

Chapter 2

History of Autophagy After 1963

Abstract This chapter gives a historical perspective of the landmark studies that have allowed the autophagy field to progress to its current status. It begins describing the early morphological and functional studies that established the pathway and reaches up to the molecular era of our understanding with the identification of the autophagy genes, at first in the yeast model system and then in mammals.

Keywords Autophagy · Lysosome · Yeast · ATG

2.1 The First Morphological Studies

Autophagy is essentially a membrane trafficking pathway that enwraps intracellular material, including organelles like ER and mitochondria, into double membrane vesicles and delivers them for degradation to lysosomes. Beginning more than 60 years ago, it took a decade of studies to establish the existence of this pathway, during which the first scientists in the field using primary cells and immunocytochemistry techniques described the characteristic membrane structures and the cargos of the pathway, all of which were identified based on their morphology. It started with Christian de Duve discovering the end point of the pathway, the lysosome. de Duve fractionated rat liver lysates and observed for the first time in the fractions a membrane enwrapped organelle with acidic pH and hydrolytic activity, which he later named lysosome (de Duve et al. 1955). Alex Novikoff used rat liver lysates enriched in lysosomes to observe by electron microscopy for the first time cytoplasmic particles that, based on their appearance, he postulated to be lysosomes and called them “dense bodies” (Novikoff 1956). He also observed the same type of organelles in various tissues suggesting that the pathway is ubiquitous and not a property of the liver tissue. Then in electron microscopy studies of the development of mouse kidneys Clark made the first observation of intracellular organelles and in particular mitochondria inside the “dense bodies” suggesting that the cargo delivered to them was not originating from outside of the cell through endocytosis

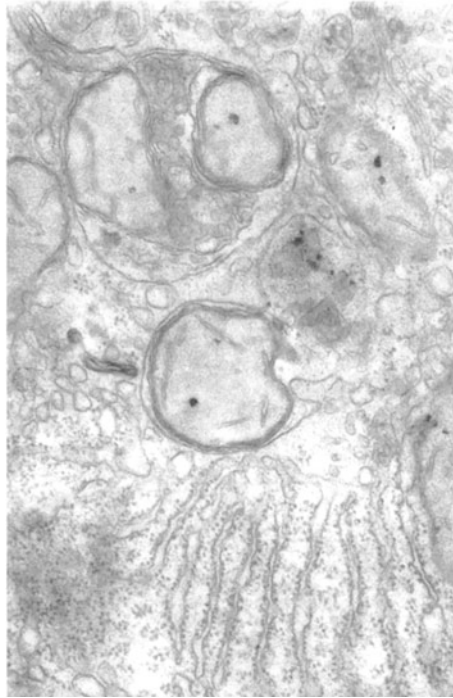


Fig. 2.1 Early images of autophagosomes seen by EM. Illustration used by de Duve and Wattiaux in 1966 to illustrate a review on lysosomes. Original caption was as follows: The autophagic vacuole on top contains two mitochondrial profiles and endoplasmic reticulum. Remnants of a second inner membrane are seen near the outer membrane which appears to be in continuity with a channel-like projection. Autophagic vacuole in center seems to have second membrane closely apposed to mitochondrial outer membrane, as noted by Novikoff and Shin (15). Outer membrane of this vacuole continues around second structure showing ferritin-like granules and spherules as seen in peribiliary dense bodies. This image could illustrate fusion between an “autophagosome” and a lysosome, to form an “autolysosome”. Note remarkable configuration of granular endoplasmic reticulum in lower part of picture: parallel channels appear to terminate in 11 common smooth-surfaced “collecting channel”. This figure first appeared in de Duve and Wattiaux (1966). Reproduced with permission of Annual Reviews

(Clark 1957). Novikoff showed that the dense bodies in mouse kidney cells contain also lysosomal enzymes proving that the “dense bodies” and the lysosomes are the same organelles and the end point of this pathway (Novikoff 1959). The first study of the regulation of this pathway was done when mitochondria and endoplasmic reticulum were observed inside cellular vesicles of rat hepatocytes after exposure to the catabolic hormone glucagon (Ashford 1962) which again Novikoff showed that they contained lysosomal hydrolases proving to be lysosomes (Novikoff and Essner 1962). After almost a decade of studies de Duve postulated the existence of a pathway present in all cell types that leads to sequestration of cytoplasmic structures into single- or double-membrane vesicles termed autophagosomes that were related

to lysosomes. He then coined the term autophagy to name the pathway from the Greek words auto (means self) and phagy (means eating) at the Ciba Foundation symposium on lysosomes in 1963 (de Duve and Wattiaux 1966). The first period of morphological studies closed with the demonstration of transient intermediates of autophagic vacuoles, i.e. double membrane vesicles enclosing cytoplasm and organelles, before fusing with the lysosome and without containing hydrolytic enzymes (Arstila and Trump 1968) (Fig. 2.1).

2.2 The First Functional Studies

The first indications that autophagy is a catabolic pathway were based on studies showing that baseline autophagy observed in rat liver cells is enhanced by nutrient deprivation or glucagon but is rapidly inhibited by insulin (Deter et al. 1967; Pfeifer 1977; Pfeifer and Warmuth-Metz 1983). Further biochemical studies demonstrated that amino acids are the nutrients that inhibit autophagy more potently in rat liver cells (Mortimore and Ward 1976; Mortimore and Schworer 1977). The authors perfused liver with amino acid containing solution and found that protein degradation was inhibited by 8 different amino acids: leucine, tyrosine, phenylalanine, glutamine, proline, histidine, tryptophan and methionine (Mortimore et al. 1983). Similar experiments from P. Seglen's lab showed that leucine is the most potent inhibitor of autophagy (Seglen et al. 1980). Autophagy was also found to be controlled by circadian rhythms in rat heart muscle and liver (Pfeifer 1981). Some first mechanistic insights into the autophagy machinery were the discoveries of autophagy inhibitors including 3-methyl-adenine (Seglen and Gordon 1982) [which was later found to be an inhibitor of the class III phosphatidylinositol-3 kinase (PI3 K) (Blommaert et al. 1997)] and also of kinase and phosphatase inhibitors (Holen et al. 1992). Nevertheless, until the discovery of the autophagy genes, progress in understanding the molecular mechanisms of the pathway remained slow.

2.3 Difficulties in the Study of Autophagy

During the first four decades of studies, the progress in the understanding of autophagy was delayed by the lack of a good model system and reliable readout methods. Autophagosomes and autolysosomes were initially identified by electron microscopy based on their morphology. However, this approach creates two problems: (i) *detection of lysosomes*: lysosomes are very heterogeneous in shape and size, especially between different cells and tissues, therefore it is difficult to distinguish them from other membrane-bound subcellular organelles and quantitate them reliably; (ii) *detection of autophagosomes before their fusion with lysosomes*: autophagosomes are transient organelles that can only be detected based on their

characteristic morphology. Autophagosomes are the only vesicles in cells that are bound by a double membrane and, in addition, they can enclose other organelles with characteristic morphology, like mitochondria. Their full lifespan is approximately ten minutes and before their closure they adopt a crescent-shaped membrane morphology only during the later stages of their expansion and just before their closure. This way they offer only a short window of opportunity to be detected, especially if any distinguishable organelles are not already enwrapped. Another indicator used for quantitating autophagy was the release of amino acids from pre-labelled cell proteins, though this approach again suffers by the lack of specificity for the autophagy pathway (Mortimore et al. 1972). Everything changed though when the genes that code the proteins of the autophagy pathway were identified.

2.4 Molecular Era—Identification of Autophagy Genes in Yeast

Although autophagy was initially discovered in mammals, the major breakthrough for understanding the pathway at the molecular level occurred after ground-breaking studies in the model organism *Saccharomyces cerevisiae*, also known as baker's yeast. Yeast cells, instead of lysosomes have a single vacuole, a large storage compartment that occupies most of their cytoplasm and accommodates various metabolites including amino acids. The yeast vacuole has acidic pH, contains hydrolytic enzymes and it is now established to be the equivalent of the mammalian lysosomes (Jones 2002). Under nitrogen starvation yeast cells undergo a dramatic morphological differentiation that includes meiotic division and generation of spores (sporulation). As this differentiation occurs in the absence of extracellular nutrients, yeast cells are forced to recycle their own macromolecules in order to generate the required building blocks. The group of Yoshinori Ohsumi in Japan capitalized on this process postulating that autophagy is the pathway that generates the required nutrients for cell differentiation under nitrogen starvation. Indeed, using light microscopy they observed that after 30 min of nitrogen starvation yeast vacuoles start accumulating and are eventually filled with single membrane spherical bodies, demonstrating for the first time the induction of autophagy in yeast (Takeshige et al. 1992). They also showed that autophagy is induced by additional modes of starvation including carbon-, sulfate-, phosphate- and single amino-acid-starvation (Takeshige 1992). Later electron microscopy studies demonstrated that yeast autophagosomes are also double membrane organelles, which are formed in the cytosol and then fuse with the vacuolar membrane to generate the single-membrane spherical bodies that were originally found to fill the yeast vacuole under nitrogen starvation (Baba et al. 1995). These ground-breaking studies established for the first time a readout to monitor autophagy in an organism ideal for genetic screens, paving the way for the dissection of the pathway at the molecular level (Fig. 2.2).

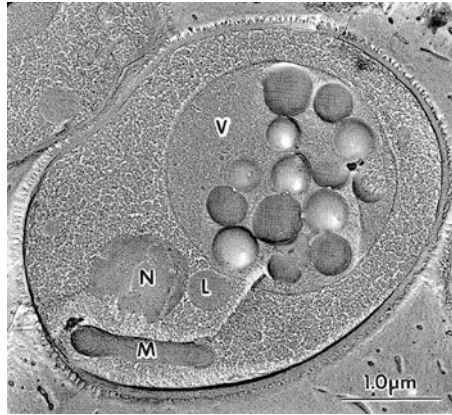


Fig. 2.2 Autophagic bodies in yeast. Autophagic bodies accumulating in the vacuole of a yeast cell that has been starved of nitrogen for 3 h and contains a mutation inactivating lysosomal/vacuolar function. Under these conditions, autophagosomes fuse with the vacuole but are not destroyed. This assay allowed identification of autophagy mutants in yeast by the work of Ohsumi and colleagues. Image was produced by rapid freezing of unfixed cells followed by freeze etching. This figure first appeared in Baba et al. (1995). Reproduced with permission of Japan Society for Cell Biology

Ohsumi's group used light microscopy and the phenotype described above as readout to design a genetic screen that identified the first autophagy gene, which they called *apg1* (Tsukada and Ohsumi 1993). Further characterization of this mutant strain revealed that, in yeast, even though autophagy is not required for survival in fed conditions it is necessary for survival under nitrogen starvation. Moreover, the mutant strain was defective in starvation-induced protein degradation and in sporulation but grew normally when cultured in rich medium, corroborating the hypothesis that autophagy promotes survival through recycling of nutrients (Tsukada and Ohsumi 1993). They then used this viability phenotype to design a genetic screen that allowed the isolation of more than 100 autophagy mutants, which belonged to 15 different complementation groups (Tsukada and Ohsumi 1993). Of note, complementation occurs if two mutant strains of the same diploid organism bearing homozygous recessive mutations to different genetic loci that give the same phenotype (in our case defect in autophagy) do not show the phenotype when they are crossed. This is a genetic test to decide whether two mutant strains bear mutations on the same or different genes without the need to know the exact function of these genes. Analysis of the mutants by electron microscopy confirmed that they were indeed defective in autophagosome formation (Tsukada and Ohsumi 1993). This first screen led to the isolation of most of the autophagy genes, making possible the study of autophagy at the molecular level (Nakatogawa et al. 2009).

The cataloguing of the autophagy genes continued with the help of more genetic screens that followed soon after: for autophagy (Thumm et al. 1994), for the transport of the vacuolar enzyme α -aminopeptidase I to the vacuole

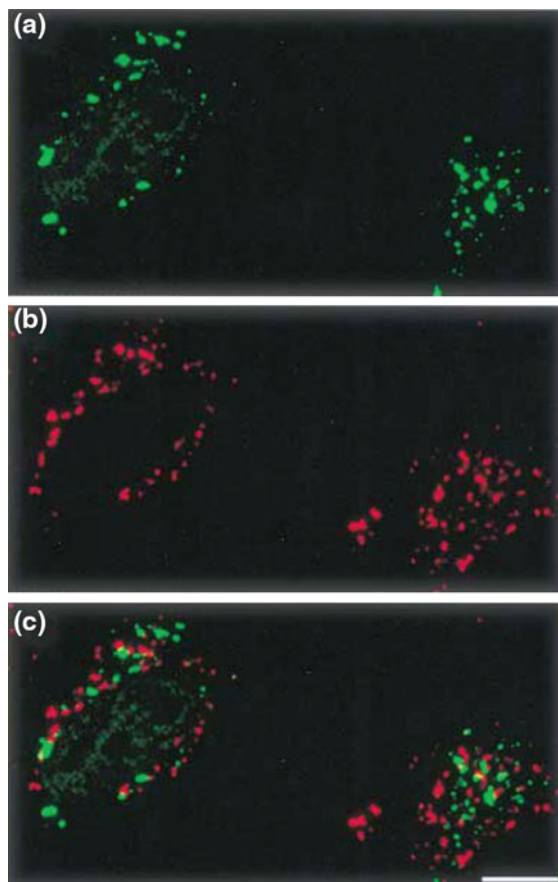
(Cytoplasm-to-vacuole targeting, Cvt) pathway (Harding 1995), for pexophagy (Titorenko et al. 1995; Sakai 1998; Mukaiyama et al. 2002) and for glucose-induced selective autophagy in *Pichia pastoris* (Yuan et al. 1997). These screens identified genes greatly overlapping with the genes identified by the first screen but also generated a range of names for the same genes. In order to facilitate communication within and outside the field, in 2003 the autophagy community adopted a unified gene and protein nomenclature based on the atg acronym, which stands for “autophagy-related” (Klionsky et al. 2003).

The yeast genome project, which made *Saccharomyces cerevisiae* the first eukaryotic organism whose genome was sequenced and became publicly available, allowed the identification of the genes from the autophagy screens in a very short period. The identified genes in turn allowed both to genetically manipulate and to accurately monitor autophagy, accelerating our understanding of the pathway. The first autophagy gene identified was also the first to be further characterized and was found to be a Ser/Thr protein kinase (Matsuura et al. 1997). A second gene, ATG6, had also been previously identified as VPS30 and was found to be involved in the vacuolar protein sorting (Vps) pathway (Kihara 2001). However, the rest of the autophagy genes had sequences that resembled no other known proteins therefore there was no available information for their function. Among these genes were surprisingly found two unique ubiquitin-like conjugation systems. Western blot analysis of Atg12 detected a protein band of higher molecular weight on top of the predicted one, which was missing in some of the mutant strains (atg5, atg7, or atg10) (Ohsumi et al. 1998). Further analysis uncovered that Atg12 is a ubiquitin-like protein which is conjugated to Atg5 using a ubiquitin-like conjugation system that consists of an E1-like enzyme (Atg7) and an E2 enzyme (Atg10). Soon after, a second ubiquitin-like conjugation system was discovered, built this time around the small ubiquitin-like protein Atg8 that was initially shown to associate tightly with forming autophagosomes. Atg8 is synthesized as a precursor, processed by a cysteine protease (Atg4) and strikingly conjugated to the phospholipid phosphatidylethanolamine (PE) by a second conjugation system that consists of an E1-like enzyme (Atg7), an E2-like enzyme (Atg3) and an E3-like enzyme (Atg12–Atg5 conjugate) (Kirisako 1999; Ichimura et al. 2000). The characterization of the main autophagy genes was completed with the discovery of an autophagy-specific phosphatidylinositol-3-kinase complex built around the lipid kinase Vps34 (Kihara 2001).

2.5 Identification of Autophagy Genes in Mammals

The identification of the autophagy genes in yeast paved the way for studies aiming to identify their mammalian homologues. The first autophagy genes identified in mammals were ATG5 and ATG12, by N. Mizushima, demonstrating for the first time that the autophagic machinery is conserved from yeast to mammals (Mizushima 1998). Soon after T. Yoshimori discovered the mammalian homolog of

Fig. 2.3 Expression of GFP-LC3 in mammalian cells. This image showed for the first time the localization of mammalian LC3 tagged with GFP and expressed in mammalian cells. The cells were also treated with bafilomycin A1 to enhance accumulation of autophagosomes before they fuse with lysosomes (stained here for Lamp-1 in the red channel). This figure first appeared in Kabeya (2000). Reproduced with permission of John Wiley and Sons



Atg8 (LC3) (Kabeya 2000), which localizes to the autophagosome membrane, allowing the widespread development of assays to monitor autophagy in biochemical and imaging studies (Fig. 2.3). The relatively low amino-acid sequence similarity between yeast and mammalian homologs delayed the identification of the Atg1 (ULK1) and Vps34 counterparts.

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Autophagy at the Cell, Tissue and Organismal Level

Karanasios, E.; Ktistakis, N.T.

2016, VI, 103 p. 15 illus., 12 illus. in color., Softcover

ISBN: 978-3-319-33143-0