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REVIEW ARTICLE

Regulation of autophagy by amino acid availability in *S. cerevisiae* and mammalian cells

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Abstract Autophagy is a catabolic membrane-trafficking process that occurs in all eukaryotic organisms analyzed to date. The study of autophagy has exploded over the last decade or so, branching into numerous aspects of cellular and organismal physiology. From basic functions in starvation and quality control, autophagy has expanded into innate immunity, aging, neurological diseases, redox regulation, and ciliogenesis, to name a few roles. In the present review, I would like to narrow the discussion to the more classical roles of autophagy in supporting viability under nutrient limitation. My aim is to provide a semblance of a historical overview, together with a concise, and perhaps subjective, mechanistic and functional analysis of the central questions in the autophagy field.

Keywords Autophagy · Macroautophagy · Microautophagy · Nitrogen starvation · Amino acids · Tor

Autophagy—introduction

Autophagy is a general name for catabolic membrane-trafficking pathways in eukaryotic cells that lead material from the cytosol into the lumen of the degradative organelle, be it the lysosome or the vacuole, depending on cell type. This review will focus on types of autophagy which depend on membrane trafficking: macroautophagy and to a lesser extent, microautophagy. During macroautophagy (Fig. 1a), intracellular membrane cisternae undergo deformation coupled with expansion to form a cup-shaped isolation membrane (sometimes termed a phagophore) that engulfs cytosolic material. The cup-shaped isolation membrane undergoes a closure step that is topologically analogous to a 'reverse gastrulation', thus forming an autophagosome. The autophagosome is a unique double-membrane vesicle which contains the engulfed cytosolic material within the lumen of the inner membrane. Autophagosomes mature either by directly fusing with the lytic compartment, as is observed in yeast cells, or, as seen in mammalian cells, by fusing with Golgi-derived vesicles carrying zymogens of lysosomal hydrolases and proton pumps as well as with elements of the endosomal network. Once exposed to active vacuolar/lysosomal hydrolyses, the inner membrane disintegrates and exposes the cytosol-derived content to degradation. Finally, biosynthetic building blocks such as amino acids are recycled back to the cytoplasm to meet metabolic demands. Microautophagy (Fig. 1b) is a related process where the lysosomal/vacuolar membrane directly invaginates to bud vesicles into the lumen of the organelle without first forming a free autophagosome.

Macroautophagy has been known since the 1960s, when work by Ashford and Porter (1962) showed that glucagon induced the proliferation of lysosomal compartments that contained cytosolic material such as mitochondrial profiles. Subsequent studies by Deter and De Duve (1967); Mortimore and Poso (1987); Seglen et al. (1991), and co-workers showed that macroautophagy is a response of mammals and mammalian-derived cell cultures to starvation conditions. Thus, Mortimore and colleagues showed induction of autophagy in starved rats, while Seglen and colleagues were able to follow the phenomenon in isolated hepatocytes and to conduct biochemical studies (Fosse et al. 1995; Holen et al. 1996).

Work in the 1990s, in the laboratories of Yoshinori Ohsumi (Baba et al. 1994; Takeshige et al. 1992; Tsukada

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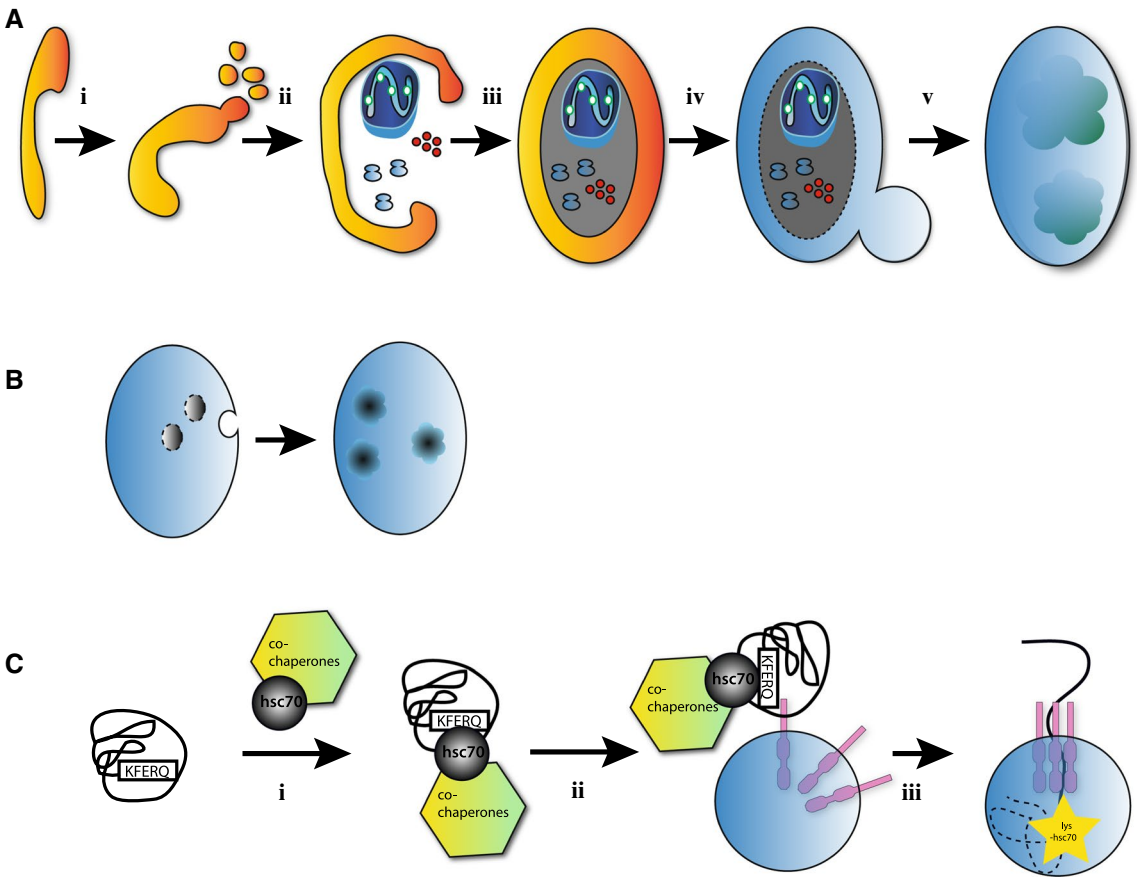

Fig. 1 Overview of macroautophagy, microautophagy, and chaperone-mediated autophagy. **a** During macroautophagy, intracellular membrane cisternae undergo expansion coupled with deformation (*i* and *ii*) to form a sequestering membrane (a.k.a. the 'phagophore') that engulfs cytoplasmic material. The sequestering membrane closes to generate an autophagosome (*iii*). The autophagosome then undergoes maturation to import active lysosomal/vacuolar hydrolases into the intermembrane space, forming an autolysosome or autophagolysosome (*iv*). Finally, the cytosolic material is degraded into biosynthetic building blocks (*v*) that are recycled into the cytoplasm. **b** Microautophagy is a variation in which the lysosomal/vacuolar membrane (*in blue*) directly invaginates to generate autophagic bodies that are degraded by resident hydrolases. **c** Chaperone-mediated autophagy is a non-membrane-trafficking pathway that directly translocates target proteins from the cytosol to the lysosomal lumen. During CMA, specific target proteins (here denoted by the degradation motif KFERQ) associate with hsc70 and additional chaperone cofactors. The hsc70 complex serves to unfold the target protein as well as to present it to the LAMP2 receptor (shown in *dirty pink*). LAMP2 molecules then self-associate to form a dynamic, transitory channel through which proteins are translocated with the aid of lysosomal hsc70 (lys-hsc70)

and Ohsumi 1993); Thumm et al. (1994); Klionsky et al. (1992); Harding et al. (1995) showed that autophagy, and specifically macroautophagy, occurred in yeast cells. This led to the relatively rapid discovery of genes that are required for this process, and to many of the mechanistic insights that we possess today.

Autophagy in *Saccharomyces cerevisiae*

In *S. cerevisiae*, macroautophagy is induced upon nitrogen starvation. The original report (Takeshige et al. 1992) also included studies on amino acid starvation, although that study did not take into account the interaction of starvation with auxotrophy effects (see below). Once mutants in macroautophagy were available, it became rapidly apparent that the process is essential for survival under nitrogen starvation (Tsukada and Ohsumi 1993). The generally accepted model is that during starvation in the presence of a carbon source, turnover of nitrogen sources allows carbon metabolism to continue apace, even in the absence of external nitrogen. This feature is technologically important; during winemaking, yeast cells grow in a very nitrogen-poor environment, and nitrogen is depleted early in the process, while fermentation can take 2–3 weeks (Gutierrez et al. 2012) implying that the fermentation occurs under nitrogen starvation. The role of macroautophagy in recycling cellular nitrogen received further support when it was discovered that Atg22, a protein previously thought to function in autophagy, was actually a transporter for amino acid

efflux from the vacuolar lumen. Thus in *atg22Δ* cells, loss of viability occurs under nitrogen starvation because amino acids released from autophagic degradation of proteins in the vacuolar lumen are not transported to the cytosol at a sufficient rate (Yang et al. 2006; Yang and Klionsky 2007).

However, significant question marks remain in our current understanding of the physiological functions of autophagy as a survival mechanism. A baffling result surfaced in 2011, when it was found that by buffering the extracellular medium, macroautophagy mutants could be made to survive nitrogen starvation for prolonged periods (Suzuki et al. 2011). This in itself could still be explained within the realm of autophagy as a recycling mechanism; in the absence of buffer, the extracellular medium becomes extremely acidic. Once it becomes more acidic than the vacuole, thermodynamic considerations lead to leaching of basic amines into the medium. Thus, upon buffering, this leaching would be stopped and nitrogen would be available despite the lack of recycling. More puzzling, however, is the fact that once autophagy mutants are analyzed after surviving starvation in buffered medium, it turns out that by some hat trick they have lost their mitochondrial DNA! It is still unclear how or why mitochondrial DNA is lost under these conditions (Suzuki et al. 2011). The authors suggest that autophagy must function to prevent oxidative damage under these conditions. This conceivably could occur through autophagic degradation of mitochondria (mitophagy). However, yeast cells starved for nitrogen in the presence of glucose do not undergo significant mitophagy even after 6 days in the starvation medium (H. Abeliovich, unpublished). In addition, glucose is known to inhibit mitochondrial ROS production in yeast by shutting down oxidative phosphorylation through the Crabtree effect (Merico et al. 2007). An alternative explanation is that cytoplasmic amino acids play a role in repressing oxidative metabolism, in the presence of glucose, and that inappropriate induction of respiration leads to loss of mitochondrial DNA integrity. In agreement with this last explanation, Hughes and Gottschling (2012) found that aging yeast cells lost mitochondrial function due to an increase in vacuolar pH, and that overexpression of the neutral amino acid transporter Avt1, or overexpression of Vma1 and Vph1, which resurrect the vacuolar pH gradient, could counteract this loss of mitochondrial function.

The general nature of the induction mechanism

An important question in the regulation of starvation-induced macroautophagy is the precise nature of the induction mechanism. While autophagy is known to be regulated by the Target of Rapamycin (Tor) kinase (Noda and Ohsumi 1998; Ravikumar et al. 2003), the signaling pathway during the induction of autophagy by starvation is far from elucidated. To try to understand how macroautophagy is regulated by amino acid withdrawal in yeast, Ecker et al. (2010) performed an analysis of the requirements for specific amino acid starvations. One question was whether nutrient sensing is achieved through extracellular receptors and channels, or whether intracellular metabolism plays a role in sensing nutrient levels. Whereas prototrophic cells can respond to full nitrogen starvation, but not to amino acid starvation, it was found that starvation for specific amino acids elicited autophagic responses solely in cells auxotrophic for that specific amino acid. This response is not simply an artifact of auxotrophy because it could be mimicked by inhibiting glutamine synthesis using methionine sulfoximine, an inhibitor of glutamine synthetase. Interestingly, starvation for nucleotides such as uracil did not elicit autophagy even in a uracil auxotroph, indicating that this is a specific response for amino acid starvation. These results imply an intracellular mechanism, not the one mediated by extracellular receptors for amino acids. The autophagic response elicited by the withdrawal of auxotrophically required amino acids was not identical to the canonical response to nitrogen starvation which is almost universally used in the study of macroautophagy in *S. cerevisiae*; autophagic flux was lower than that observed under full nitrogen starvation, and more importantly, the genetic requirements for induction of autophagy by amino acid starvation are distinct from those observed by full nitrogen starvation. While deletion of *GCN2* fully inhibited autophagy under amino acid starvation, it had no effect on autophagic flux in response to full nitrogen starvation. *GCN2* encodes a protein kinase, Gcn2, which is activated by unconjugated tRNA in the cytoplasm (Dever et al. 1992), and regulates a downstream cascade of events in response to amino acid limitation. Central to this response downstream of Gcn2 is the up-regulation of Gcn4, a transcription factor that induces the expression of genes required for the biosynthesis of amino acids and nucleotides (Hinnebusch 2005). Importantly, this response is conserved in animal cells (Harding et al. 2000), and has been shown to be responsible for dietary choices made in the mouse brain (Hao et al. 2005; Maurin et al. 2005, 2014).

The effect of the *gcn2Δ* and *gcn4Δ* mutations on amino acid starvation-induced autophagy in auxotrophic yeast appears complex, but one aspect of this effect seems to be that the gcn mutants display and absence of Atg8 induction in response to amino acid starvation. This is in contrast to the WT cells, in which a robust increase in Atg8 levels occurs under the same conditions. In agreement with the autophagy phenotype observed in these mutants, full nitrogen starvation does elicit Atg8 induction in *gcn2Δ* and *gcn4Δ* cells. Interestingly, wild-type cells show lower levels of lipidated Atg8 during amino acid starvation than in

full nitrogen starvation, again in agreement with the idea that amino acid starvation is distinct from full nitrogen starvation-induced macroautophagy.

These conclusions were corroborated by the work of Graef and Nunnari (2011). They studied the involvement of mitochondrial respiration in signaling events that regulate the induction of autophagy in response to starvation. While cells devoid of respiratory capacity induced normal autophagic responses in response to total nitrogen starvation, they were unable to induce autophagy in response to amino acid starvation. Although the role of mitochondrial respiration in regulating amino acid starvation-induced autophagy is not clear, it is conceivable that defects in glutamine and or aspartate synthesis from TCA cycle intermediates may affect the induction of autophagy in response to amino acid starvation. The role of Gcn2 in the regulation of autophagy seems conserved, as the mammalian interferon-inducible eIF2α ortholog PKR is required for autophagy in mammalian cells (Talloczy et al. 2002). The fact that this role is conserved at the level of amino acid starvation-induced autophagy, but not at the level of nitrogen starvation-induced autophagy in yeast, further supports the idea that the latter process is an adaptation of the single-cellular lifestyle choices of *S. cerevisiae*.

It is instructive to compare these results with mammalian cells. In complex multicellular organisms, individual cells almost never encounter conditions of nitrogen scarcity. However, under specific circumstances they do encounter amino acid starvation. Dietary problems can definitely lead to amino acid starvation at the cellular level, as mammals, for example, are auxotrophic for specific amino acids. On the other hand, amino acid starvation per se cannot occur in wild-type yeast cells, as they are capable of synthesizing the full complement of amino acids from carbon sources and simple nitrogen sources such as ammonia. Instead, what we perceive as amino acid starvation responses in yeast must correspond to homeostatic mechanisms that balance and regulate metabolism.

There are many instances in which data obtained in mammalian cells and whole animals disagree with results from yeast cells. Some of these discrepancies may be due to bona fide mechanistic differences between these cells, and others may be the result of very different types of assays used to measure autophagy in mammalian cells and in yeast. However, it stands to reason that at least some of the differences may be due to a comparison of full nitrogen starvation conditions in yeast, versus amino acid starvation in mammals. An additional complication of mammalian cells is that nutrient availability is only partially cell autonomous. Thus, the concentration of amino acids in the medium is not the sole determinant of the cell's physiological status vis-a-vis amino acid availability. Lum et al. (2005) showed that withdrawal of growth factors in Bax−/− Bak−/− cells (which cannot induce apoptosis in response to growth factor deprivation) will induce autophagy, and that autophagy is essential for cell survival under these conditions. Importantly, growth factor deprivation also leads to down-regulation of cell-surface nutrient transporters, including amino acid permeases (Edinger and Thompson 2002; Edinger et al. 2003).

Amino acid limitation and Tor-mediated signal transduction during the induction of autophagy in yeast and mammals

The Tor1 and Tor2 kinases in *S. cerevisiae*, originally identified as targets of the immunosuppressant rapamycin (Heitman et al. 1991; Kunz et al. 1993) and their orthologs in other eukaryotes, have been central to our understanding of eukaryotic responses to nutrient availability. They function within protein complexes, TORC1 (Kim et al. 2002; Loewith et al. 2002) and TORC2 (Jacinto et al. 2004; Loewith et al. 2002; Sarbassov et al. 2004), which are (respectively) rapamycin-sensitive and rapamycin-insensitive complexes of these kinases together with ancillary regulatory subunits. Tor is highly conserved among eukaryotes, although in most organisms it is expressed from a single gene, in contrast with the situation in *S. cerevisiae*. It was recognized early on that the response to rapamycin (which inhibits the TORC1 complex) closely mimics nitrogen starvation responses in *S. cerevisiae* (Barbet et al. 1996; Schmidt et al. 1998) and in *S. pombe* (Alvarez and Moreno 2006; Weisman et al. 2007). Indeed, rapamycin induces macroautophagy in yeast (Noda and Ohsumi 1998). TORC1 is localized to the limiting membrane of the vacuole in yeast. In mammalian cells, it localizes to the lysosome upon activation. It is tethered to the vacuole/lysosome through the Ego1/3–Gtr1–Gtr2 complex—the "Ego" complex, in yeast, and the "Ragulator" complex in mammalian cells (Binda et al. 2009; Sancak et al. 2008, 2010). These complexes are thought to regulate TORC1 activity in response to nutrient levels. In mammalian cells, nucleotide occupancy on the Rag protein GTPases (components of the Ragulator complex), modulates mTORC1 localization to the lysosome membrane and its activation in response to amino acid levels, while the Rheb GTPase directly regulates TORC1 kinase activity in response to hormone and growth factor stimulation via the TSC1/2 GAPs (Saucedo et al. 2003; Smith et al. 2005; Zhang et al. 2003).

When active, TORC1 phosphorylates components of the autophagic machinery (among numerous other substrates), and this phosphorylation functions in turn to inhibit the induction of autophagy when nutrients are present or as dictated by hormonal signals. Direct phosphorylation of Atg1/Ulk1 and of Atg13 by TORC1 has been reported in

yeast and mammals (Kim et al. 2011; Kraft et al. 2012; Abeliovich et al. 2000; Klionsky et al. 1992). Originally, studies on yeast cells suggested that dephosphorylation of Atg13 leads to the nucleation of a holoenzyme comprising, minimally, of Atg1, Atg13, and Atg17, and that this increases the downstream activity of the Atg1 kinase (Kamada et al. 2000). However, several results have questioned this model. First, unlike the original data from yeast, the ULK1–mAtg13 complex is constitutively assembled in mammalian cells (Hosokawa et al. 2009). In addition, more recent studies, using non-overexpressed proteins, demonstrated that the Atg13–Atg1 complex is constitutively assembled in yeast as well, and that only its phosphorylation state is modulated (Kraft et al. 2012). The overall phosphorylation level of both Atg13 and Atg1 in *S. cerevisiae* is reduced upon induction of autophagy (Abeliovich et al. 2003; Matsuura et al. 1997; Scott et al. 2000). Importantly, kinase-dead Atg1 is not completely defective for autophagic trafficking of aminopeptidase I (Abeliovich et al. 2003), and experiments with analog-sensitive (Shokat and Velleca 2002) Atg1 alleles also supported the idea of a structural role for Atg1, as opposed, or in addition to, a signal-transducing role (Abeliovich et al. 2003), although these last results have been controversial (Kabeya et al. 2005). In fact, no convincing Atg1 substrate found in yeast has been shown to be crucial for the induction of autophagy, other than Atg1 itself. A recent study in yeast has shown that Atg2, Atg1, and Atg9 are phosphorylated in an Atg1-dependent fashion and that mutation of all 6 Atg1-dependent phosphorylation sites on Atg9 blocked autophagy. However, this result should be treated with caution, as the mutation of six serine residues to alanine could have gross structural effects, and this has not been ruled out (Papinski et al. 2014). In addition, the autophagy defect of the 6-ala *atg9* mutant is not complete (Papinski et al. 2014), implying that kinase activity does not fully explain Atg1 function, since the *atg1* deletion mutant is completely defective in autophagy.

A structural study on the yeast Atg1 complex has suggested a role for the C-terminus of the protein in membrane binding and curvature sensing (Ragusa et al. 2012), suggesting an alternative, if not mutually exclusive role, for Atg1 in autophagosome biogenesis, as a structural component of autophagosomes. Such a role is consistent with the finding that in yeast cells, Atg1 is degraded in the vacuole during starvation-induced macroautophagy, through its interaction with Atg8 (Nakatogawa et al. 2012).

Confusingly, a different substrate has been identified for the Atg1 ortholog, ULK1, in mammalian cells; it was recently shown that beclin1 (Liang et al. 1999), a subunit of Vps34, the type III PtdIns3 kinase which is essential for autophagy and endo-lysosomal trafficking (Juhasz et al. 2008; Kihara et al. 2001; Matsunaga et al. 2009), is phosphorylated on serine 14 in a Ulk1/2-dependent fashion and that this phosphorylation is essential for the induction of autophagy by amino acid starvation (Russell et al. 2013). It remains to be determined whether an analogous mechanism occurs in yeast cells. If correct, this would imply that Atg1/Ulk functions to regulate localized phosphatidylinositol 3-phosphate (PtdIns3p) synthesis during the induction of autophagy. PtdIns3p synthesis by the Vps34 type III PtdIns3 K is required for the formation of key early autophagosomal intermediates termed "omegasomes", which are precursors of the autophagic sequestering membrane (Axe et al. 2008).

Role of the Atg8/LC3 family proteins and associated factors

Expansion of the sequestering membrane also involves Atg8/LC3, a ubiquitin-like protein (UBL). Unlike other UBLs, however, Atg8 family members are conjugated to a phospholipid, phosphatidylethanolamine (Ichimura et al. 2000). This conjugation reaction requires a host of autophagy-specific factors, including an E1 (Atg7), E2 (Atg3), and apparently also an E3 activity. The E3 activity is carried out by a complex of several proteins, which include Atg12, Atg5, and Atg16 (Hanada et al. 2007; Noda et al. 2013; Romanov et al. 2012). Atg12 is an additional autophagy-specific UBL, which is selectively conjugated to Atg5 through the actions of Atg7 and Atg10 (a specific E2-like activity) (Mizushima et al. 1998). The Atg5–Atg12 conjugate recruits Atg16 and the trimeric Atg5–Atg12–Atg16 complex is potentially capable of forming a lattice (Fujioka et al. 2010; Kuma et al. 2002) in addition to providing an E3 activity for Atg8/LC3 conjugation to PE. Recent data from in vitro work with giant unilamellar vesicles suggest that the Atg5–12–16 complex initially interacts reversibly with membranes, leading to formation of Atg8–PE (Kaufmann et al. 2014). The membrane-bound Atg8–PE in turn nucleates the Atg5–12–16 lattice, which seems to immobilize Atg8 in the membrane. Interestingly, attachment of selective autophagic cargo to Atg8 is mutually exclusive with participation in the lattice (Kaufmann et al. 2014), and this result may have important implications for our understanding of autophagosome formation. The sequestering membrane of autophagosomes can be divided into a convex face and a concave face (see Fig. 1a). The Atg5–12–16 lattice is only found on the convex face, while Atg8 is found on both faces (Mizushima et al. 2001, 2003; Kirisako et al. 1999). If Atg8–PE on the concave face is cargo bound, this could be a key for understanding the formation of asymmetry between two faces, which is essential for autophagosome formation. Membrane-bound Atg8

on the convex face functions in autophagosome expansion: in yeast, *atg8Δ* and hypomorphic atg8 mutants form small autophagosomes (Abeliovich et al. 2000; Xie et al. 2008), and in vitro studies demonstrate that recombinant Atg8 can promote liposome tethering and hemifusion (Nakatogawa et al. 2007), although a role for SNARE proteins has also been invoked in autophagosome expansion (Abeliovich et al. 1999; Nair et al. 2011). Unlike ubiquitin, Atg8 is synthesized as a precursor lacking a C-terminal glycine; the functional C-terminal glycine is exposed following proteolysis of the encoded C-terminal arginine by Atg4, an Atg8-specific protease/amidase. It has been suggested that Atg4 is regulated by redox and that redox signaling also plays a role in the induction of autophagic trafficking (Scherz-Shouval et al. 2007). Interestingly, ectopic expression of a truncated Atg8 with an exposed glycine does not complement an *ATG4* deletion because Atg4 is also required for hydrolysis of Atg8–PE, and recycling of free Atg8. An additional open question is whether the large number (currently 8) of mammalian Atg8-like proteins reflects different types of autophagosomes, or a specialization in promoting different stages in autophagosome biogenesis (Weidberg et al. 2011).

In yeast cells, completed autophagosomes directly fuse with the vacuole, in a manner that is dependent on classic endosome to vacuole-trafficking factors such as SNAREs and Rab GTPases. This was demonstrated by EM analysis of yeast cells expressing a temperature-sensitive allele of the vacuolar t-SNARE Vam3. Under nonpermissive temperature and in response to starvation (Darsow et al. 1997) or rapamycin treatment (Abeliovich et al. 1999), these cells accumulate completed cytoplasmic autophagosomes. In mammalian cells, autophagosomes undergo maturation steps which include fusion with endosomes to form amphisomes (Berg et al. 1998), which then undergo fusion with lysosomes, although direct fusion of autophagosomes with lysosomes in mammalian cells cannot be ruled out (Itakura et al. 2012).

Membrane sources

Another important mechanistic question involves the identification of membranes and cellular compartments which contribute to the formation of the autophagosomal membrane. Early studies by Bill Dunn (1990a, b) strongly suggested that the ER plays a central role in the formation of the sequestering membrane and the autophagosome itself. Twenty odd years later, this is still the prevailing consensus, although strong arguments have been made to suggest that the plasma membrane (Ravikumar et al. 2010), Golgi (Ohashi and Munro 2010; Reggiori et al. 2004), endosomes (Ohashi and Munro 2010), and mitochondria (Hailey et al. 2010) may also contribute. The fact that Atg9, the only integral membrane protein that is absolutely and specifically required for autophagy, resides in the trans-Golgi and endosomes in both mammalian cells and in yeast (Young et al. 2006); (Ohashi and Munro 2010) strongly argues that these compartments may donate membranes to the process. Recent data from yeast and mammalian cells suggest that ER exit sites and the ER–Golgi intermediate compartment (ERGIC) are the point of autophagosome assembly (Ge et al. 2013; Graef et al. 2013), and that COPII components play a role in the process (Graef et al. 2013). The same authors also pointed out that the nascent autophagosomes may also maintain a physical junction with the vacuole/lysosome, but the role of this junction is unclear at this point.

Vps34, and correspondingly PtdIns3p, are known to localize to the endo-lysosomal system. In fact, the presence of PtdIns3p on membranes is a hallmark of endosomal and lysosomal compartments (Odorizzi et al. 2000; Simonsen et al. 2001). Surprisingly, work with the mammalian PtdIns3p-binding protein DFCP1, revealed that it is an ER protein which, upon induction of autophagy, rapidly localizes to unique ER-associated, ring-shaped structures that were dubbed "omegasomes" (Axe et al. 2008). The re-localization of DFCP1 depends on its twin FYVE PI3P-binding domains, implying either an unusual transfer of PI3P from the endosomal system into the ER, or equally unusual, direct PI3P synthesis on the ER membrane. LC3 was then observed to be recruited to omegasome-engulfed complexes on this ER-associated membrane patch, cementing the idea that omegasomes are a very early intermediate in the nucleation of autophagosomes. Originally, Vps34 was not observed on the ER, but it was noted that omegasomes form very near to Vps34-carrying lysosomes and endosomes (Axe et al. 2008). Subsequent studies revealed, however, that Atg14L, an autophagy-specific subunit of the type III PtdIns3 K holoenzyme, does localize to the ER and that this localization is essential for recruitment of PtdIns3 K to specific locations on the ER (Matsunaga et al. 2010). This was corroborated by in vitro reconstitution of starvation-induced Atg14 recruitment to membranes, which demonstrated recruitment of Atg14 to the ERGIC compartment (Ge et al. 2013). When combined with the more recent data on the origin of autophagosomes, it stands to reason that omegasomes are forming around ER exit sites or the ERGIC, in a COPII-dependent fashion, upon localized activation of the Vps34 PtdIns3 K. It is also conceivable that the conserved proximity of autophagosome formation sites to lysosomes in mammals, and to the vacuole in yeast reflects lipid transfer between these organelles, although no direct evidence for this has been presented to date.

Physiological role of autophagy in mammalian nutrient responses

The role of autophagy in mammalian amino acid homeostasis is underscored by the phenotype of atg5−/− mice. These pups are born apparently normal, yet die within 24 h of delivery due to low serum amino acid levels. The accepted explanation for this result is that the disruption of placental nutrition at birth leads to a starvation effect and that autophagic recycling and release of amino acids is essential for neonate survival, although additional explanations are also possible (Kuma et al. 2004). However, while standard autophagy KO animals have an apparently normal or near-normal embryogenesis, it appears that autophagy also has an essential role in early embryogenesis, and that maternally inherited Atg proteins in atg−/− oocytes can cover up for the genetic deletion in the zygote. Thus, it was found that autophagy is induced upon fertilization, in a burst that continues into the four-cell stage. Parental gonad-specific ablation of *atg5* causes early embryonic lethality (Tsukamoto et al. 2008). The currently accepted explanation for this phenomenon is that massive recycling occurs during early embryogenesis, due to the need to degrade maternal components, and that this in turn greatly increases the demand for free amino acids, which are required for the synthesis of new, embryo-specific proteins. However, it cannot be excluded that insufficient degradation of maternal factors or oocyte-specific structures hinders further development in this system (Tsukamoto et al. 2008).

Chaperone-mediated autophagy (CMA)

In the preceding paragraphs, I discussed the mechanisms of autophagy which rely on membrane trafficking. In the 1980s, it was suggested by the late Fred Dice that, in addition to macroautophagy and microautophagy, proteins are also directly translocated across the limiting membrane of the lysosome for degradation in the lumen (Dice 1987). The support for this view was that starvation-induced autophagic degradation did not occur at identical rates, as would be expected if they are "harvested" from the cytosol by nonspecific engulfment (Dice et al. 1978). Indeed, it proved feasible to reconstitute a lysosomal protein translocation pathway in vitro, analogous to the translocation of proteins across the endoplasmic reticulum membrane or the mitochondrial inner membrane (Chiang et al. 1989). These experiments demonstrated translocation-dependent degradation of proteins, initially RNAse A and later additional substrates, into the lumen of isolated lysosomes. The mechanism required a specific pentapeptide degradation motif in the substrate protein, KFERQ, although later work showed more relaxed rules for the motif centering on conservation of charged residues (Dice 1992). Unlike macroautophagy, studies in yeast were unable to demonstrate a convincing and screenable assay for CMA in yeast, and as a result we do not possess the level of molecular understanding that we have with respect to macroautophagy and related processes (Horst et al. 1999). Historically, this translocation-mediated degradation pathway was termed "chaperone-mediated autophagy" only in 2000.

In vitro work using mammalian cells showed that translocation of CMA substrates requires initial binding to cytosolic hsc70 (Chiang et al. 1989), followed by binding of the substrate to LAMP2-A, one of three splice variants of the ubiquitous lysosomal membrane protein LAMP2 (Cuervo and Dice 1996). In agreement with this, knockdown of LAMP2-A was found to result in diminished in vitro degradation of specific substrates (Massey et al. 2006). The substrate is then translocated via a channel in the lysosomal membrane. Current work suggests that the channel is a dynamic multimeric complex of several LAMP2A molecules with at least two additional, unidentified proteins (Bandyopadhyay et al. 2008). Translocation through the lysosomal membrane has been shown to depend on Hsp70 activity in the lumen, apparently acting as a molecular ratchet (Cuervo et al. 1997), by analogy to translocation of proteins into the mitochondrial lumen.

Like other forms of autophagy, CMA is also up-regulated by amino acid starvation (Cuervo et al. 1995). However, the signaling mechanism(s) which regulate CMA in response to nutrient cues has not been resolved. Numerous studies have identified a role for CMA in aging (Cuervo and Dice 2000; Kiffin et al. 2007), neurodegenerative disease (Cuervo et al. 2004; Mak et al. 2010; Martinez-Vicente et al. 2008; Orenstein et al. 2013; Vogiatzi et al. 2008; Wang et al. 2009; Liu et al. 2009), and cancer (Kon et al. 2011; Lv et al. 2011).

Conclusion

Classical autophagy is a catabolic membrane-trafficking event that occurs in response to starvation, through the deformation of cellular membranes upon the combined localized actions of lipid-modifying enzymes such as Vps34, together with coat-like structures formed by a subset of Atg proteins. As such, it may theoretically mobilize many types of intracellular membranes, although most studies indicate the ER as a major source. It must be borne in mind, however, that there may be a near-continuum of autophagic responses in response to different specific stimuli, and accordingly one expects that the specific mechanism, such as target membranes, genetic requirements, and the like will vary accordingly, as in the differences

observed between nitrogen starvation-induced autophagy and amino acid starvation-induced autophagy in yeast cells.

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