Mammalian macroautophagy at a glance

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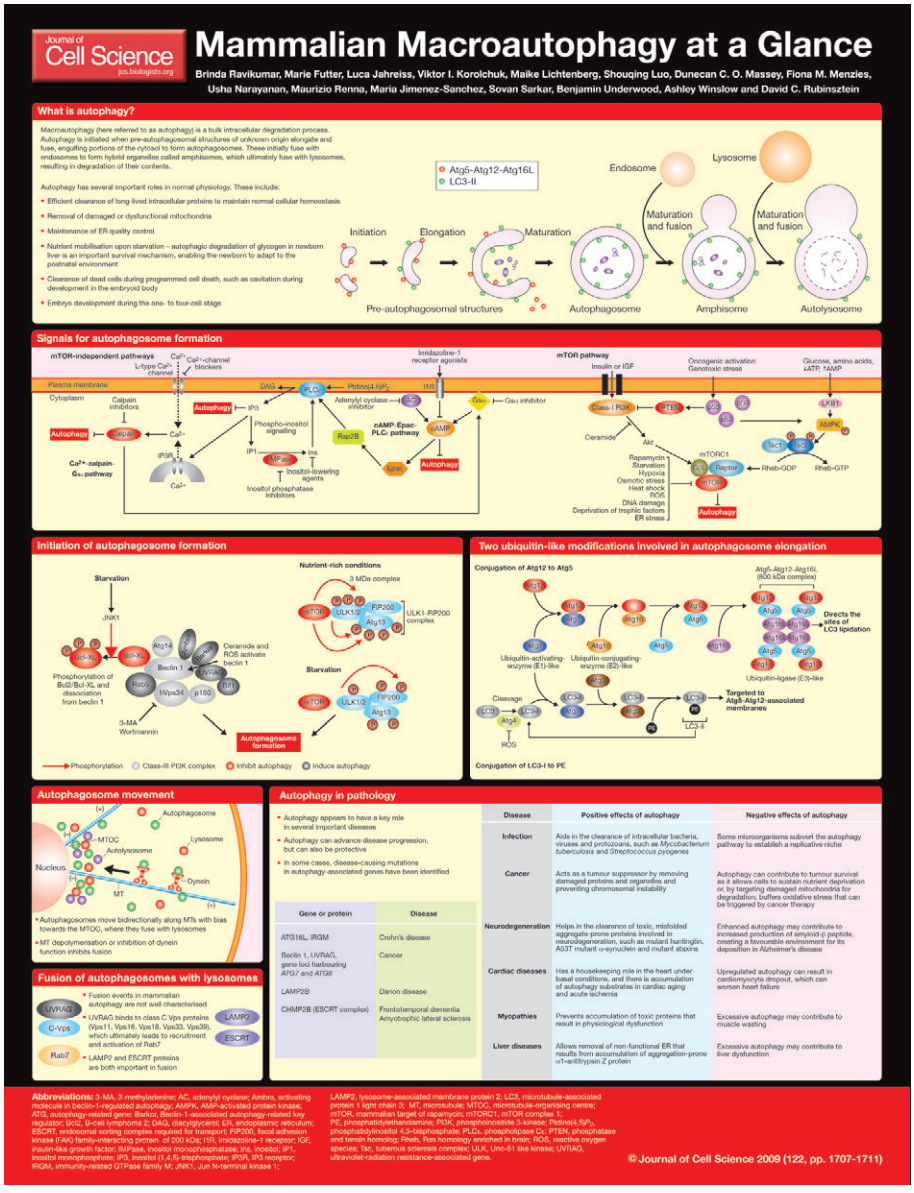
Autophagy refers to a set of non-specific bulk degradation processes in which cells deliver cytoplasmic substrates for lysosomal degradation. Types of autophagy include macroautophagy, chaperone-mediated autophagy and microautophagy. Chaperone-mediated autophagy is selective for specific cytosolic proteins that contain a pentapeptide motif. This motif is recognised by the chaperone heat shock cognate 70 (Hsc70), which transfers protein substrates to the lysosomal membrane where, through binding to the receptor lysosome-associated membrane protein 2A (LAMP2A), they are translocated into the lysosomal lumen and degraded. In microautophagy, lysosomes can also directly engulf cytoplasm by invagination, protrusion and/or septation of the lysosomal limiting membrane. The focus of this poster article, however, is mammalian macroautophagy.

Macroautophagy (referred to here as autophagy) is a process in which cells form double-membrane vesicles, called autophagosomes, around a portion of cytoplasm. These autophagosomes ultimately fuse with lysosomes, resulting in degradation of their contents. Autophagy is upregulated under physiological stress conditions such as starvation. However, mammalian cells undergo autophagy at a basal level that might be important for the clearance of normally occurring, misfolded, ubiquitylated proteins. Autophagy has also been implicated in several human diseases, such as cancer, certain neurodegenerative disorders and infectious diseases. Although autophagy is thought to be predominantly a cell-survival mechanism, some evidence points towards a role in cell death. Our understanding of mammalian autophagy, in terms of the molecular machineries and signalling cascades that are involved in its regulation, has grown considerably over the last few years. In particular, there has been an explosion of data in the last 3 years trying to address the importance of mammalian autophagy in physiology and pathophysiology. Here, we aim to summarise the recent advances in mammalian macroautophagy in terms of its regulation, which will include newly identified molecules and signalling pathways, and we will address the ever-growing roles of autophagy in human physiology and pathology.

Autophagy machinery

The foundation for understanding the mammalian autophagy machinery was provided by a series of genetic screens in the yeast *Saccharomyces cerevisiae*; these screens identified around 30 so-called autophagy-related (ATG) genes that are required for autophagy (Xie and Klionsky, 2007). About half of these genes have obvious mammalian counterparts, and many of the core aspects of the process are conserved. It is possible that some genes that do not have obvious orthologues in mammals still exist but have been missed, as there might be structural and functional



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orthology without obvious sequence similarity.

Initiation of autophagosome formation

Autophagosome formation appears to start at phagophore (autophagosome precursor; also known as pre-autophagosomal structures)-assembly sites (PAS). The source of the membrane is unclear, although recent data support a contribution from the endoplasmic reticulum (ER) (Axe et al., 2008). The formation of the phagophore requires the activity of the class-III phosphoinositide 3-kinase (PI3K) Vps34, which forms phosphatidylinositol 3-phosphate [PtdIns(3)*P*]. Chemical inhibition of Vps34 by 3-methyladenine or wortmannin blocks the formation of new autophagosomes. Vps34 is part of a large macromolecular complex specific for autophagy that contains the proteins Atg6 (also called beclin 1), Atg14 and Vps15 (p150) (Itakura et al., 2008). Other proteins that are involved in the initiation stage of autophagosome formation include Atg5, Atg12, Atg16, the newly identified autophagy-related protein focal adhesion kinase (FAK)-family interacting protein of 200 kD (FIP200), which interacts with Atg1 (also called ULK1) (Hara et al., 2008), and the mammalian orthologue of Atg13 (Chan et al., 2009; Hosokawa et al., 2009; Jung et al., 2009).

Autophagosome elongation

Atg5 and Atg12 are involved in the first of two ubiquitylation-like reactions that control autophagy. Atg12 is conjugated to Atg5 in a reaction that requires Atg7 [ubiquitin-activating-enzyme (E1)-like] and Atg10 [ubiquitin-conjugating-enzyme (E2)-like]. Atg5-Atg12 conjugates are localised onto the PAS and dissociate upon completion of autophagosome formation. The process of Atg5-Atg12 conjugation depends on Vps34 function, and Vps34 activity, along with autophagy, is positively regulated by the small GTPase Rab5 (Ravikumar et al., 2008). Atg5-Atg12 interacts non-covalently with Atg16L (Atg16-like) to form a complex of approximately 800 kDa (Ohsumi and Mizushima, 2004).

The second ubiquitylation-like reaction involves the conjugation of microtubule-associated protein 1 light chain 3 (MAP1-LC3; also known as Atg8 and LC3) to the lipid phosphatidylethanolamine (PE). LC3 is cleaved at its C-terminus by Atg4 to form cytosolic LC3-I, which is covalently conjugated to PE to form membrane-associated LC3-II, a process that requires the activities of Atg7 (E1-like) and Atg3 (E2-like) (Ohsumi and Mizushima, 2004). The Atg5-Atg12 conjugate seems to modulate LC3-I conjugation to PE by acting in an E3-like fashion (Hanada et al., 2007) and the Atg5-Atg12-Atg16L complex also determines the sites of LC3 lipidation (Fujita et al., 2008). In this way, LC3 is specifically targeted to Atg5-Atg12-associated membranes, which are expanded phagophores.

Crosstalk between the two ubiquitylation-like systems has also been suggested. Atg10 can interact with LC3-I and facilitate LC3-I conjugation to PE (Nemoto et al., 2003). Similarly, Atg3 co-immunoprecipitates with Atg12, and overexpression of Atg3 increases Atg5-Atg12 conjugation (Tanida et al., 2002). Although Atg5-Atg12 is lost upon completion of autophagosome formation, LC3-II remains associated with autophagosomes even after fusion with lysosomes, after which LC3-II on the cytosolic face of autolysosomes can be delipidated and recycled (to LC3-I). Conjugated LC3 is a substrate for Atg4, and Atg4 delipidates LC3-II and removes it from the membrane. Furthermore, the production of reactive oxygen species (ROS; specifically H_2O_2) during starvation results in oxidation and inactivation of Atg4. This inhibits LC3-II delipidation and might promote autophagosome formation (Scherz-Shouval et al., 2007).

LC3-II is the only known protein that specifically associates with autophagosomes and not with other vesicular structures. Thus, LC3-II levels correlate with autophagic vacuole numbers, which can also be assessed by scoring LC3-positive vesicle numbers (Kabeya et al., 2000). LC3-II can mediate membrane tethering and hemifusion, and these functions might be crucial for the expansion of autophagosome membranes. These data also raise the possibility that LC3 assists the final fusion of the pre-autophagosomal double-membrane 'cups' into fused vesicles (Nakatogawa et al., 2007).

Autophagosome maturation and fusion

Autophagosomes form randomly in the cytoplasm. They are then trafficked along microtubules in a dynein-dependent manner to lysosomes, which are clustered around the microtubule-organising centre (MTOC; located near the nucleus). There, autophagosomes tether, dock and fuse with lysosomes, and the contents of the two vesicles are mixed (Jahreiss et al., 2008). The details of this aspect of mammalian autophagy are still unclear, although the SNARE proteins that are involved in yeast autophagosome-vacuole fusion have been characterised (Ishihara et al., 2001). En route to fusion with the lysosomes, autophagosomes can fuse with endosomes to form amphisomes. It is currently not clear whether amphisome formation is a prerequisite for autophagosome-lysosome fusion. Reduced activity of some ESCRT (endosomal sorting complex required for transport) proteins disrupts autophagosome-lysosome fusion, resulting in the accumulation of autophagosomes (Skibinski et al., 2005). Similarly, LAMP2 deficiency has been associated with the accumulation of autophagosomes, which is consistent with a defect in fusion (Eskelinen et al., 2002).

Signals for autophagosome formation

mTOR-dependent pathway

One of the classical pathways that regulate autophagy is controlled by the mammalian target of rapamycin (mTOR). mTOR activity inhibits mammalian autophagy, but its activity is inhibited under starvation conditions, which contributes to starvation-induced autophagy. Autophagy is also induced by inhibiting mTOR with drugs such as rapamycin (Rubinsztein et al., 2007). Mammalian Atg13, ULK1 and ULK2 have recently been identified as direct targets of mTOR (Chan et al., 2009; Hosokawa et al., 2009; Jung et al., 2009). Atg13 binds to ULK1 and ULK2 (ULK1/2) and mediates their interaction with FIP200. Under nutrient-rich conditions, mTOR is associated with this complex. Inhibition of mTOR leads to its dissociation from the complex and results in the partial dephosphorylation of Atg13 and ULK1/2; this activates ULK1/2 to phosphorylate FIP200 and thereby induce autophagy (Chan et al., 2009; Hosokawa et al., 2009; Jung et al., 2009).

Several signalling molecules regulate autophagy via mTOR. Insulin or insulin-like growth factor (IGF) signalling activates mTOR via PI3K and Akt. Activation of adenosine-monophosphate-activated protein kinase (AMPK) inhibits

mTOR activity via tuberous sclerosis complex 1 (Tsc1), Tsc2 and Ras homology enriched in brain (Rheb) (Hall et al., 2008). p53, a commonly mutated gene in human cancers, can positively and negatively regulate autophagy via the mTOR pathway. Oncogenic or genotoxic stress stabilises and activates p53. This can stimulate autophagy by activating AMPK or by upregulating phosphatase and tensin homologue (PTEN) and Tsc1. Genetic or chemical inhibition of p53, however, also activates autophagy (Levine and Abrams, 2008).

Recently, further insights have been provided into the mechanisms behind starvation-induced autophagy. Autophagy can be inhibited by the binding of the apoptosis-related proteins B-cell lymphoma 2 (Bcl2) or basal-cell lymphoma-extra large (Bcl-XL) to beclin 1 (Atg6). Starvation induces Jun N-terminal kinase 1 (Jnk1) activity, which phosphorylates Bcl2, thereby disrupting the interaction between beclin 1 and Bcl-2 and inducing autophagy (Wei et al., 2008). This mechanism might also account for the upregulation of autophagy after proteasome inhibition or ER stress, as one study showed that ER-stress-induced autophagy is Jnk1-dependent and that proteasome inhibition can induce ER stress (Ding et al., 2007). Several other beclin-1 binding partners such as activating molecule in beclin-1-regulated autophagy (Ambra), Rab5, ultraviolet-radiation resistance-associated gene (UVRAG) and beclin-1-associated autophagy-related key regulator (Barkor) can activate beclin 1.

mTOR-independent pathway

An mTOR-independent pathway for the induction of autophagy has recently been discovered (Sarkar et al., 2005). In this pathway, the inhibition of inositol monophosphatase (IMPase) reduces free inositol and myoinositol-1,4,5-triphosphate (IP3) levels, which leads to an upregulation of autophagy. Lithium, carbemazepine and valproate – drugs that are used to treat a range of neurological and psychiatric conditions – induce autophagy via this pathway (Sarkar et al., 2005). Further studies have shown that this pathway is regulated by intracellular Ca^{2+} levels and cyclic AMP (cAMP), and have revealed additional drugs that might induce autophagy, such as verapamil (an L-type Ca^{2+}-channel antagonist) and clonidine (an imidazoline-receptor agonist that reduces cAMP levels) (Williams et al., 2008). In this pathway, autophagy is inhibited when intracellular cAMP levels are increased by adenylyl cyclase (AC), which influences autophagy by activating the guanine nucleotide exchange factor Epac. Epac then activates Rap2B, which inhibits autophagy by activating phospholipase Cε (PLCε), resulting in the production of IP3, which mediates the release of Ca^{2+} from ER stores. Increased intracytosolic Ca^{2+} blocks autophagy by activating calpains (a family of Ca^{2+}-dependent cysteine proteases), which mediate their effects on autophagy through Gsα, which is activated after calpain cleavage. This, in turn, increases AC activity to increase cAMP levels, thus forming a loop. Other compounds that induce autophagy include ceramide, sphingosine, trehalose, fluspirilene, trifluoperazine, pimozide, nicardipine, penitrem A, niguldipine and amiodarone (Zhang et al., 2007), but their mechanisms of action are not well understood.

Physiology and pathology

Starvation

One of the ancestral purposes of autophagy has been as a means of recycling macromolecules to provide new nutrients at times of starvation. This is a key role for autophagy in single-cell organisms such as yeast, and is a crucial function in mammals as well. For instance, in the immediate newborn period there is relative starvation before breastfeeding is established. Autophagy-deficient mice are apparently normal at birth but die soon thereafter, owing to an inability to recycle nutrients (Kuma et al., 2004).

Autophagy appears to be important for mammalian development as well. Fertilisation induces autophagy, and autophagy-defective oocytes derived from oocyte-specific Atg5-knockout mice failed to develop beyond the four- and eight-cell stages if they were fertilised by *Atg5*-null sperm (Tsukamoto et al., 2008). Autophagy also appears to regulate the clearance of apoptotic corpses during development [for instance, during embryonic cavitation (Qu et al., 2007)].

Aggregation-prone proteins

The key proteolytic systems in mammalian cells are autophagy and the ubiquitin-proteasome system. The proteasome can only process unfolded proteins, as it has a narrow opening; however, oligomers and organelles, which cannot enter the proteasome, are accessible to autophagy. Indeed, autophagy appears to be a crucial route for the degradation of aggregation-prone proteins. These include neuro-degenerative-disease-associated proteins such as mutant huntingtin (which causes Huntington's disease), and mutant forms of α-synuclein (which causes forms of familial Parkinson's disease). The clearance of such proteins is impaired when autophagy is compromised. However, autophagy upregulation is a possible therapeutic strategy for such diseases, as drugs that induce autophagy by mTOR-dependent or mTOR-independent routes enhance the clearance of such mutant proteins and alleviate their toxicity in a range of animal models (Sarkar et al., 2008).

This function of autophagy might also be relevant physiologically, because protein aggregates form in the brain and some other tissues (from endogenous 'normal' proteins) in Atg5- or Atg7-knockout mice, which have compromised autophagy (Hara et al., 2006; Komatsu et al., 2006). Such a mechanism might also contribute to other neurodegenerative diseases. For instance, recent studies suggest that autophagosome-lysosome fusion is impaired in a form of frontotemporal dementia that is caused by mutations in a gene in the ESCRT complex, and by mutations in the dynein-complex component dynactin that cause a form of motor neuron disease (Munch et al., 2004; Skibinski et al., 2005). This will impair the clearance of autophagic substrates and predispose cells to aggregate formation.

Infectious agents and immunity

Autophagy is also a crucial clearance route for a range of infectious agents, including *Mycobacterium tuberculosis* (the causative agent of tuberculosis), the group A streptococcal bacterium *Streptococcus pyogenes* (the causative agent of streptococcal pharyngitis, toxic shock syndrome and necrotising fasciitis) and viruses such as herpes simplex virus type I. Conversely, some bacteria and viruses have also evolved to subvert the autophagic system and use autophagy for replication (Levine and Deretic, 2007).

Autophagy might have implications in diseases that are linked to infection, as it is a route that enables the presentation of cytosolic antigens by major

histocompatibility complex (MHC) class II molecules. Recent studies suggest a wider role in immunity, as autophagy in thymic epithelial cells regulates the T-cell repertoire and is crucial for the development of immunological tolerance (Levine and Deretic, 2007). The immunological roles of autophagy might also account for the robust associations of variants in two autophagy genes, *Atg16L* and immunity-related GTPase family-M (*IRGM*), with the autoimmune condition Crohn's disease (Massey and Parkes, 2007).

Autophagy and cell death

Although increased numbers of autophagosomes sometimes occur in dying cells, this increase is often not the result of autophagy-mediated cell death. Indeed, autophagy has cytoprotective roles. Blocking autophagy increases the susceptibility of cells to pro-apoptotic insults, whereas enhancing autophagy is anti-apoptotic. This might be because autophagy enhances the clearance of mitochondria and reduces mitochondrial load, thereby diminishing the amount of cytochrome *c* release and subsequent caspase-9 activation that occur after an apoptotic insult (Ravikumar et al., 2006).

Because of its anti-apoptotic effects, it might seem that the upregulation of autophagy predisposes cells to cancer. Although the situation is likely to be complex, the existing view is that autophagy downregulation predisposes cells to carcinogenesis, which is consistent with observations that hemizygous loss of beclin 1 is associated with increased tumorigenicity (Levine et al., 2008). However, blocking autophagy might aid certain chemotherapeutic regimes for established cancers by enhancing their killing effects.

Although the relationship between apoptosis and autophagy is still unclear, there are crucial proteins that are relevant to both processes, implying some degree of crosstalk: Bcl2 and Bcl-XL are anti-apoptotic as well as being blockers of autophagy (as discussed above), and Atg5 has been proposed to be a pro-apoptotic protein (Maiuri et al., 2007).

Conclusions and perspectives

Over the last 5 years there has been a dramatic increase in interest in mammalian autophagy. This is partly due to a growing understanding of the process, coupled with improved tools for its detection. Perhaps the most exciting areas of development have come from the realisation that autophagy might be a crucial process that governs a wide range of physiological processes and diseases in humans. Besides the neurodegenerative diseases, infectious agents, immunological disorders and cancer scenarios that have been discussed, recent data suggest that autophagy might be important in other conditions ranging from cardiomyopathies to pancreatitis. Further understanding of the physiological and pathological roles of this process is crucial, but will require more knowledge of the basic cell biology and signalling pathways that regulate autophagy.

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