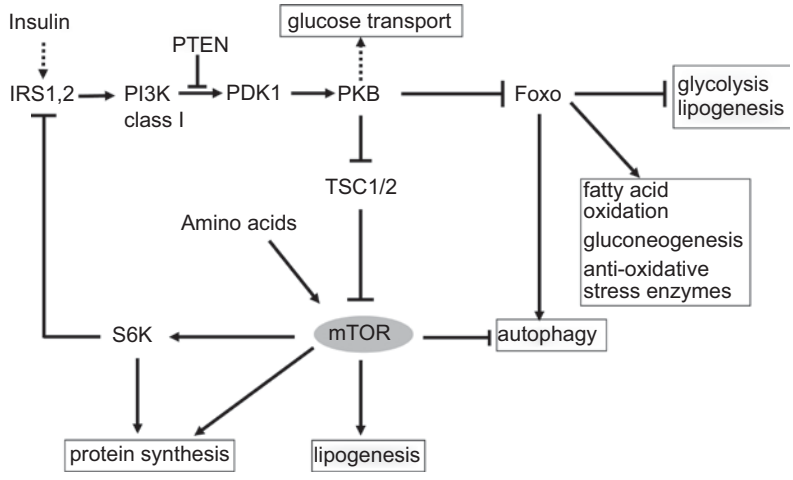

Figure 5. Insulin- and amino acid-dependent signaling and the regulation of metabolic pathways.

One possible mechanism is the degree of loading of tRNA with amino acids, which, in mammalian cells, controls the activity of the eIF2α kinase GCN2 (general control non-derepressible-2),[157] needed for autophagy.[158] Although localized downstream of mTOR, eIF2α kinase stimulates autophagy independently of mTOR[158,159] (Figure 3), possibly through eEF2 kinase.[117] Other putative amino acid-sensing mechanisms involve the participation of diadenosine polyphosphates,[68] by-products of the amino acyl-tRNA synthetase reaction, and phosphatidic acid, which is required in the process leading to amino acid-induced mTOR activation and which is located downstream of Rheb.[67,160–162]

Data obtained with mammalian cells have indicated that amino acids, leucine in particular, activate both total class III PI3K and Beclin 1-associated class III PI3K without affecting the class III PI3K-Beclin 1 association *per se*; this results in mTOR activation.[56,163–165] The effect is mediated by an amino acid-induced rise in cytoplasmic Ca^{++}, which results in increased binding of Ca^{++}/calmodulin to an evolutionarily conserved motif in class III PI3K.[166] Its product, PtdIns(3)P, may then recruit FYVE-domain-containing proteins to endosomes. These proteins are required to build a mTORC1 signalosome.[164,166] A recent study, however, failed to show any requirement for Ca^{++} for class III PI3K activity in mammalian cells.[167]

There are three major problems with this mechanism. First, as we have seen, PtdIns(3)P is essential for autophagosome formation. Knock-down of class III PI3K blocks autophagy in several cell systems[31,168–171] but, at least in *Drosophila* and *C. elegans*, it does not affect phosphorylation of S6K.[67,168] The possibility cannot be excluded, however, that amino acid signaling through class III PI3K may occur only in vertebrates and mammals.[67] It is difficult to reconcile the involvement of PtdIns(3)P in both mTOR activation, which inhibits autophagy, and autophagosome formation, unless one postulates two distinct class III PI3K protein complexes, one associated with autophagosomes and one with endosomes, the latter acting downstream of amino acids to up-regulate mTOR.[97,164,166] Class III PI3K is indeed known to occur in several protein complexes,[97] and furthermore, a significant portion of class III PI3K is not bound to Beclin 1[172], suggesting that amino acids may stimulate the unbound class III PI3K. The second problem is the inability of amino acids to affect the class III PI3K-Beclin 1 complex, which is apparently inconsistent with the observation that the association of class III PI3K and Beclin 1 is decreased by amino acids,[95,96] as discussed above. The third problem is the increase in cytoplasmic Ca^{++}, seen in the presence of amino acids. Amino acids are known to stimulate glycogen synthesis from glucose by activating glycogen synthase.[173,174] This is incompatible with a rise in cytoplasmic Ca^{++} which actually increases glycogen phosphorylase activity. Similarly, amino acids inhibit autophagy, whereas an increase in cytoplasmic Ca^{++} stimulates autophagy, mediated by Ca^{++}/calmodulin-dependent kinase kinase-β and AMPK.[99] There is no clear answer to these apparent inconsistencies but, clearly, they need to be solved in the future.

Another interesting potential mechanism of amino acid sensing is suggested by the observation that amino acids, leucine in particular, are able to promote the association of Rheb with mTOR.[175] Although the effect has been said not to be due to increased loading of Rheb with GTP,[67,175] other data indicate that amino acids do in fact promote the loading of Rheb with GTP.[161,176–178]

Rag (Ras-related small GTP-binding protein) proteins, a family of four Ras-related small GTPases in mammalian cells, have been shown to interact with mTORC1 by binding to raptor in an amino acid-sensitive manner.[179,180] Expression of constitutively active Rag^{GTP} in mammalian cells eliminates the requirement for amino acids for mTOR to be activated. Conversely, the expression of dominant-negative Rag^{GDP} inhibits mTOR in the presence of amino acids.[179,180] Like amino acids, Rag^{GTP} does not activate mTOR directly but rapidly targets mTOR from discrete locations throughout the cytoplasm to a perinuclear region that also contains Rheb and the late endosomal/lysosomal marker Rab7. Moreover, experiments with *Drosophila* have demonstrated that the effects of the Rag proteins on TOR activity are accompanied by predicted effects on cell growth, autophagy, and animal viability under starvation conditions.[180]

The observation that in the presence of amino acids mTOR is localized to the endosomal/lysosomal compartments is in agreement with other studies reporting the association of mTOR with the cellular endomembrane system containing ER, Golgi, and endosomes.[181–183] This compartment also contains the autophagosomal marker LC3.[182]

An attractive putative mechanism for the regulation of autophagy can now been envisaged: autophagy may be considered a constitutively active process, deriving its membranes from an ER/endosome-containing compartment (see section, **Formation of autophagosomes**). When nutrients are present in excess, a brake is applied to the autophagic system by the movement of mTOR into this compartment where the autophagosomes are formed. However, because the inhibition of mTOR by rapamycin does not affect its translocation,[179] mTOR activity is essential for this inhibition of autophagy to occur, for example, by phosphorylating Atg13 and Atg1, as discussed above (see section, **Insulin, amino acids and mTOR-mediated signaling**). Simultaneously,

ER-associated mTOR can stimulate protein synthesis. Several years ago it was predicted that the same signaling mechanism may have contrary impacts on protein synthesis and autophagic protein degradation.[63] Amino acid stimulation of the translocation of mTOR to the ER appears to account for this mechanism.

Hypothesis: Could glutamate dehydrogenase be the amino acid sensor?

As we discussed recently elsewhere,[184] the observation that amino acids increase the loading of Rag and of Rheb with GTP[179] (see above) may provide a clue to the mechanism of amino acid sensing in mTOR signaling. In most cell types, leucine is the amino acid that stimulates mTOR signaling and inhibits autophagy most potently, whereas the other branched-chain amino acids do not share this characteristic. Any proposed mechanism must account for this high specificity. One attractive hypothesis is that glutamate dehydrogenase could be the actual amino acid sensor. This mitochondrial enzyme is known to be specifically activated by leucine.[185] In this context, it is of importance to note that in pancreatic β-cells the ability of leucine (but not of valine or isoleucine) to stimulate production of insulin has been ascribed to stimulation of glutamate dehydrogenase.[155,186–188] Studies of various leucine analogs have revealed a close link between their ability to stimulate mitochondrial metabolism via the activation of glutamate dehydrogenase and their ability to stimulate rapamycin-sensitive S6K phosphorylation.[155] Moreover, a combination of glutamine (a glutamate donor) and leucine, which maximizes the flux through glutamate dehydrogenase, is most effective in stimulating phosphorylation of S6K in β-cells and mimics the effect of a complete mixture of all amino acids.[155] Strikingly, in perifused hepatocytes, leucine combined with glutamine, proline, or alanine, which also rapidly produce glutamate in the course of their metabolism, is also an effective inhibitor of autophagic proteolysis.[189] Leucine, combined with glutamine, also strongly inhibits autophagy in HeLa cells.[152]

A possible connection between glutamate dehydrogenase and mTOR signaling could be the production of 2-oxoglutarate, which, upon further metabolism in the citric acid cycle, produces GTP in the succinyl-CoA synthase reaction (Figure 6). This GTP may then be used to activate Rheb and Rag. It is important to note that this GTP is produced within the mitochondria. However, mechanisms can be envisaged that would transport it into the cytosol.[68]

As we will discuss below (see **Mitophagy**), 2-oxoglutarate is also required in the process that leads to the degradation of HIF-1α (hypoxia inducible factor), a transcription factor that promotes autophagy.

Further support for the possibility that glutamate dehydrogenase may be an amino acid sensor is related to NADPH, one of its other reaction products. The mitochondrial respiratory chain is an important producer of reactive oxygen species (ROS)[190,191] and can potentially harm the cell by oxidizing lipid, DNA, and proteins. NADPH, derived from the glutamate dehydrogenase reaction, may be used to scavenge ROS via the glutathione-glutathione reductase system. In addition to NADPH, 2-oxoglutarate can also act as a scavenger of ROS, which oxidizes 2-oxoglutarate to succinate non-enzymically.[192] There is ample evidence that ROS is also involved in initiating autophagy in a

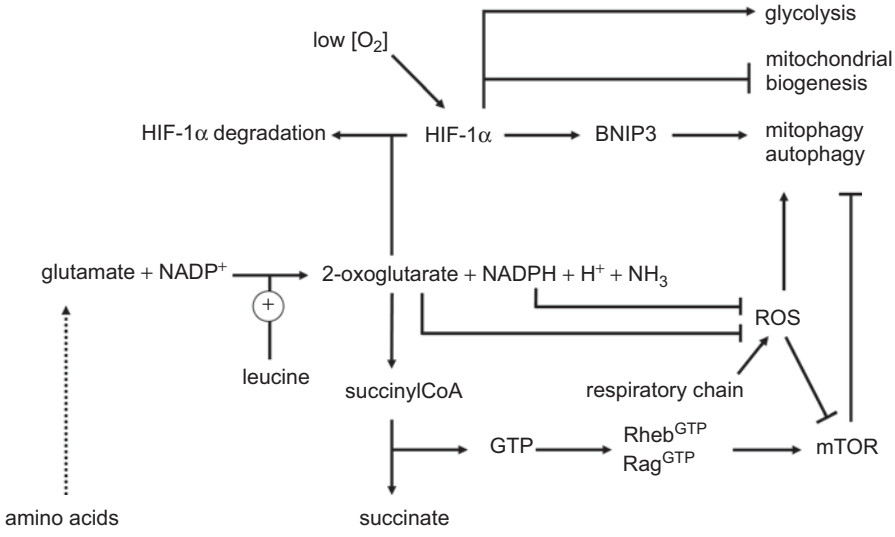

Figure 6. Is glutamate dehydrogenase an amino acid sensor? Activation of flux through glutamate dehydrogenase by leucine results in the activation of mTOR and the inhibition of autophagy via GTP produced in the succinyl-CoA synthase reaction, the scavenging of ROS by NADPH and 2-oxoglutarate, and the promotion of the degradation of the transcription factor HIF-1α by 2-oxoglutarate.

manner that is sensitive to anti-oxidants,[193–198] perhaps because of the oxidation of a critical cysteine residue in Atg4.[195] ROS also appears to initiate autophagy in starvation.[195] Atg4 is involved in the deconjugation of LC3-II, and oxidation of Atg4 inactivates Atg4, which favors the formation of autophagosomes. The evidence available regarding the effect of ROS on the activity of mTOR is controversial, with increased or decreased activity being observed depending on the conditions and cell type used.[58] Relatively high levels of ROS activate AMPK[199] and in this way may inhibit mTOR. Interestingly, and again in β-cells, it has been postulated that the NADPH produced by isocitrate dehydrogenase may be involved in maintaining the cellular redox state during glucose-stimulated insulin production;[200] there is no reason to believe that NADPH derived from glutamate dehydrogenase could not serve the same function.

All these observations make glutamate dehydrogenase a highly attractive candidate as a sensor of amino acids in the control of both signaling and of autophagy, at least in mammalian cells. Future experiments with mutant glutamate dehydrogenase may provide direct experimental evidence to support this proposal.

Ceramide, NFκB, and autophagy

Ceramide and related lipid molecules are important signaling molecules that are involved in cell differentiation, proliferation, apoptosis, and senescence.[201] A number of studies have indicated that ceramide stimulates autophagy.[202–205] Mechanisms responsible for the stimulation of autophagy by ceramide include: a. the induction of BNIP3;[203] b. the upregulation of Beclin 1 and decreased phosphorylation of protein kinase B, resulting in mTOR inhibition;[202,206] and c. the involvement of JNK1,[206,207] which mediates dissociation of the Beclin 1-Bcl-2 complex by ceramide-induced phosphorylation of Bcl-2.[207] Ceramide is also known to reduce intracellular amino acid concentrations by inhibiting amino acid transport and to reduce mTOR-dependent signaling,[208,209] and the down-regulation of amino acid transporters by ceramide is another mechanism that contributes to activating autophagy under these conditions.[209] Importantly, genetic or pharmacological inhibition of autophagy makes cells exquisitely sensitive to ceramide toxicity, and pyruvate protects against cell death under these conditions. This demonstrates that, in the presence of ceramide, autophagy produces substrates for energy generation and serves to support cell survival.[209]

Ceramide can stimulate the production of ROS,[210,211] and this may be yet another mechanism that may contribute to the activation of autophagy by ceramide (Figure 7). This possibility is supported by the observation that TNFα (tumor necrosis factor α), which stimulates ceramide production by sphingomyelinase,[201,212] induces ROS-mediated autophagy in sarcoma cells.[194] As we have seen, Atg4 is redox controlled,[195] and so it would be of interest to evaluate Atg4 activity under these conditions. It is noteworthy that TNFα-induced ROS production and autophagy are both suppressed by activation of the transcription factor NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), a phenomenon that is accompanied by stimulation of mTOR.[194] Inhibition of autophagy by NFκB activation has also been reported in other cancer cells.[213] The inhibition of autophagy suggests a hither-to unknown mechanism for the anti-apoptotic function of NFκB, which is overactivated in tumorigenesis and in many infectious and inflammatory diseases.[214] It has been suggested that stimulation of autophagy may provide a way of evading the resistance of cancer cells to anti-cancer agents that activate NFκB.[194] NFκB inhibits autophagy, but conversely, autophagy stimulates the degradation of signaling regulatory proteins that activate NFκB signaling.[215] These observations underscore the importance of NFκB in autophagy regulation.

Another example of ROS-mediated stimulation of autophagy by TNFα, and also by lipopolysaccharide (LPS, which induces TNFα), has recently been reported in cardiomyocytes, a phenomenon accompanied by severe total glutathione depletion; the anti-oxidant N-acetylcysteine suppresses autophagy under these conditions.[198] Activation of autophagy by rapamycin protects the cells against ROS production and LPS toxicity, which highlights the importance of autophagy as a cytoprotective process.[198]

Sphingosine 1-phosphate, a sphingolipid metabolite, also stimulates autophagy, albeit by a mechanism different from that of ceramide in that it inhibits mTOR in a protein kinase B-independent manner and does not upregulate Beclin 1.[216] An increase in

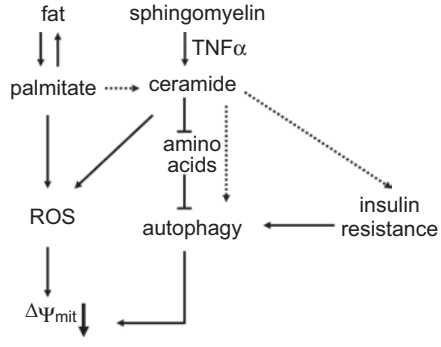

Figure 7. Ceramide, autophagy, and cell survival. Ceramide protects against damaged mitochondria by stimulating the removal of mitochondria with a low mitochondrial membrane potential ($\Delta\psi_{mit}$). For further details, see the text.

cytosolic Ca^{++} by sphingosine 1-phosphate[217] may contribute to this effect.

Selective autophagy

Although starvation-induced autophagy is non-specific in that cytoplasmic components are sequestered randomly,[218,219] autophagy can also specifically recognize and target redundant or damaged organelles for elimination. Although relatively little is known about the signals that allow the autophagic system to recognize these structures, some progress in this exciting field is now being made.

Pexophagy

In yeast, specific autophagic elimination of peroxisomes (pexophagy) is observed when the cells are switched to culture media in which the peroxisomal function is no longer required for growth.[220–223] Similar observations have been made for mammalian cells in which peroxisomal function was made redundant experimentally.[224–226] Autophagy also plays a role in the turnover of glycosomes, peroxisome-like organelles, during the differentiation of *Trypanosoma brucei,* the parasite responsible for human sleeping sickness in Africa.[227]

In yeast, two peroxisomal membrane proteins that are also involved in peroxisome biogenesis, Pex3 and Pex14 (Pex, peroxisomal import protein), are specifically required for the recognition of peroxisomes to be degraded by autophagy,[223,228] in addition to Atg30, which needs to be phosphorylated by an as yet unknown protein kinase.[229] In mammalian cells, the recognition signal for pexophagy is entirely unknown. It could perhaps involve a 69-kDa peroxisomal membrane protein that becomes specifically palmitoylated in the presence of low concentrations of the peroxisomal substrate palmitate.[38] However, the identity of this protein has not been established.

Mitophagy

When *Saccharomyces cerevisiae* cells are switched from a medium containing lactate, which needs the mitochondria for oxidation, into a low-nitrogen medium, the mitochondria are specifically eliminated by autophagy (mitophagy) except in cells in which the Uth1 protein in the mitochondrial outer membrane has been deleted.[230] The protein phosphatase, Aup1p,[231] present in the mitochondrial intermembrane space, and the mitochondrial inner membrane protein, Mdm38,[232] involved in the K^+/H^+ exchange across the inner membrane and essential for the maintenance of the proper mitochondrial morphology, are other candidate proteins involved in the autophagic recognition of mitochondria in yeast. One of the autophagy-related proteins, Atg11, which is essential only for selective autophagy, is also essential in mitophagy.[233]

In mammalian cells, the pro-apoptotic mitochondrially associated protein, BNIP3,[203,234] which is involved in the opening of the mitochondrial permeability transition pore,[235] and a low mitochondrial membrane potential have been proposed as possible mitophagic signals.[236–240] BNIP3 has been shown to promote autophagic mitochondrial degradation in reticulocytes by inducing the loss of mitochondrial membrane potential,[241,242] and Ulk1 appears to be essential in reticulocyte mitophagy.[243] Interestingly, mitochondria appear to undergo frequent cycles of fusion and fission in a "kiss and run" pattern; however, mitochondrial fission products with a low membrane potential are less likely to be involved in a fusion event and are destined for autophagic degradation.[239]

We do not know exactly how BNIP3 and a low mitochondrial membrane potential are linked to, for example, the mTOR pathway and the autophagic machinery. As discussed above, BNIP3 may titrate Bcl-2 away from Beclin 1 (see section, **Insulin, amino acids, and mTOR-mediated signaling**). BNIP3 can also directly bind Rheb, lower the GTP loading of Rheb, and, in this way, inhibit mTOR activity.[244] Other, or perhaps additional, mechanisms of BNIP3 action are also possible.[245] A low mitochondrial membrane potential is likely to result in a decrease in the local ATP concentration, which would activate AMPK. Because mTOR is associated, at least in part, with the mitochondria,[246] this would inhibit mTOR, as discussed above.

The expression of BNIP3 is under the control of the hypoxia-inducible factor 1 (HIF-1), the transcription factor that serves as the master regulator of oxygen homeostasis in all metazoans.[247] HIF-1-deficient cells do not induce BNIP3 or mitophagy under hypoxic conditions, and so they die because they are unable to protect themselves against the ROS that continue to be produced by the respiratory chain in spite of the low oxygen concentrations,[248,249] presumably because of the increased reduction pressure on the respiratory chain under these conditions. HIF-1 not only stimulates mitophagy but also simultaneously inhibits mitochondrial biogenesis;[248] thus it acts as a double-edged sword by providing greater adaptation at low oxygen concentrations. The production of ATP continues because HIF-1 also stimulates the transcription of glycolytic enzymes.[247,248] The α-subunit of HIF is degraded by the proteasome, but for this to occur the transcription

factor needs to be hydroxylated by prolyl hydroxylase domain-containing proteins (PHD), which require 2-oxoglutarate, possibly derived from glutamate dehydrogenase, to be active.[250] Thus, degradation of HIF-1α may be another mechanism, in addition to mTOR activation, by which amino acids can inhibit autophagy (Figure 6).

Recent studies have provided evidence that the protein Parkin, a ubiquitin ligase and the product of the *PARK2* (Parkinson disease 2) gene that is frequently mutated in Parkinson's disease, may be involved in targeting depolarized mitochondria for autophagic elimination.[251] For example, when oxidative phosphorylation is uncoupled or complex 1 of the respiratory chain is inhibited, Parkin is recruited from a cytosolic location to the mitochondria in a manner that is independent of mitochondrial fission. Fission of the depolarized mitochondrial fragment occurs only after Parkin has been recruited to the mitochondrial outer membrane, where the fragment is sequestered and degraded by autophagy.[251,252] It is not clear how Parkin recognizes defective mitochondria, but ROS or aggregation of proteins in the mitochondrial outer membrane may be involved.[252]

Mitophagy is also protective in a cell model of Parkinson's disease involving mitochondrial PINK1 (PTEN-induced kinase 1),[253] another protein that has been associated with Parkinson's disease and neuropsychiatric disorders. Knock-down of PINK1 induces mitochondrial fragmentation and mitophagy, which is potentiated by overexpression of Parkin, and which proceeds through ROS production and activation of mitochondrial fission.[253]

Because damaged mitochondria are a major source of ROS and, as will be discussed later, because ROS production contributes to aging, selective degradation of damaged mitochondria provides protection against aging [237,254,255] and apoptosis.[256]

Reticulophagy

Stress caused by the accumulation of misfolded proteins in the ER activates the unfolded protein response and stimulates the selective autophagic degradation of ER membranes.[257–260] This is accompanied by Atg1 kinase activation, and it is likely that TOR kinase activity is reduced under these conditions.[261] The ER stress sensors that are linked to the autophagy system include Ire1 (inositol-requiring enzyme 1), JNK, and the eIF2α kinase PERK (PKR-like ER kinase).[257–260,262]

α1-Antitrypsin deficiency, which is associated with chronic liver inflammation and carcinogenesis, provides an example of how autophagy attempts to remove a mutant protein that is retained in the ER because it is not properly folded and where it is prone to form aggregates rather than being secreted into the blood and body fluids where it normally acts as an inhibitor of neutrophil proteases.[263]

Ribophagy

It has been known for a long time that cytoplasmic RNA can be selectively degraded by autophagy, as demonstrated in rat liver during amino acid or insulin deprivation.[264] Recent data obtained with *S. cerevisiae* has demonstrated that, in response to nitrogen starvation, mature ribosomes can be selectively degraded by autophagy and that the catalytic activity of the Ubp3/Bre5 ubiquitin protease is required for the degradation of the entire 60S ribosomal subunit, but not that of the 40S ribosomal subunit.[265] This suggests that de-ubiquitination of still unknown targets is required for selective engulfment of ribosomes by autophagosomes to occur. Although cells in which the genes that encode these proteins have been deleted display defective ribophagy during starvation or after rapamycin treatment, they are still able to remove ribosomes by non-specific general autophagy.[265] In eukaryotic cells, ribosomal protein constitutes about 50% of the total cell protein. This indicates that, under starvation conditions, both non-selective and selective ribophagy are required in order to mobilize sufficient protein for the production of amino acids and allow the cells to survive.

Although both ribosomal subunits are degraded by selective macroautophagy, only the regulated turnover of the 60S subunit requires the Ubp3/Bre5 ubiquitin protease.[265] The signal that targets the 40S ribosomal subunit for specific autophagic degradation remains to be identified. Early experiments with hepatocytes suggested that autophagy may be inhibited by phosphorylation of ribosomal protein S6.[63] It is possible that phosphorylation of S6 by Sch9, the yeast homolog of mammalian S6K,[132] protects the ribosomal 40S subunit against autophagy and that S6 dephosphorylation is required to initiate 40S ribophagy.

Autophagy of glycogen

In newborn mammals, when the maternal supply of nutrients is interrupted and before suckling begins, the breakdown of glycogen in liver, heart, and muscle by specific autophagy occurs under the influence of glucagon and adrenalin in order to maintain glucose homeostasis.[266,267] In the autophagolysosomes, glycogen is hydrolyzed by α-glucosidase to release glucose. Glycogen autophagy is probably required

to assist the degradation of hepatic glycogen to glucose 6-phosphate by phosphorylase in the cytosol, followed by conversion to glucose, because glucose 6-phosphatase may not be sufficiently active at birth. Nothing is known about the mechanism by which glycogen is recognized by the autophagic system. However, we cannot exclude the possibility that the autophagic sequestration of glycogen may occur randomly, but that the rate of production of glycogen-derived glucose becomes significant because of the large stores of glycogen present in the liver and muscles at birth.

In contrast to the situation in newborn liver, heart, and muscle tissue, autophagy does not appear to be required for the delivery of glycogen to the lysosomes in adult muscle; under these conditions, glycogen may reach the lysosomes through microautophagy, which involves direct uptake of glycogen by invagination of the lysosomal membrane.[268]

Autophagy and cell death

Because autophagy has been reviewed recently in relation to cell death,[269–273] we will briefly discuss only a few highlights here.

For many years it has been thought that autophagy is not only cytoprotective but also that, under some conditions, it can kill the cell. This type of mechanism has been called type-II cell death, as opposed to type-I cell death, which is caused by apoptosis, and type-III cell death, caused by necrosis. In many cases, however, this conclusion has been founded on the detection of autophagosomes or of the accumulation of LC3-II in dying cells. As outlined in several recent papers,[5,23,41,273–275] this conclusion is based on the erroneous assumption that the concentration of a pathway intermediate equals autophagic activity, or flux. This is incorrect because the accumulation of autophagic structures is observed not only when the rate of autophagosome formation is increased but also when the fusion of autophagosomes with lysosomes is inhibited or when lysosomal function is affected, *e.g.* by a decrease in the activity of the ATP-dependent lysosomal proton pump. Clearly, in the latter case, the number of autophagosomes in the cell increases due to the decreased flux through the system. This analysis is entirely analogous to that of metabolic pathways, where the distinction between concentrations and fluxes has been known for decades.[41] A possible complication, surprisingly apparently never considered in the literature, is that in long-term cell cultures relatively large amounts of NH_3 may accumulate in the medium. This NH_3 is produced by glutaminase from glutamine, present in culture media at millimolar concentrations, and/or derived from the α-amino group of amino acids (glutamine included) in the course of their oxidative metabolism. Because NH_3 is a weak base that can permeate biological membranes in the unprotonated form (but not when protonated), it increases the pH of acidic cellular compartments, including the lysosomes, and thus affects lysosomal function.

To illustrate this point we mention a recent study showing a large accumulation of autophagic vacuoles and of LC3-II in antibody-producing CHO cells that had completely consumed 2 mmol/L glutamine, and died, in the course of a few days of culture.[276] Under these conditions, the ammonia concentration in the medium must have reached a concentration of at least 4 mmol/L, more than sufficient to affect the lysosomal pH, but this complication was not considered. Another study, in this case carried out with intestinal epithelial cells that had been exposed to glutamine for 24 h, likewise noted a considerable increase in LC3-II and in the number of autophagosomes.[277] Although it was concluded that glutamine had stimulated autophagy in these cells, the possibility cannot be ignored that, here too, accumulating ammonia may have been responsible for the phenomena observed.

Unfortunately, the erroneous assumption that the level of autophagosomes present reflects the autophagic flux has led to tremendous confusion in the literature. An example is that of cell death induced by the caspase inhibitor zVAD in mouse L929 cells.[278] An important complication in these experiments has been that zVAD also blocks lysosomal cathepsins so that both apoptosis and autophagy are inhibited under these conditions; this leaves necrosis as the only cell-death mechanism.[275,279] Although the large amounts of ROS observed in the presence of zVAD have been ascribed to specific autophagic elimination of catalase,[280] it is more likely that mitochondrial rupture was the cause of ROS production. Although autophagic compartments and LC3-II accumulated in the presence of zVAD, the activation of autophagy by either rapamycin or serum starvation offered protection against zVAD-induced cell death.[279] In contrast, knock-down of Beclin 1, Atg5, or Atg7, or the addition of acidotropic chloroquine, sensitized the cells to the killing effect of zVAD, and it was concluded that autophagy was promoting cell survival rather than cell death.[279]

Overexpression of Atg1 in *Drosophila* is capable of inducing apoptosis.[86] It remains to be established, however, whether in this context Atg1 triggers apoptosis by stimulating autophagy or by an autophagy-independent effect of Atg1.

Autophagy-induced cell death has also been reported in mammalian cells. One example of autophagy-induced cell death in mammalian cells is

that of $Bax^{-/-}/Bak^{-/-}$ (Bax, Bcl-2-associated X protein; Bak, Bcl-2 homologous antagonist/killer), immortalized MEF (mouse embryonic fibroblasts) cells treated with apoptotic stimuli.[281,282] It is noteworthy that this has been ascribed to inhibition of PTEN by Bax/Bak,[283] and it was concluded that inhibiting pro-apoptotic proteins and inducing autophagy could be used to sensitize cancer cells to therapy.

A second example of autophagy-induced cell death in mammalian cells was found in MCF7 human breast carcinoma cells carrying a mutation in the *Beclin 1* gene that renders the mutant Beclin 1 unable to bind Bcl-2; in contrast, the expression of wild-type Beclin 1 in these cells increases autophagy without causing cell death.[96]

Although these examples do show that autophagy can induce cell death in certain experimental settings, it remains to be demonstrated that autophagy actually does so under normal physiological conditions *in vivo*. One has to realize that, in the experiments referred to above, autophagy was artificially increased in a way that bypassed the normal physiological control mechanisms that prevent overactivation of autophagy. However, examples of autophagy-induced cell death have been identified during the development of *Drosophila* and *Dictyostelium*.[284,285] Likewise, genetic overactivation of autophagy in *C. elegans* can induce cell death during starvation, whereas inhibiting starvation-induced autophagy decreases survival of wild-type animals.[286]

Autophagy and disease

Aging and type-2 diabetes

ROS production by mitochondria, with the attendant loss of mitochondrial function, has long been implicated in the aging process.[287,288] Indeed, transgenic mice in which catalase, normally present in peroxisomes, has been targeted to the mitochondria, live longer.[289] Because autophagy, which declines with age,[290,291] can eliminate damaged intracellular structures, including mitochondria, it is not surprising that activating autophagy can increase longevity. Indeed, the ability of calorie restriction to increase longevity may be due not only to decreased ROS production because of diminished pressure on the respiratory chain but also to activation of autophagy,[288,292] particularly of mitochondria.[293] Direct evidence that maintaining appropriate autophagic activity throughout the life span slows down the functional organ failure associated with aging was provided by recent experiments with transgenic mice encoding Lamp-2A that was expressed in the liver only. Expression of this protein not only prevented the age-related decline in mitochondrial function and cellular ATP levels but also decreased the intrahepatic accumulation of oxidized and polyubiquitinated proteins.[294]

Because autophagy is inhibited by insulin-dependent mTOR-mediated signaling, it is anticipated that diminished signaling would extend the life span. There is now overwhelming evidence that this may, indeed, be the case. Apart from the fact that defective insulin signaling can induce life-span extension,[295–299] inhibitory mutations of individual components of the signaling pathway have also been shown to increase longevity in various organisms, including yeast, *C. elegans*, *Drosophila*, and mammals, and knockdown of *Atg* genes prevents this (see[5,75,291,300] for extensive recent reviews on this topic). In this context, it is of great interest that amino acid signaling in rats declines with age.[301] Although not considered in these studies, perhaps this can be seen as an attempt by the body to activate autophagy and thus to protect itself against cell damage.

Oxidative stress and decreased mitochondrial function not only are involved in aging but also contribute to the development of type-2 diabetes.[302,303] Autophagy in β-cells of diabetic rats and mice was found to be increased and to provide protection against cell damage by oxidative stress.[304–306] Likewise, the *in vitro* exposure of β-cells to palmitate, the fatty acid that is thought to play an important role in the etiology of type-2 diabetes through the synthesis of ceramide,[307] stimulates autophagy and thus provides protection against palmitate toxicity (Figure 7).[308] Whether increased ceramide concentrations were responsible for the activation of autophagy under these conditions was not studied.

These recent findings clearly indicate that autophagy in β-cells increases in a context of insulin resistance. Whether this also applies to the insulin-sensitive peripheral tissues remains to be demonstrated, but it seems likely. Rather than concluding that autophagy is an adaptive response to increased insulin resistance,[306] the possibility must be considered that insulin resistance itself could be the adaptive response that stimulates autophagy, which, in turn, protects against cell death. We realize that this is a provocative and speculative view, especially because insulin resistance, *i.e.* reduced responsiveness of the insulin-sensitive tissues to normal plasma concentrations of insulin, is one of the risk factors in metabolic syndrome. These risk factors, which also comprise adiposity, dislipidemia (high plasma triglycerides, high low-density lipoprotein cholesterol, low high-density lipoprotein cholesterol), and high blood pressure, predispose to cardiovascular disease and type-2 diabetes.[309] The latter is also characterized

by glucose intolerance, with decreased glucose consumption in muscle and adipocytes and increased hepatic glucose production, resulting in high plasma glucose concentrations,[309] which are toxic.[310] Thus, even though both lipid and glucose metabolism are greatly disturbed during insulin resistance and are potentially very harmful,[311] perhaps increasing autophagy as a protection mechanism against oxidative stress takes priority in order to prevent an even worse outcome.[312]

Reasoning along the same lines, one may speculate that the onset of insulin resistance in elderly people[311,313] is, in fact, an adaptive mechanism intended to increase autophagy and thus help to prolong life. In this context, it is of interest to note that the Klotho protein, which has strong anti-aging properties in mammals, confers insulin resistance.[314] Likewise, NAD^+-dependent ADP-ribosylation of glutamate dehydrogenase by the mitochondrially located sirtuin (silent mating type information regulation 2 homolog), Sirt4, which is important in life-span extension (see also next paragraph), downregulates glutamate dehydrogenase and inhibits insulin production in β-cells.[315] It is also noteworthy in this context that minor defects in the insulin/IGF1 (insulin-like growth factor) signaling pathway at the level of the IGF1 receptor, due to genetic variation, may contribute to human longevity in centenarians.[316] In addition, certain genetic variants of the *FoxO3A* gene are strongly associated with human longevity.[317] Although not discussed in these studies, it is tempting to relate these observations to increased autophagy.

Other factors to consider in life-span extension are the sirtuins, a family of NAD^+-dependent protein deacetylases (with the exception of Sirt4, which is an ADP-ribosyltransferase), responsible for the deacylation of histones, transcription factors, and many other proteins involved in the control of metabolism. The sirtuins can be viewed as energy sensors because their activity is increased when the NAD^+/NADH ratio increases due to a fall in energy level (Figure 3), by analogy with AMPK, which senses the cellular energy state from changes in the AMP/ATP ratio.[318,319] The sirtuins are involved in activating a variety of metabolic processes, such as lipolysis, fatty acid oxidation, mitochondrial biogenesis, and gluconeogenesis, and inhibiting glycolysis and lipogenesis.[318–320] In yeast, *C. elegans*, and *Drosphila*, Sir2 is needed for the longevity effect of calorie restriction to occur, and in mammals the same is true for its ortholog Sirt1.[318,320] The effects of sirtuin activation resemble many of the effects of calorie restriction. Because calorie restriction provides protection against many diseases, including cardiovascular disease, cancer, metabolic syndrome, type-2 diabetes, and neurodegenerative diseases, strategies intended to activate sirtuins may be used to combat these pathologies.[318,321]

Sirt1, which increases in starvation, also appears to stimulate autophagy by deacetylating Atg5, Atg7, and Atg8, and $Sirt1^{-/-}$ mice resemble $Atg5^{-/-}$ mice in that they accumulate damaged organelles in their tissues and die early in the perinatal period.[322] Conversely, acetylation of these Atg proteins by the p300 acetyltransferase has the opposite effect and inhibits autophagy.[323] Interestingly, p300 and Atg7 physically interact in a manner that is promoted by high nutrient availability. Whether this is a direct effect of nutrients on the association of p300 and Atg7 or whether it occurs indirectly, *e.g.* through AMPK activation and/or mTOR inactivation, remains to be established.

Enhanced turnover of mitochondria, *i.e.* increased autophagy of damaged mitochondria and increased biogenesis of mitochondria, and, consequently, decreased ROS production, may contribute to life-span extension.[320] In addition to deacetylation of Atg proteins, it is also possible that Sirt1 activates autophagy by stimulating AMPK, via deacetylation of LKB1,[324] which is the upstream kinase of AMPK, and via deacetylation and activation of FoxO3,[325,326] which controls the synthesis of Atgs (Figure 3). The ability of the polyphenol resveratrol, found in red wine, grapes, and nuts, to extend life span in several species has been ascribed, at least in part, to its ability to activate Sirt1.[319,327,328] Resveratrol can also stimulate autophagy,[329,330] although in this case autophagy appears to be non-canonical in that it does not appear to require the entire set of Atg proteins, in particular Beclin 1, to form the autophagosome.[330,331] It should be pointed out that resveratrol has other effects, too, such as the inhibition of PDK1[332] and weak inhibition of mitochondrial electron transport,[333,334] which may possibly activate AMPK. These effects may result in the activation of autophagy by mechanisms that are independent of Sirt1 (Figure 3). Another interesting observation is that depletion of NAD in neuroblastoma cells by pharmacological means increases the accumulation of LC3-II.[335] For reasons discussed above (see section, **Formation of autophagosomes**), this is insufficient to prove that the autophagic flux is activated, especially because NAD depletion can result in serious energy depletion. Nonetheless, it is intriguing to think that Sirt1 (due to an increased NAD^+/NADH ratio) could possibly be involved in autophagy under these conditions.

Another interesting aspect of autophagy in relation to disease (*e.g.* metabolic syndrome) is found in the observation that cholesterol depletion can activate autophagy[336] and that statins, which inhibit cholesterol synthesis at the level of HMGCoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase, inhibit mTOR

signaling[336,337] and activate autophagy.[336,338–340] An earlier study with isolated hepatocytes incubated in the absence of amino acids revealed a 20% increase in autophagy by lovastatin and simvastatin, but no attention was paid to this observation.[113] This relatively low level of stimulation may be explained by the fact that, under these experimental conditions, autophagy was already almost maximal, and also by the short incubation period (2 h), during which only slight changes in cholesterol concentration probably occurred.

Interestingly, a recently published large-scale trial (JUPITER study) with rosuvastatin showed that this compound significantly reduces the incidence of major cardiovascular events in apparently healthy individuals without hyperlipidemia but with elevated levels of C-reactive protein, an inflammatory biomarker that predicts future vascular events.[341] It would be of great interest to find out whether increased autophagy contributes, at least in part, to this finding and provides protection under these conditions.

Statins also ameliorate insulin sensitivity both in small animal models of insulin resistance and in man. Animal experiments have shown that they improve insulin signaling.[342,343] Again, one wonders whether stimulated autophagy underlies this effect, although, due to statin-induced inhibition of mTOR (see above), the possibility that feedback inhibition of IRS1 by S6K is relieved (Figure 3) also cannot be ruled out here.

Neurodegeneration

As indicated above in the section **Regulation of autophagy**, even in the presence of excess nutrients, a basal level of autophagy has to continue for cellular house-keeping. For example, mice with neuron-specific knock-out of Atg5 or Atg7 display severe neurodegeneration, have deficits in motor function, and accumulate aggregates of polyubiquitinated proteins in cytoplasmic inclusion bodies.[43–45] This demonstrates that basal autophagy is essential for normal neuronal function. In several neurodegenerative diseases, such as Huntington's, Alzheimer's, and Parkinson's, autophagy tries to protect the cells from the accumulation and aggregation of defective misfolded cytosolic proteins caused by mutations in their genes, *e.g.* the polyglutamine-tract at the N-terminus of the Huntingtin protein in the case of Huntington's, β-amyloid precursor protein and the microtubule-associated protein tau in Alzheimer's, and α-synuclein in Parkinson's disease.[290,344,345] Under normal conditions, these proteins are degraded by the ubiquitin-proteasome system, but in these neurodegenerative diseases this system becomes overwhelmed and autophagy acts as a rescue system. However, autophagy, too, is overwhelmed, either because its intrinsic capacity is too low or because its capacity somehow becomes compromised by the accumulation of the protein aggregates. In the case of Huntington's, the protein aggregates themselves appear to sequester mTOR, and this may be considered an attempt by the cell to eliminate these aggregates by autophagy.[346] However, these aggregates can also bind Beclin 1, which would be counterproductive for autophagy.[347] It is noteworthy that stimulation of autophagic sequestration by rapamycin, at least in experimental models of Huntington's, helps to clear the mutant Huntingtin protein.[79,346] However, inhibition of the synthesis of the Huntingtin protein by rapamycin may also contribute to its clearance.[348]

In Alzheimer's disease, the affected neurons accumulate autophagosomes, suggesting that autophagy becomes limited either at the level of the fusion of the autophagosomes with the lysosomes and/or at the level of the lysosome itself. Therapeutic modulation of autophagy in this case would need to focus on these steps.[349,350] The major challenge now facing the search for treatments for these neurodegenerative diseases is to find ways to stimulate autophagy in the affected neurons *in vivo*.

The autophagic removal of the ubiquitinated protein aggregates may be a selective process that requires the adaptor protein p62, or sequestosome 1 (SQSTM1). p62 not only binds to both the protein aggregates and LC3 to target them for autophagic degradation[351,352] but also is involved in the formation of the protein aggregates themselves.[352,353]

Familial amyotropic lateral sclerosis (ALS), another neurodegenerative disorder, selectively affects motor neurons. A deficiency in SOD1, a Cu/Zn superoxide dismutase, is found in about 20% of these patients. Once again, autophagy is an attempt to provide protection, in this case against the deleterious effects of ROS.[354,355]

As discussed in the section on **Mitophagy,** in Parkinson's disease, mitophagy protects against dysfunctional mitochondria.

Heart disease

As in other tissues, autophagy is also essential for proper function of the heart, where the process functions both as a mechanism to supply nutrients for survival and as a cellular quality-control mechanism.[37,356–358] For example, cardiac-specific loss of Atg5 in mice leads to cardiac hypertrophy, left ventricular dilatation, and contractile dysfunction, together with the accumulation of ubiquitinated proteins and abnormal mitochondrial and sarcomere

structure.[357] Autophagy becomes of particular importance in stressful situations, such as pressure overload, ischemia/reperfusion, cardiac hypertrophy, and heart failure.[5,356–359] Although there has been some debate about whether autophagy has a protective or detrimental (*i.e.* by autophagy causing the death of failing cardiomyocytes) effect on heart function,[5,359,360] most of the evidence suggests that the process is protective.[198,356–359,361]

Cancer

As discussed earlier (see section, **Insulin, amino acids, and mTOR-mediated signaling**), the insulin-growth factor-amino acid-mTOR-signaling pathway regulates protein synthesis and autophagy in opposite ways. The role of overactivation of this pathway in tumor growth has been discussed in several papers.[362–365] There is now ample evidence that suppression of autophagy can also promote tumorigenesis, and many of the components involved in activating autophagy are tumor suppressors, *e.g.* Beclin 1, UVRAG, PTEN, TSC1/2, p53, and ceramide.[5,366–372] With regard to p53, however, the problem is that, depending on the conditions, both p53 activation and p53 inhibition can stimulate autophagy in a manner that proceeds via AMPK activation and mTOR inhibition.[373–375] This apparent paradox needs to be resolved.

Many chemotherapeutic drugs not only inhibit protein synthesis but also stimulate autophagy. Such drugs include rapamycin, tamoxifen (via ceramide production), arsenic trioxide, resveratrol, and temozolamide.[366,376] In some cases, this stimulation of autophagy is an attempt by the cell to protect itself.[377,378]

A well-known example of the involvement of autophagy genes in tumor suppression is that of *Beclin1*, which is monoallelically deleted in human breast, ovarian, and prostate cancers. Haploinsufficiency of *Beclin 1* promotes tumorigenesis in various tissues in transgenic mice.[379,380]

An increase in autophagy can stop tumor growth by two mechanisms. First, it may tip the balance between protein synthesis and degradation towards degradation, which reduces cell growth. Second, it may remove defective mitochondria and other intracellular structures that are potential sources of ROS, which can cause DNA damage, genome instability, and tumor initiation. This mechanism connects the tumor-suppressor and anti-aging functions of autophagy and, as autophagy decreases during aging, explains the increased incidence of cancer as people age.[367,368] Unfortunately, in some cases, autophagy can also act as a survival mechanism for the tumor. This is during the initial stages of rapid tumor growth or in solid tumors, when insufficient vascularization limits nutrient and oxygen supply to the cancer cells.[367,368] Although the signal that stimulates autophagy under these conditions is not known, one may speculate that the activation of AMPK and HIF-1 plays a role.[381] It is also possible that autophagy in the hypoxic environment serves to eliminate damaged mitochondria that might otherwise overproduce ROS and kill the cancer cells.[367,382] Indeed, shifting cancer cell metabolism from glycolysis to glucose oxidation by adding dichloroacetate, an inhibitor of pyruvate dehydrogenase kinase, activates mitochondrial pyruvate oxidation and causes ROS-induced cancer cell death that can be prevented by inhibiting the respiratory chain.[383]

In summary, it is not yet possible to predict the outcome on tumor growth of manipulating autophagy as a strategy for cancer therapy.

Lysosomal disorders

The autophagic pathway becomes severely compromised in diseases involving a primary defect in lysosomal function. An example of this is Danon's disease, in which there is a deficiency in the lysosomal membrane protein Lamp-2, leading to the massive accumulation of autophagosomes in several tissues, *e.g.* muscle, heart, and liver, because of retarded fusion of autophagosomes and lysosomes.[12] Fusion blockade is also observed in lysosomal storage disorders, when the substrate to be degraded accumulates inside the lysosomes, once again interfering with the fusion of autophagosomes and lysosomes. This results in the accumulation of other intracellular material, unrelated to the primary enzyme defect, in the autophagosomes, *e.g.* aggregate-prone ubiquitinated proteins and organelles such as mitochondria.[12,384,385] For example, in Pompe's disease, which involves deficient lysosomal α-glucosidase, there is massive accumulation of autophagosomes in muscle and heart, filled not only with glycogen but also with ubiquitinated proteins.[268] Likewise, deficiencies in lysosomal cathepsins result in accumulation of autophagosomes,[384] and impaired autophagosome-lysosome fusion may underlie neuronal ceroid lipofuscinosis (Batten's disease), caused by mutations in the endosomal/lysosomal membrane protein Cln3 (ceroid-lipofuscinosis, neuronal 3).[386,387] In Batten's disease, subunit c of mitochondrial ATP synthase accumulates in autophagosomes, and this can be mimicked in mice expressing the mutated form of Cln3 in their neurons, which suggests that autophagic turnover of mitochondria is affected under these circumstances.[386] Similarly, in the lysosomal storage

disorders, including mucopolysaccharidosis type IIIA (Sanfilippo A) and multiple sulfatase deficiency, severe neurodegeneration is accompanied by autophagosome accumulation caused by defective fusion between autophagosomes and lysosomes.[385]

Niemann-Pick's disease, another neurodegenerative lysosomal storage disorder, characterized by the accumulation of unesterified cholesterol and sphingolipids in late endosomes and lysosomes, is caused in most cases by a loss of function mutation in the *NPC1* (Niemann-Pick type C disease 1) gene encoding a late-endosomal protein involved in lipid sorting and vesicle trafficking.[388] Npc1-deficient human fibroblasts display considerable accumulation of autophagosomes and an increased level of LC3-II, but also increased autophagic flux, together with upregulation of Beclin 1. In contrast to the other lysosomal storage disorders mentioned above, the fusion between autophagosomes and lysosomes appears to be intact, and it has been proposed that, in Niemann-Pick, the enhanced autophagic flux in fibroblasts may be an attempt to promote cell survival.[388]

Another situation where lysosomal function may be compromised is that of hepatic encephalopathy (HE). HE is characterized by neuropsychiatric manifestations, which can range from mild abnormalities of mental state to coma. The disease is caused by a failure of liver function as the result of acute or chronic liver disease.[389] Under such conditions, the plasma ammonia concentration rises because ammonia is no longer properly converted in the liver into either urea or glutamine. The mechanism of ammonia toxicity in the brain has been a matter of debate for years; possible mechanisms that have been proposed include astrocyte swelling, opening of the mitochondrial membrane pore, ROS production, and protein nitration.[389–391] Although never previously considered as a possible mechanism for the toxicity of ammonia, compromised autophagy may also contribute. Two facts suggest this hypothesis. First, increased intracellular glutamine (produced from ammonia) not only increases astrocyte volume by osmosis in the short term, which probably accounts for the brain swelling and increase in intracranial pressure,[389] but also does so in the long term by stimulating mTOR signaling, which is known to increase astrocyte cell size.[392] This would lead to suppression of autophagy, and thus to an impaired capacity of the astrocyte to remove aberrant cell structures such as mitochondria. Second, the rise in intracellular ammonia concentration in HE may directly affect the intralysosomal pH. In the future, experimental evidence may reveal more about this intriguing, but so-far hypothetical, mechanism of ammonia toxicity.

Infection and immunity

Work in the last decade has provided strong evidence that autophagy not only contributes to maintaining cellular homeostasis but also can be used to combat bacterial, parasitic, and viral infections. Because excellent reviews on these topics have appeared recently (see [5,350,393,394]), we will only briefly summarize some highlights here.

As part of the innate immunity system, professional phagocytes (macrophages and dendritic cells) and non-professional phagocytic cells are equipped with degradative, mainly lysosomal, enzymes that kill and degrade pathogens once they have been phagocytosed. The autophagic pathway may assist in this cellular defense mechanism. Different groups of bacteria and parasites have developed different strategies to evade these cellular defense mechanisms. For example, they can puncture the membrane of the phagosome and so escape into the cytosol where they can multiply, or they can prevent the fusion of phagosomes with lysosomes so that they can replicate inside the non-lytic phagosomal compartment. In such cases, stimulating the autophagic sequestration of the bacteria could help to deliver them to the lysosomes for final killing. There are also cases, however, in which the bacteria can actually benefit from autophagy because they are able to survive in autophagosomes after inhibiting their fusion with lysosomes. With regard to viral replication, autophagy can similarly be either detrimental or beneficial (by permitting survival in initial autophagosomes). In the case of HIV (human immunodeficiency virus)-1 infection, autophagy appears to be required for viral envelope protein-mediated apoptosis in uninfected bystander CD4+ T lymphocytes[395,396] while, in infected cells, autophagosome formation is downregulated, probably as part of a strategy to avoid virus degradation, to enhance viral replication, or to evade the immune system.[397] The immune signaling molecules that activate autophagic removal of pathogens include Toll-like receptors, TNF, interferon γ, and the eIF2α kinase PKR (double-stranded RNA-dependent protein kinase) (against viral infection).[5,350,393,394]

Apart from being involved in the elimination of pathogens, autophagy can also contribute to adaptive immunity. Lysosomes generate the antigenic peptides that are exposed on the cell surface in association with the major histocompatibility complex class II (MHC II), for presentation to CD4+ T cells, and autophagosomes often fuse with MHC II-loading compartments. Another mechanism by which autophagy can influence adaptive immunity is its ability to promote the development and survival of B and T cells.[393,394]

A possible role of autophagy in the pathogenesis of Crohn's disease, a chronic inflammatory bowel disease affecting most commonly the small intestine and colon, has recently been revealed in studies showing a strong genetic association between a single-nucleotide polymorphism in the *ATG16L1* gene, in which alanine is substituted for threonine at position 300, and enhanced susceptibiliy to Crohn's disease.[398] Although it is still unclear to what extent this mutation affects autophagy,[398,399] Paneth cells appear to be the target of the mutant protein.[400] One possibility is that autophagy in Paneth cells is involved in the specific recognition and elimination of certain intestinal bacteria.[399] These intestinal epithelial cells exocytose lysozyme and other antimicrobial peptides packaged in granules. In Paneth cells, autophagy appears to function as an important brake on the expression of proinflammatory genes, as indicated by experiments in mice with hypomorphic (*i.e.* decreased) expression of the Atg16L1 protein, and similar results have been obtained with intestinal epithelial cell-specific deletions of Atg5 or Atg7.[398,400] Strikingly, the same cell abnormalities, *i.e.* reduction in granule number, abnormal mitochondria, and a dramatic increase in cytoplasmic vesicles at the expense of ER, as observed in Paneth cells of autophagy-deficient mice, are also seen in Paneth cells from Crohn patients who are homozygous for the *ATG16L1* risk allele.[398] Paneth cells may be more susceptible than other intestinal epithelial cells to autophagy deficiency as a result of their abundant ER, which may make them particularly sensitive to ER stress. Experiments with a knock-in mouse in which the endogenous *Atg16L1* has been replaced by the Crohn's disease risk allele of *ATG16L1* may provide direct information about the precise effect of *ATG16L1* polymorphism.[398]

Conclusion

For decades, autophagy has lurked in the literature disguised as a rather insignificant process. However, with the identification of the *ATG* genes and the signaling pathways that control it, autophagy has come out of the shadows and is now recognized as a major cell biological process, of similar importance to protein synthesis and other major pathways. Its function both as a catabolic pathway in response to energy shortage and as a cellular quality-control mechanism in response to stress and its disregulation in many diseases has caused autophagy to take center stage in today's cell biological research. However, it is important to stress that Atg proteins not only may be engaged in autophagy but also may have other functions in cell biology. For example in neutrophils, prior to being coupled to Atg12, Atg5 can be cleaved by calpain to form an aminoterminal polypeptide fragment with pro-apoptotic properties, which translocates to the mitochondria.[401] In phagocytic cells, however, Atg5 may be engaged in cellular immunity and the intracellular killing of pathogens by mechanisms that are independent of autophagosome formation.[402,403]

As we have indicated throughout this review, many questions remain to be answered. The most important task that lies ahead, however, is to translate the experimental findings into clinical practice in a wide range of pathologies, including cancer, neurodegeneration, diabetes, and infectious diseases, as well as in aging, so that *in vivo* manipulation of autophagy can be used to benefit patients.

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