TIMELINE

Autophagy: from phenomenology to molecular understanding in less than a decade

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Abstract | In 2000, it was suggested to me that "Autophagy will be the wave of the future; it will become the new apoptosis." Few people would have agreed at the time, but this statement turned out to be prophetic, and this process of 'self-eating' rapidly exploded as a research field, as scientists discovered connections to cancer, neurodegeneration and even lifespan extension. Amazingly, the molecular breakthroughs in autophagy have taken place during only the past decade.

Ten years ago, a seminar speaker who talked about autophagy could almost be guaranteed that no one in the audience, with the possible exception of the host, would have even heard of the term, let alone be familiar with any of the research. This situation is certainly changing, although there is still some level of unawareness. For example, at the first Keystone Symposium on Autophagy in Health and Disease, held in 2007, Seiji Kondo noted that in the field of cancer research, his colleagues' response when discussing the efficacy of modulating autophagy to enhance anti-cancer drugs is usually "What's autophagy?"

The term 'autophagy' was chosen to distinguish the lysosomal degradation, or 'eating' (phagy), of part of the cell's self (auto) from the breakdown of extracellular material (heterophagy). In brief, autophagy is a ubiquitous process in eukaryotic cells that results in the breakdown of cytoplasm within the lysosome in response to stress conditions and that allows the cell to adapt to environmental and/or developmental changes (BOX 1). Although initially considered simply a degradative process, recent studies have revealed an integral role for autophagy in human pathophysiology. Accordingly, there has been a tremendous increase in autophagy research in the past 10 years, marked by a substantial increase in the number of autophagy-related papers (FIG. 1). Similarly, our knowledge regarding the connections between autophagy and human physiology has continued to expand, so that researchers in fields as wide-ranging as cancer, neurodegeneration and microbial pathogenesis have begun to study this process.

Amazingly, although autophagy was first described approximately 40 years ago, our molecular understanding of it only started in the past decade. This Timeline covers the history of autophagy research, with a focus on the key events that have led to its increasing prominence.

Christian de Duve — the beginning

Most people in the field of autophagy consider Christian de Duve to be the founding father of this research area. After all, autophagy is a degradative mechanism that is part of the lysosomal system, and it was de Duve who carried out the pioneering biochemical work that helped lead to the discovery of the lysosome as a distinct entity in 1955 (see TIMELINE). He coined the term 'lysosome'[1], and received the Nobel Prize in Physiology or Medicine in 1974, mostly for his work on this organelle. de Duve also came up with the term 'autophagy', which he introduced at the CIBA Foundation Symposium on Lysosomes in 1963. According to de Duve, "I was in a word-coining mood, and proposed the terms 'endocytosis' and 'exocytosis' at this same time".

The descriptive name 'autophagy' was intended to illustrate observations from electron microscopy studies, which showed novel single- or double-membrane vesicles that contained parts of the cytoplasm, including organelles, in various degrees of disintegration[2–5]. At the time, de Duve and others suggested that the sequestering organelle, or 'autophagosome', was derived from a preformed membrane such as the smooth endoplasmic reticulum, but the current view is that the autophagosome forms in a largely *de novo* process, starting with a core membrane that expands through vesicular addition. Whereas autophagy was, and still is, considered to be primarily non-specific[6], de Duve suggested the possibility of selective types of autophagy that might allow the targeted degradation of abnormal cellular constituents, an aspect of autophagic function that has gained considerable prominence in recent years.

The period from the 1960s through to the 1980s marked the 'golden days' of morphological analysis of autophagy. Whereas the initial studies of de Duve and others primarily examined the terminal stages of the process, and focused on steps just before or after fusion with the lysosome, Per Seglen's laboratory began to study the early and intermediate steps using electroinjected radioactive probes (reviewed in REF. 7). These analyses identified the phagophore[8], the initial sequestering organelle that develops into an autophagosome (BOX 1), as well as the amphisome[9], which marks the convergence of the autophagic and endocytic pathways.

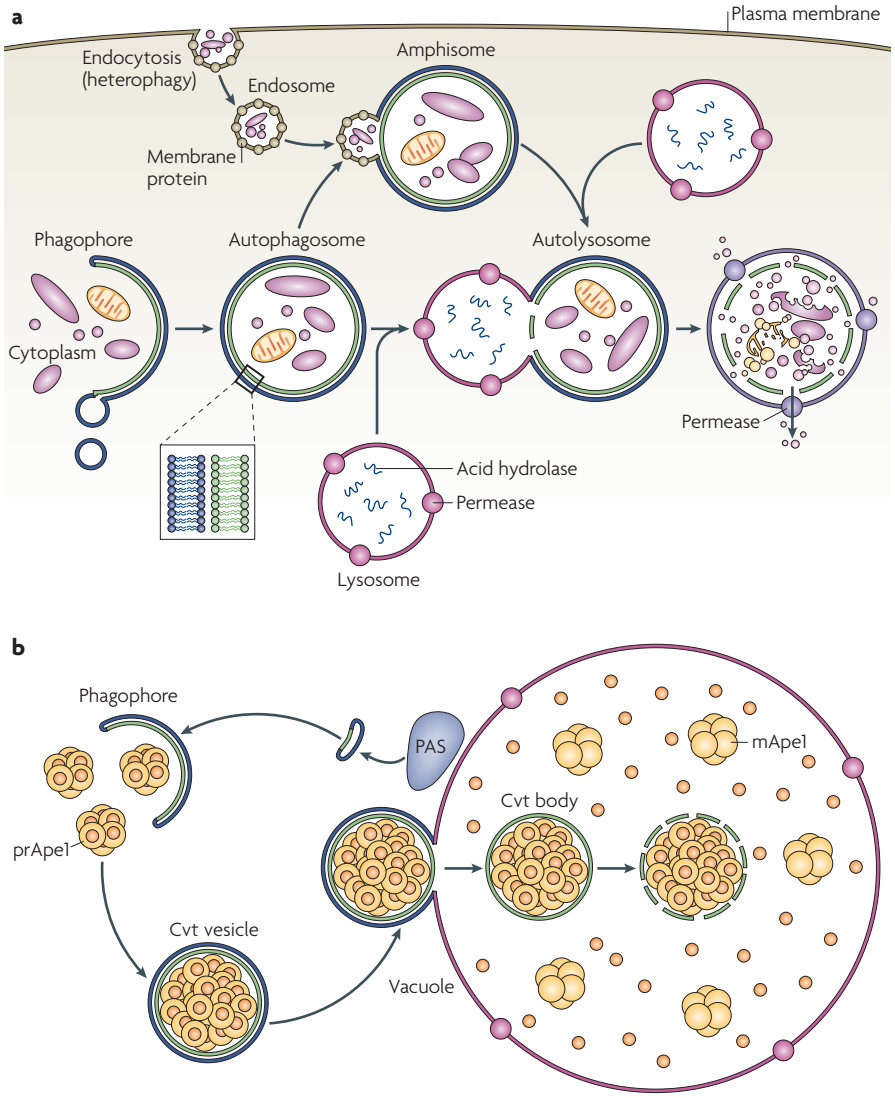
The identification of autophagy genes in higher eukaryotes (see below) made it possible to analyse mammalian cells that express autophagy proteins tagged with fluorescent markers. Time-lapse studies provide compelling images which suggest that autophagosome formation proceeds in a step-wise manner, marked by the expansion of the sequestering membrane[10] (Supplementary information S1 (movie)).

Analysis of autophagy in the yeast system lagged behind considerably until 1992, when a study by Yoshinori Ohsumi's laboratory demonstrated that the autophagy morphology in yeast was similar to that documented

Box 1 | **Specific and nonspecific autophagy**

During nonspecific autophagy (see figure part **a**), sequestration begins with the formation of a phagophore that expands into a double-membrane autophagosome while surrounding a portion of the cytoplasm. The autophagosome may fuse with an endosome (the product of endocytosis), which is a form of heterophagy (in a heterophagic process, the cell internalizes and degrades material that originates outside of the cell, in contrast to autophagy, in which the cell consumes part of itself). The product of endosome–autophagosome fusion is known as an amphisome. The completed autophagosome or amphisome fuses with a lysosome, which supplies acid hydrolases. The enzymes in the resulting compartment, an autolysosome, break down the inner membrane from the autophagosome and degrade the cargo. The resulting macromolecules are released through permeases and recycled in the cytosol.

The yeast cytoplasm-to-vacuole targeting (Cvt) pathway is one example of specific autophagy (see figure part **b**), and the only example of a biosynthetic autophagy-related pathway. The overall morphology is identical to nonspecific autophagy; however, the sequestering Cvt vesicles are smaller than autophagosomes and they appear to exclude bulk cytoplasm. The phagophore assembly site (PAS) either becomes the sequestering vesicle or generates it. The precursor form of aminopeptidase I (prApe1) forms oligomers in the cytosol, and is targeted through the action of a receptor, Atg19, and the adaptor or scaffold protein Atg11 to allow selective cargo recognition and packaging. The completed vesicle fuses with the vacuole, the yeast analogue of the mammalian lysosome. The larger size of the vacuole allows the release of the inner single-membrane compartment of the Cvt vesicle, which is now termed a Cvt body; during nonspecific autophagy in yeast, this structure is termed an autophagic body. The Cvt body is lysed and prApe1 is matured by proteolytic removal of a propeptide to generate the active, resident hydrolase mApe1.



in mammals[11] (FIG. 2). This result was crucial as a foundation for further studies in this genetically tractable organism. In yeast, the autophagy machinery is concentrated at a perivacuolar (the vacuole is the yeast equivalent of the lysosome) site termed the pre-autophagosomal structure, which acts as a phagophore assembly site (PAS)[12,13].

Hormonal and enzymatic regulation

Early analyses of the endomembrane system revealed striking and dynamic morphological changes in response to hormones. For example, as early as 1962, Thomas Ashford and Keith Porter noted the presence of sequestered organelles in rat hepatocytes following their exposure to glucagon[2], although the concept of autophagy had not yet been formulated. A few years later, de Duve and colleagues confirmed that glucagon induces autophagy[14] and, in a corollary analysis 10 years later, Ulrich Pfeifer demonstrated the converse result with insulin[15]. These results with metabolic hormones fit with our understanding of autophagy as a catabolic, energy-generating mechanism, and continued studies over the past three decades have added many additional details to our understanding of the regulatory mechanism (BOX 2).

In the same year that the insulin analysis was reported, Glenn Mortimore and colleagues began a series of studies that demonstrated that amino acids inhibit autophagy[16], and Per Seglen and his collaborators examined the regulatory role of amino acids, as well as various other pharmacological reagents. For example, Seglen and Paul Gordon discovered the inhibitory action of 3-methyladenine[17], and provided the first evidence for the regulatory effects of protein kinases and phosphatases on autophagy[18].

The target of rapamycin (*TOR*) gene was isolated from yeast in 1993 (REF. 19) and from mammals in 1994. The TOR kinase was soon linked to cell growth, cell-cycle progression and protein synthesis. In 1995, Fred Meijer's group made the critical observation that rapamycin, an inhibitor of TOR, acts as an autophagy inducer[20]. According to Meijer, "The demonstration that autophagy regulation involves the TOR kinase provided a major breakthrough in the understanding of signalling control." Many advances have been made in the process of identifying TOR-interacting proteins, and these results have provided important insights into the regulation of autophagy. Meijer's laboratory also demonstrated that amino acids stimulate TOR, providing a connection between the studies on amino-acid-dependent and TOR-dependent regulation.

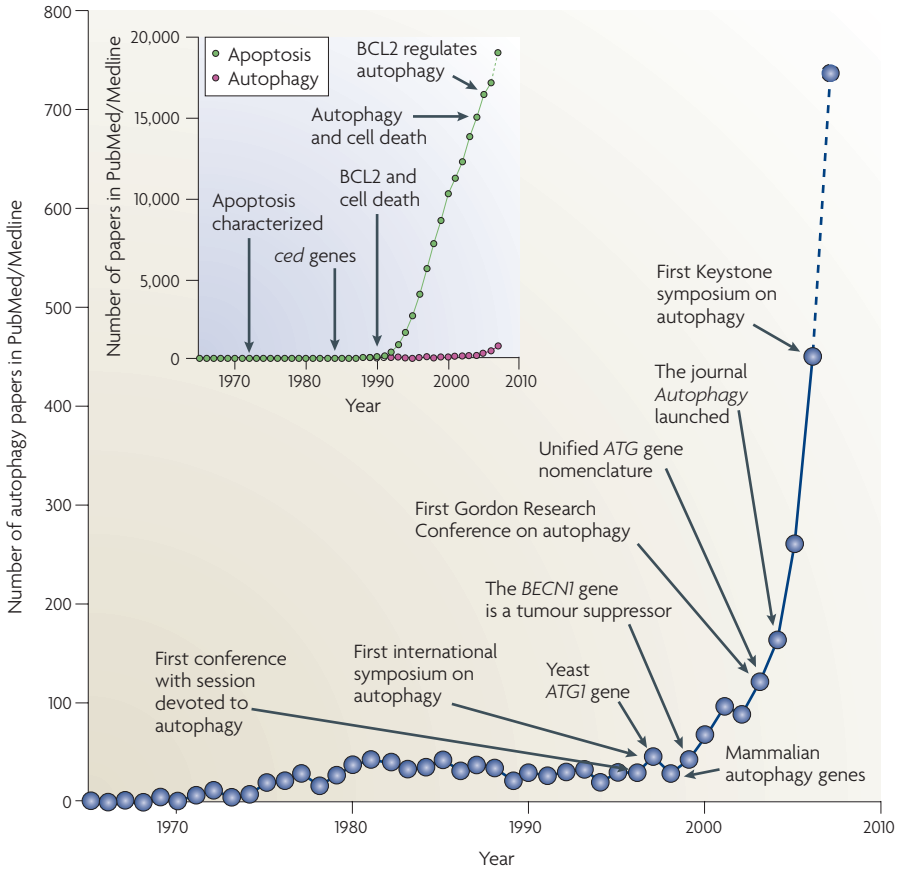


Figure 1 | **A dramatic increase in autophagy research.** The main graph shows the number of papers retrieved by a PubMed/Medline search for 'autophagy', with key events noted during the indicated timeline. Autophagy research has expanded dramatically in the past few years, spurred on by the identification of the *ATG* genes. If this field is indeed the 'new apoptosis', it appears that we are only witnessing the tip of the iceberg. The inset displays the number of records retrieved for 'apoptosis' compared with those for 'autophagy'. Research in apoptosis accelerated rapidly after the identification of the *ced* genes in *Caenorhabditis elegans*, suggesting that progress in the autophagy field will continue on an upward trend, especially considering the relationship between these two processes. Results as of 1 May 2007; the final point in each graph is extrapolated.

Another important observation by the same group was that wortmannin, a phosphatidylinositol 3-kinase (PI3K) inhibitor, inhibited autophagy[21]; in the same study, 3-methyladenine was also shown to inhibit PI3K.

At this time, 3-phosphoinositide-dependent protein kinase-1 (PDK1) was identified, providing a link between insulin signalling, phosphoinositides, protein kinase B (PKB)/AKT and TOR (BOX 2). Nonetheless, the observation that rapamycin stimulated autophagy, whereas 3-methyladenine and wortmannin were inhibitory, seemed counterintuitive, because both sets of compounds block cellular signalling upstream of, or at, TOR. One explanation for this apparent contradiction was the presence of two different classes of phosphoinositide[21]. Indeed, Patrice Codogno's laboratory, in collaboration with Meijer's group, showed that the class I PI3K product PtdIns(3,4,5)P_3 is inhibitory for autophagy, whereas PtdIns(3)P generated by the class III enzyme is essential; both classes of enzymes are blocked by 3-methyladenine and by wortmannin[22]. In agreement with these results, overexpression of PTEN, which reduces the level of PtdIns(3,4,5)P_3, stimulates autophagy[23]. Again, research in the yeast system initially lagged behind the mammalian field, but Ohsumi's laboratory showed that rapamycin stimulates autophagy in yeast, similar to the observation in mammals[24].

Selective autophagy

Although de Duve suggested in 1966 that autophagy could be selective[4], it was not until 1973 that Robert Bolender and Ewald Weibel provided the first evidence for specific sequestration of an organelle (the smooth endoplasmic reticulum) by autophagy[25]. In 1977, Jacques Beaulaton and Richard Lockshin suggested that mitochondria could be selectively removed during insect metamorphosis[26], and four years later Marten Veenhuis's laboratory demonstrated that excess peroxisomes could be degraded in a selective manner in the yeast *Hansenula polymorpha*[27]. Many subsequent studies confirmed these results in various other yeasts, as well as in higher eukaryotes, and led to the conclusion that autophagy has a role in cellular remodelling, allowing the removal of superfluous organelles. This finding posed an immediate question as to whether autophagy could also target organelles that were damaged.

John Lemasters and colleagues provided evidence that the depolarization of mitochondria during the mitochondrial permeability transition leads to the induction of autophagy[28], and subsequent studies confirmed that autophagy could selectively remove this organelle[29,30]. Lemasters proposed a model that foreshadowed much recent work on the connection between autophagy and apoptosis — autophagy could function as a cytoprotective mechanism, for example, to remove damaged mitochondria, but if the damage is too extensive, the cell may undergo apoptosis. Along these lines, it is interesting to consider that autophagy might be upregulated in the presence of reactive oxygen species, which might in turn indicate the presence of a damaged organelle.

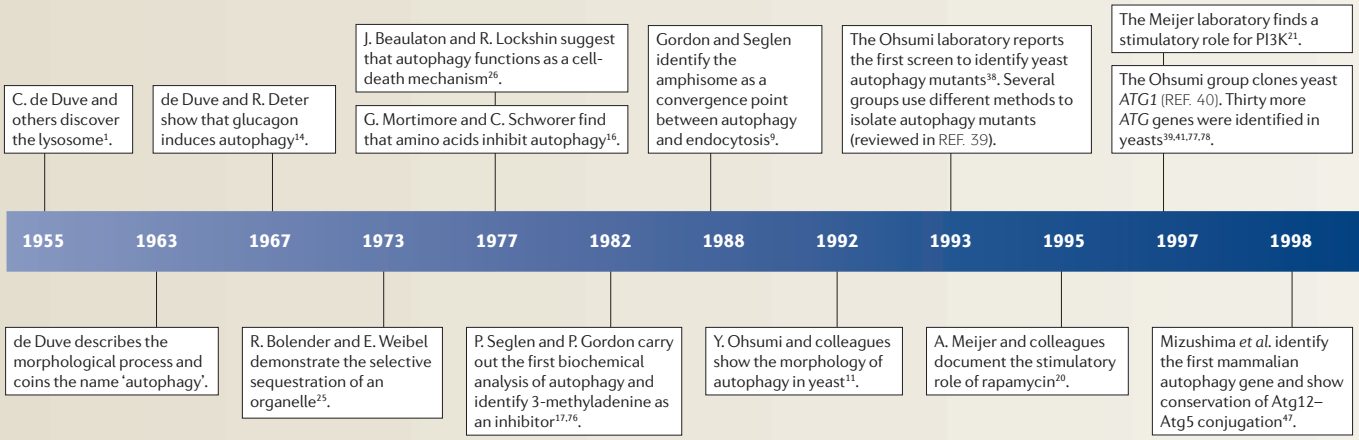
Another clear example of selective autophagy is seen in the yeast cytoplasm-to-vacuole targeting (Cvt) pathway[31] (BOX 1). The Cvt pathway overlaps extensively with nonspecific autophagy, both in terms of their morphology and the protein machinery involved[32–35]. One of the most notable features of the Cvt pathway in this regard is that it currently represents the only example of a biosynthetic autophagy-like pathway; at least two resident hydrolases are delivered to the vacuole by this route[36,37].

Although the Cvt pathway is the only known biosynthetic use of autophagy, selectivity represents an important aspect of many autophagic processes. For example, specific organelle degradation has been documented in mammals, and the elimination of certain pathogenic microorganisms from infected host cells is likely to involve a directed targeting-and-sequestration mechanism.

The start of the molecular era

Autophagy research clearly has its roots in the mammalian system, however, significant breakthroughs in our understanding of the molecular basis of autophagy only occurred following analyses in the genetically facile

Timeline | **A history of autophagy**



ATG1, autophagy-related gene-1; BCL2, anti-apoptotic protein B-cell lymphoma-2; *BECN1*, beclin-1; LC3, mammalian Atg8 homologue; PI3K, phosphatidylinositol 3-kinase.

yeast system. Ohsumi's group carried out the first genetic screen for autophagy mutants[38], and this was rapidly followed by various similar screens (reviewed in REF. 39). The demonstrated overlap among these mutants[33,35] was followed by a concerted effort to clone the complementing genes — the first, *ATG1* (autophagy-related gene 1), being published in 1997 (REF. 40). Ten years on, the thirty-first gene (*ATG31* according to the unified nomenclature[39]) was recently identified[41].

The *ATG* gene products are needed for nonspecific macroautophagy and the Cvt pathway, and also for selective degradation of peroxisomes and mitochondria[42,43]. It is not possible to describe in this Timeline the wealth of information that has been gleaned from analyses of the Atg proteins, and besides, much of this information has been covered in various reviews elsewhere[44,45]. Perhaps the most intriguing feature of the autophagic machinery, however, is the presence of two novel conjugation systems that involve ubiquitin-like proteins: Atg8 and Atg12 (REF. 46).

One point worth noting is that the identification of the *ATG* genes in yeast allowed the subsequent explosion of research into the molecular analysis of autophagy in higher eukaryotes (FIG. 2). Noboru Mizushima and colleagues identified the first mammalian autophagy genes, *ATG5* and *ATG12*, and demonstrated that the Atg12–Atg5 conjugation system is conserved from yeast to human[47]. Another critical step for autophagy analysis in higher eukaryotes was the identification of the mammalian Atg8 homologue MAP1LC3 (also known as LC3) by Tamotsu Yoshimori and Noboru Mizushima, and the subsequent development of LC3-based assays for monitoring autophagy in mammals and other higher eukaryotic systems[48]. It is important to note, however, that steady-state LC3 levels alone are not sufficient for evaluating autophagy, as it is crucial to follow flux through the entire pathway[45].

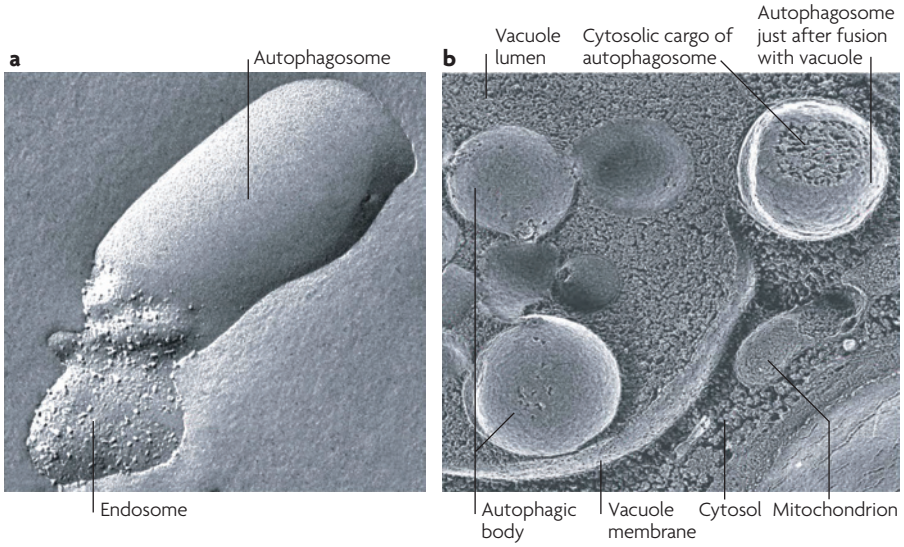
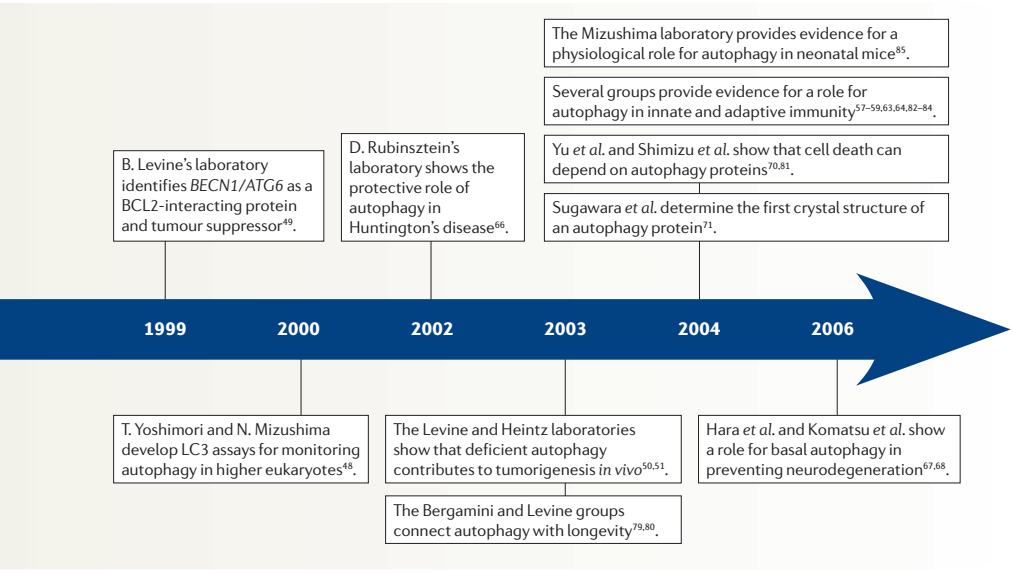


Figure 2 | **Autophagy visualized by freeze-fracture electron microscopy. a** | The fusion of an autophagosome, with its typical smooth limiting membrane that is devoid of transmembrane proteins, and an endosome with a particle-studded limiting membrane in a rat hepatocyte. The resulting structure is an amphisome. **b** | The fusion of an autophagosome with a yeast vacuole. The outer membrane of the autophagosome becomes continuous with the vacuole limiting membrane following fusion. The cargo of the autophagosome is essentially identical to the surrounding cytosol. Part **a** reproduced with permission from REF. 7 © (2004) Landes Bioscience. Image in part **b** courtesy of M. Baba, Japan Women's University, Tokyo.

Health and disease

A seminal point in connecting the molecular basis of autophagy with disease came with a 1999 paper from the laboratory of Beth Levine, which demonstrated that human cells that carry monoallelic deletions of the *BECN1*/*ATG6* gene are tumorigenic[49]. Subsequent studies showed that mice that are heterozygous for *Becn1* display higher rates of spontaneous tumour formation, confirming an *in vivo* role for autophagy as a tumour suppressor[50,51]. Beclin-1 can interact with the anti-apoptotic protein BCL2, and its binding state may play a key part in coordinating the cellular decision to undergo autophagy[52]. Finally, recent data



from the laboratories of Eileen White and Shengkan Jin provide further evidence that autophagy is a protective mechanism against DNA damage that could otherwise lead to tumorigenesis[43,53].

The paper by Liang *et al.* was soon followed by an ever-increasing number of studies that connected autophagy to various pathophysiological conditions. For example, as early as 1984, Yasuko Rikihisa demonstrated that autophagy is initiated during host infection by *Rickettsia* bacteria[54], and other studies supported the connection between autophagy and invasive microorganisms based on cytological criteria[55,56]. However, it was not until 2004, when analyses of *Streptococcus pyogenes* in *atg5*$^{-/-}$ embryonic stem cells by Tamotsu Yoshimori's group, and of *Mycobacterium tuberculosis* by the laboratories of Vojo Deretic and Maria Colombo, that clear evidence was provided for the role of autophagy in eliminating intracellular bacteria[57,58] — a finding that was again soon verified by additional studies[59].

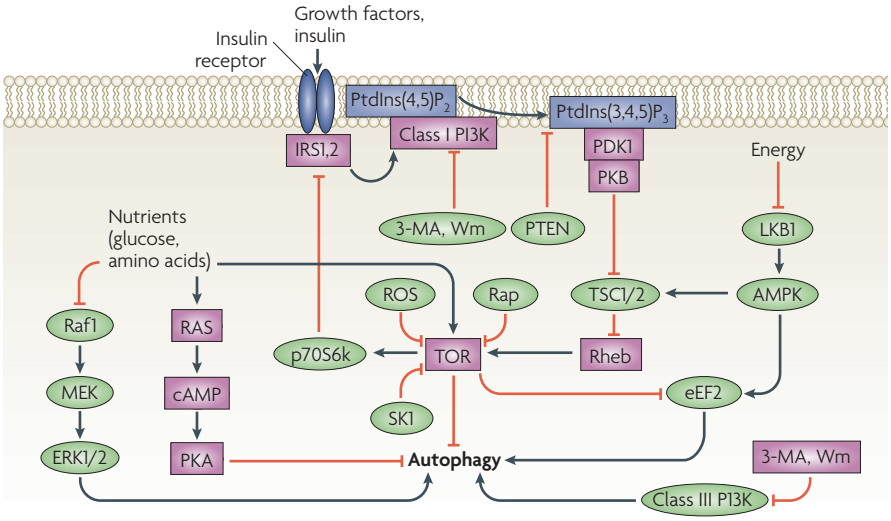
Furthermore, experiments with alphaviruses and herpes simplex virus showed that autophagy can also protect cells and mice against viruses[60–62]. It is worth noting that some of these analyses also indicate that certain microorganisms evolved to subvert the autophagic defence mechanism and, in some cases, that they rely on autophagy to establish a replicative niche. Finally, in addition to a role in innate immunity[57,58], autophagy also functions in the adaptive immune response[63,64]. In particular, Christian Münz's laboratory provided the first evidence for a pathogen-derived antigen that undergoes endogenous MHC class II processing by autophagy: the Epstein–Barr virus nuclear antigen 1 (EBNA1)[64].

At approximately the same time, a series of studies was initiated that connected autophagy to some types of neurodegenerative disease (reviewed in REF. 65). For example, a study by David Rubinsztein's laboratory showed that inhibition of TOR and induction of autophagy reduced the toxicity of certain aggregation-prone proteins, such as those involved in Huntington's disease[66]. Two parallel studies that were carried out in mice, using specific knockouts of *Atg5* or *Atg7* in neurons, demonstrated an important role for basal, constitutive autophagy in preventing the onset of the symptoms of neurodegeneration in otherwise healthy organisms[67,68].

Finally, a significant conundrum that has emerged in recent years revolves around the observation that there might be coordination between apoptosis (type I programmed cell death) and autophagy (type II programmed cell death). As early as the 1970s, Beaulaton and Lockshin suggested a role for autophagy in cell death[26], and many subsequent studies have confirmed this hypothesis. In most situations, autophagy probably functions initially as a cytoprotective mechanism, but if cellular damage is too extensive, or if apoptosis is compromised, excessive autophagy may be used to kill the cell[69,70]. An understanding of this dual role of autophagy is essential for learning how the process is controlled. As noted by Codogno, "Our knowledge of the balanced role of autophagy in cell survival and cell death will benefit studies in regulation."

Box 2 | Regulation of autophagy in mammalian cells

In the figure, the green circles represent components that stimulate autophagy, whereas the purple boxes correspond to inhibitory factors. 3-methyladenine (3-MA) and wortmannin (Wm) also inhibit class I phosphatidylinositol 3-kinases (PI3K), but the overall effect of these compounds is a block in autophagy (because they inhibit the downstream class III enzyme that produces phosphatidylinositol-3-phosphate (PtdIns(3)P), which is needed for autophagy). The regulation of autophagy is complex and far from understood. Historically, TOR (target of rapamycin) has been considered to be the central regulator of autophagy, because TOR inhibition with rapamycin (Rap) induces autophagy. However, it is now clear that there are also TOR-independent types of regulation. For example, beclin-1 and Atg4 might be regulated by the c-Jun N-terminal kinase (JNK) and reactive oxygen species (ROS), respectively. Additional information is available in recent reviews (for example, see REFS 74,75).



Concluding remarks

One of the most recent advances in the molecular era of autophagy is the structural analysis of autophagy proteins, which began with the structure of the mammalian Atg8 homologue[71]. Structure–function analyses will probably be useful in understanding the mechanism of autophagy and, combined with continued studies of the regulatory process, they might allow the rational design of drugs and the precise modulation that will be needed to use autophagy effectively in therapeutic intervention[72]. Along these lines, we will continue to advance our knowledge of the crosstalk that occurs between autophagy and apoptosis, as well as their regulation[73]. Further information on the selective nature of autophagy will be important as we continue to uncover additional examples of its specificity. Finally, we will need to address the fundamental biochemical questions that concern the functions of the Atg proteins and the mechanism of sequestering vesicle formation. The increasing prominence of autophagy in scientific research is slowly being reflected by the inclusion of this topic in textbooks. Perhaps one day soon, seminar audiences will be filled with people anxious to learn more about this already familiar topic.

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Competing interests
The author declares no competing financial interests.

DATABASES
Entrez Gene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
ATG1 | *ATG5* | *Atg7* | Atg8 | Atg12 | *ATG12* | *ATG31* | *BECN1* | MAP1LC3 | PDK1 | PKB | PTEN | *TOR*
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/sites/entrez?Db=genomeprj
Mycobacterium tuberculosis | *Streptococcus pyogenes*

FURTHER INFORMATION
Daniel J. Klionsky's homepage: http://www.biology.lsa.umich.edu/research/labs/klionsky/klionskylab.html
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