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Virginia Todde, Marten Veenhuis, Ida J. van Der Klei. Autophagy: Principles and significance in health and disease. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2008, 1792 (1), pp.3. <10.1016/j.bbadis.2008.10.016>. <hal-00501596>

HAL Id: hal-00501596

<https://hal.science/hal-00501596v1>

Submitted on 12 Jul 2010

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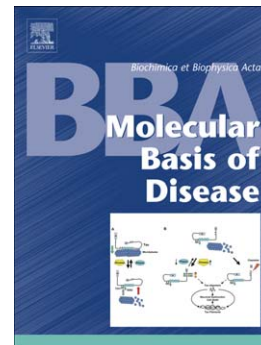
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PII: S0925-4439(08)00204-4
DOI: doi: [10.1016/j.bbadis.2008.10.016](https://doi.org/10.1016/j.bbadis.2008.10.016)
Reference: BBADIS 62877

To appear in: *BBA - Molecular Basis of Disease*

Received date: 5 June 2008
Revised date: 15 October 2008
Accepted date: 17 October 2008



Please cite this article as: Virginia Todde, Marten Veenhuis, Ida J. van der Klei, Autophagy: Principles and significance in health and disease, *BBA - Molecular Basis of Disease* (2008), doi: [10.1016/j.bbadis.2008.10.016](https://doi.org/10.1016/j.bbadis.2008.10.016)

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Autophagy:

Principles and significance in health and disease

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Keywords: yeast, autophagy, *ATG* genes, vacuole, lysosome, autophagosome

Abstract

Degradation processes are important for optimal functioning of eukaryotic cells. The two major protein degradation pathways in eukaryotes are the ubiquitin-proteasome pathway and autophagy. This contribution focuses on autophagy. This process is important for survival of cells during nitrogen starvation conditions but also has a house keeping function in removing exhausted, redundant or unwanted cellular components.

We present an overview of the molecular mechanism involved in three major autophagy pathways: chaperone mediated autophagy, microautophagy and macroautophagy.

Various recent reports indicate that autophagy plays a crucial role in human health and disease. Examples are presented of lysosomal storage diseases and the role of autophagy in cancer, neurodegenerative diseases, defense against pathogens and cell death.

1. Introduction

Cells continuously adapt to changing environmental conditions by adjusting their content to prevailing needs. This so called homeostasis involves continuous biosynthesis and turnover processes. Here we deal with a specific process of degradation, termed autophagy (from auto phagos: self eating). During autophagy portions of the cell content are delivered to the lysosome (mammals) or vacuole (plant, fungi) for degradation. For simplicity, we will use the term vacuole throughout this contribution, unless we describe a process that is confined to lysosomes.

Autophagy is among others strongly induced at nutrient starvation conditions and leads to bulk degradation of cytoplasmic components (proteins, organelles), whose building blocks are used for energy supply and the synthesis of components essential for survival at nutrient starvation conditions [1]. In cells defective in autophagy the total intracellular pool of amino acids is strongly reduced, leading to the inability of the cells to synthesize proteins that are essential for survival [2]. Similar results were observed in mutant cells in which autophagy was still normally functioning, but amino acid efflux to the cytosol was compromised due to the absence of the putative vacuolar amino acid transporter Atg22 [3]. Autophagy also may serve other functions and is important for cellular housekeeping as it may remove exhausted, redundant or unwanted components. In this way autophagy can act as an anti-ageing mechanism [4], support cell remodeling during development [5] or contribute to the cellular defense against pathogens [6].

In this contribution we present an overview of the current knowledge of the molecular mechanisms of autophagy and discuss its significance in human health and disease.

2. Modes of autophagy.

Reflecting their importance in maintaining cell vitality, various quality control machineries exist together with mechanisms removing misfolded, exhausted or redundant proteins/organelles, even at the sub-organellar level [for reviews see refs.7,8,9]. The currently known autophagy processes are sub-divided in three pathways: chaperone

mediated autophagy (CMA), macro- and microautophagy (Fig. 1). Of these, CMA is as yet only described in mammals and involved in degradation of single, soluble proteins. In contrast, macro- and microautophagy occur in a wide range of eukaryotes including mammals, plants and fungi and lead to the degradation of portions of the cytoplasm, which may include cell organelles.

Whether autophagy occurs by macro- or microautophagy and whether or not the process is selective, highly depends on the organism, the inducer of autophagy and environmental conditions. Also, within the same organism macro- and microautophagy may occur.

Illustrative for this is the nitrogen starvation induced autophagic degradation of mitochondria in *Saccharomyces cerevisiae*. When cells are pre-grown on lactate, mitochondria are selectively degraded by microautophagy, whereas when glucose is used as carbon source the organelles are predominantly degraded in a non-selective way together with other cellular components by macroautophagy [10]. Moreover, micro- and macro-autophagy processes may occur at the same time. This was for instance observed in the yeast *Hansenula polymorpha* upon shifting cells from media containing methanol/ammonium sulphate to media containing glucose and lacking any nitrogen source [11].

Further details of the CMA and micro- and macroautophagy machineries are presented below.

2.1 Molecular mechanisms involved in chaperone mediated autophagy

Chaperone mediated autophagy (CMA) is a process that results in selective degradation of individual, cytosolic proteins. Import of CMA substrate proteins into the lysosome is not mediated by vesicular transport but involves a protein translocation pathway through a proteinaceous pore in the lysosomal membrane [for a recent review see ref. 12] (Fig.1). Key proteins involved in CMA are members of the Hsp70 family of chaperones in the cytosol (Hsc70) and the lysosomal lumen (Hsc73) and the lysosomal membrane protein LAMP-2A. Cytosolic Hsc70 recognizes and unfolds substrate proteins that have a

specific motif (the KFERQ-like motif), which consists of a Q flanked on either side by four amino acids of defined composition [13]. In mammals such sequences occur in approximately 30% of the cytosolic proteins [14]. Degradation of these proteins however only occurs when CMA is activated (e.g. at nitrogen starvation conditions). Hsp70 co-chaperones (like Hsp40, Hsp90, Hip, Hop) are also required for CMA as they regulate the activity of Hsc70. The Hsc70-cargo complex is targeted to the lysosomal membrane where it binds to the cytosolic domain of LAMP-2A. LAMP-2A is an integral lysosomal membrane protein with a single transmembrane region, a short cytosolic tail and a large, highly glycosylated luminal region. LAMP-2A can form oligomers (up to octamers) which are proposed forming the protein translocation pore. LAMP-2A is the translation product of one of the three splicing variants of the LAMP-2 gene. The functions of the other two variants (LAMP-2B and LAMP-2C) are not yet known [reviewed in 12]. Inside the lumen of the lysosome, Hsc73 most likely is important to pull the substrate protein into the lysosome, by analogy to the function of Hsp70 proteins in the ER lumen (BiP) or mitochondrial matrix (MtHsp70) in protein translocation into these organelles. Cytosolic Hsc70 dissociates from its cargo prior to translocation of the substrate and recycles to the cytosol.

2.2 The molecular mechanisms of macroautophagy

During macroautophagy a double membrane structure sequesters random cell material (e.g. organelles, soluble cytosolic proteins, protein aggregates) for degradation. This structure is designated the autophagosome. Subsequently, the outer membrane of the autophagosome fuses with the vacuole membrane resulting in uptake of the cargo enclosed by the inner autophagosomal membrane (the autophagic body) in the vacuole (Fig. 2). Various proteins have been identified that play a role in macroautophagy. Most of these have been identified in screenings of yeast mutants defective in autophagy or the related Cvt pathway (see below) and have been designated *ATG* genes [15]. Based on their analysis a hypothetical model has been proposed for macroautophagy (Fig.2). Here

we confine to the key steps and conserved proteins involved in macroautophagy in higher and lower eukaryotes [for detailed reviews including all components see refs. 16,17,18]. The Ser/Thr protein kinase TOR plays a key role in signaling of nutrient limitation [19]. In addition to autophagy TOR regulates cell growth, cell cycle progression, nutrient import and protein synthesis. At normal growth conditions TOR is active and responsible for the hyperphosphorylation of Atg13, a protein that modulates Atg1 activity [20,21]. At nitrogen starvation conditions however, TOR is inactivated resulting in reduced levels of Atg13 phosphorylation (hypophosphorylation), a process that leads to an increased affinity of Atg13 for Atg1 [22]. Atg1 also is a Ser/Thr protein kinase, which functions down-stream of TOR regulating different steps in autophagosome formation [23]. Hence, nitrogen starvation stimulates the formation of Atg1-Atg13 complexes, which are part of a larger regulatory protein complex required for induction of macroautophagy.

A second important protein complex in macroautophagy is the phosphatidylinositol 3-kinase (PI 3K) complex, which mediates vesicle nucleation. This complex contains three highly conserved proteins namely the protein kinase Vps15, the phosphatidylinositol 3-kinase Vps34 [24] and Atg6 [25].

After induction of autophagy by activation of the Atg1 complex, a cascade of reactions occurs resulting in initiation of autophagosome formation. In yeast this is assumed to start at the pre-autophagosomal structure PAS [26]. The PAS has been identified by fluorescence microscopy as a fluorescent spot in the cell, localized to the peri-vacuolar region, where almost all Atg proteins assemble. In *S. cerevisiae*, 16 ATG genes have been identified that are essential for autophagosome formation and most of these proteins have been localized to the PAS or are at least transiently associated with this structure [27]. In this complex Atg17, a protein that associates with Atg13, is a core protein. However, Atg17 homologues have not yet been identified in higher eukaryotes [17].

The biochemical composition and ultrastructure of this putative structure is unknown and may represent a protein/lipid complex or harbor a small membrane vesicle to which proteins are bound. It is hypothesized that the PAS facilitates the nucleation or expansion

of the autophagosome through the recruitment of Atg proteins. However, a PAS has not been identified in mammals and hence the origin of autophagosomes in mammals is still under debate [28].

The PI 3K complex at the PAS is important for association of other Atg proteins as it is responsible for the formation of PI 3P phosphate to which these proteins are recruited. Once the initial autophagosomal vesicle (also designated phagophore) has been formed (by a yet unknown mechanism), the membrane structure expands to sequester material to be degraded (Fig. 2). This process involves two reactions that show similarity to the reactions catalyzed by E1- and E2-enzymes in ubiquitin conjugation. The first one is the addition of phosphatidyl ethanolamine (PE) to the ubiquitin-like protein Atg8, a major factor in autophagosome expansion. For its function the C-terminus of Atg8 must be proteolytically processed by Atg4 [29,30,31,32]. Subsequently, Atg8 is lipidated by the E1-like enzyme Atg7 and the E2-like enzyme Atg3 [31,33,34,35,36]. Binding of Atg8-PE to the autophagosome depends on a second series of ubiquitin conjugation like reactions. This requires the function of Atg12 as ubiquitin-like protein, which is conjugated to Atg5 via the function of Atg7 (E1) and Atg10 (E2). To this Atg12-Atg5 conjugate Atg16 associates forming a complex that has a coat like function in formation of the autophagosome [32,37,38].

Atg8-PE, associated to the developing double autophagosome membrane, is located at both the inner and outer membrane of this structure. Upon closure of the autophagosome, Atg8 protein in the outer membrane is recycled to the PAS, whereas the portion trapped inside the autophagosome is degraded together with the cargo [29]. The Atg12-Atg5-Atg16 complex however covers only the outside of the developing autophagosome and is recycled upon completion of autophagosome formation.

Retrieval of Atg8 from the outer surface of the autophagosome to the PAS is mediated by the protease Atg4. Another protein that is localized to the autophagosome and recycled to the PAS is the integral membrane protein Atg9. This process involves Atg1, Atg2 and Atg18 [39].

The final step, fusion of the outer membrane of the autophagosome to the vacuole, does not involve a specific set of Atg proteins but instead depends on proteins also required for other heterotypic and homotypic membrane fusion events of the vacuole (e.g. Vam3, Vam7, Ypt7) [40]. The outer membrane of the autophagosome fuses with the vacuolar membrane, whereas the inner membrane together with the cargo is degraded by vacuolar hydrolases (Fig. 2).

2.3 Selective macroautophagic processes and the related Cvt pathway

The Cvt pathway

The Cvt pathway (cytoplasm to vacuole pathway) is a protein transport route that so far has only been identified in the yeast species *S. cerevisiae* [17] and *Pichia pastoris* [41]. The molecular mechanisms of this pathway largely overlap with macroautophagy. However, the Cvt pathway is not an autophagic process, but a biosynthetic pathway responsible for sorting of two hydrolases, aminopeptidase I and α -mannosidase I, to the vacuole (Fig. 3). These proteins are synthesized in the cytosol as inactive, propeptide containing zymogens, where they fold and assemble into oligomers that associate into a large complex. Upon recognition by the cargo receptor protein Atg19 [42], the so called Cvt complex is formed. Next, Atg19 interacts with Atg11, a coiled coil protein that plays a role in recruiting the Cvt complex to the PAS to form the Cvt vesicle [43]. Ultimately, inside the vacuole the Cvt vesicle is degraded and the zymogens are processed into the enzymatically active form via cleavage of the pro signal by vacuolar resident proteinases. Several genes involved in the Cvt pathway overlap with those required for macroautophagy and, in addition, require Atg19 and Atg11 for cargo selection. Because of this property, yeast mutants defective in the Cvt pathway have been highly instrumental in the identification of *ATG* genes required for macroautophagy [44].

Macropexophagy

Peroxisomes constitute the most recent class of subcellular organelles that have been discovered in eukaryotic cells, but are the first organelles for which selective organelle degradation by autophagy has been described [45,46,47]. Selective peroxisome degradation by autophagy (also termed pexophagy) has been mainly studied in yeast species that can grow on methanol, namely *Candida boidinii* (Fig. 4), *H. polymorpha* (Fig. 5) and *P. pastoris*. During growth on methanol peroxisomes develop that contain key enzymes of methanol metabolism, which become redundant for growth upon placing these cells in media supplemented with excess glucose. Hence, this autophagic process is not induced by nutrient limitation, but occurs during a rapid intracellular remodeling process to removing organelles that catalyze redundant metabolic pathways.

In the methylotrophic yeast *H. polymorpha* peroxisomes are degraded by macropexophagy when cells are shifted from methylotrophic growth conditions to conditions in which the synthesis of peroxisomal enzymes involved in methanol metabolism is fully repressed (e.g. glucose or ethanol) [46,47] (Fig. 5A,B). In *P. pastoris*, two types of pexophagy have been described. In this organism macropexophagy is induced when cells are shifted from methanol to ethanol, whereas micropexophagy occurs when methanol-grown cells are placed at glucose excess conditions [48,49] (for details see below).

The first step of macropexophagy in *H. polymorpha* is the recognition of the organelle to be degraded. Like in the Cvt pathway, Atg11 is important for selection of peroxisomes as cargo. An Atg19 homologue is absent in this yeast species [17] and an Atg19-like protein present in this organism is not essential for macropexophagy (Todde unpublished results). At the organelle surface two peroxisomal membrane proteins (Pex14 and Pex3) are crucial for macropexophagy [50,51]. These are key components in import of peroxisomal matrix proteins (Pex14) or formation of peroxisomal membranes (Pex3). Both proteins play a role in the initial steps of macropexophagy, probably for signaling and/or recognition of the organelles to be degraded. Upon induction of macropexophagy, the

peroxisomal membrane protein Pex3 is removed from the organelle, possibly by the ubiquitin proteasome system [51]. The presence of Pex14, specifically the extreme N-terminus of this protein and its phosphorylated variant, are essential for the onset of pexophagy [50,52]. Five other components of the peroxisomal translocon (Pex2, Pex10, Pex12, Pex13, Pex17) were recently analyzed and shown being redundant for pexophagy [53].

During vegetative reproduction of yeast, peroxisomes are predominantly formed by fission of pre-existing ones [54,55]. As a consequence, not all peroxisomes should be degraded upon induction of pexophagy, thus allowing a rapid adaptation of the cell to new conditions that require novel peroxisome functions for growth. Indeed, in *H. polymorpha* at least one organelle escapes degradation. Consistent with this are data of Leao-Helder et al. [56] who showed that deletion of the transcription factor *MPP1* leads to cells that invariably contain only one peroxisome that appeared to be protected from degradation by macropexophagy. However, the molecular details of this protection mechanism are not yet understood.

Following recognition, the target organelle is sequestered by autophagosomes (also designated pexophagosomes). This process starts at one site at the peroxisomal surface and its completion is essential for the proper delivery of the cargo to the vacuole. The number of sequestering membrane layers may vary between 2 to 12 (Fig. 5B). Genes that have been shown to be involved in peroxisome sequestration overlap with those involved in the formation of autophagosomes [17].

In mammals peroxisome degradation is induced upon removal of peroxisome proliferators, compounds that induce peroxisome development. This process indeed represents a macroautophagy pathway as it is blocked in Atg5-deficient livers [57].

Reticulophagy

The endoplasmic reticulum (ER) is an important site of folding and modification of proteins (e.g. secretory proteins, vacuolar proteins and plasma membrane proteins). The

protein folding capacity of the ER is regulated via the unfolded protein response (UPR) and thereby carefully adjusted to cellular needs. In case of folding stress, the volume of the ER as well as its content of proteins involved in protein folding and modification increase. During this stress response, part of the ER is selectively sequestered by double membrane vesicles, similar to autophagosomes. Surprisingly, the nature of these sequestering membranes was shown to be the ER itself as suggested by the presence of ribosomes on this membrane. The so formed “ER-containing autophagosome” subsequently fuses with the vacuole and releases its content into the vacuole for degradation [58]. Electron microscopy suggested that this process called “reticulophagy” was highly selective as no cytosol and other organelles were included into the degradation vesicles [58]. Not much is known yet about ER degradation and its role on ER maintenance. Possibly, this selective pathway may serve to degrade damaged portions of the ER or resize it after the folding stress induced enlargement. The resizing hypothesis resembles the regulation of peroxisome abundance, which also involves organelle proliferation and selective degradation.

2.4 The molecular mechanisms of microautophagy

During microautophagy the components to be degraded are sequestered by the vacuolar membrane without their prior sequestration in autophagosomes. For small particles the vacuolar membrane forms tubular invaginations from which small vesicles pinch off (see Fig. 1). When relatively large structures are taken up by microautophagy, finger like protrusions of the vacuole, eventually together with vacuolar fragments, surround the cellular components to be degraded which, after homotypic fusion of the vacuolar membranes, ultimately end up in the vacuolar lumen.

Microautophagy is responsible for degradation of various cellular components including the vacuolar membrane (possibly as a mechanism to reduce the organellar size). Reducing the vacuolar size is crucial during macroautophagy, when large autophagosomes fuse

with the vacuole. To compensate for this, the vacuolar membrane may be resized via microautophagy [59].

Like macroautophagy, microautophagy is induced in yeast cells that experience nitrogen starvation via the TOR signaling complex. In addition to TOR, microautophagy is controlled by a second regulatory complex, the EGO complex. This complex consists of three proteins, Ego1, Ego3 and the GTPase Gtr2 [60].

The dissection of the steps and the identification of proteins involved in the microautophagy pathway are still in its infancy relative to the knowledge of the principles of macroautophagy. Uttenweiler and colleagues developed elegant *in vitro* assays to reconstitute microautophagy [61]. Using purified yeast vacuoles and cell extracts, the uptake of a reporter protein was monitored. Using various chemical inhibitors the process of microautophagy could be blocked at different stages *in vitro*.

Another important protein complex that is crucial for microautophagy is the VTC complex (Vacuolar Transport Chaperone), which is composed of four proteins, Vtc1, Vtc2, Vtc3 and Vtc4. This complex localizes to the vacuolar membrane, but also to other cellular membranes. The absence of one of the subunits of this complex severely reduces microautophagy activity *in vitro*. *In vivo* however the frequency of autophagic tube formation is not reduced in mutant cells lacking one of the Vtc proteins. This suggests that the Vtc proteins act in autophagic tube organization or vesicle scission, but are not required to form the invaginations. In line with this assumption is the observation that VTC protein complexes concentrate inside microautophagic tubes and at the tips of these tubes [62]. This contrasts the general distribution of transmembrane proteins in the vacuolar membrane, which show a high concentration at the vacuolar surface and at the base of the tubules, but a low concentration inside the tubes [63].

In vitro Atg proteins are apparently not required for the microautophagy process described above. However, *atg* mutants appear partly defective in certain selective microautophagic processes (e.g. in mitophagy or micropexophagy), possibly because this involves the uptake of larger particles by the vacuole.

2.5 Selective microautophagy related pathways

Micropexophagy

In *P. pastoris* glucose-induced micropexophagy clusters of peroxisomes are taken up by vacuolar sequestering membranes (compare Fig. 4). Remarkably, in this process a second membrane structure, called the MIPA (micropexophagic membrane apparatus) appears to be important for fusion of the sequestering vacuolar membranes [for a recent review see ref. 64].

For micropexophagy in *P. pastoris* both general Atg proteins and a subset of unique proteins are required. An example of the latter ones is Pfk1, the alpha subunit of phosphofructokinase, a component of the glycolysis. Null mutants of *PFK1* were shown to be impaired in micropexophagy independently of its glycolytic enzyme activity [65]. Hence, *P. pastoris* Pfk1 serves multiple functions similar as for instance described for pyruvate carboxylase in *H. polymorpha* [66]. In *P. pastoris pfk1* cells peroxisome degradation was blocked at the stage prior to vacuolar membrane fusion. Unlike general microautophagy, various *ATG* genes have been shown to be required for micropexophagy in *P. pastoris*, i.e. *ATG1*, *ATG11*, *ATG26* and *ATG28*. Their role is suggested to be related to the early steps of the process: recognition and sequestration of organelles to be degraded. Moreover, Atg8 is localized to the MIPA and required for micropexophagy. As Atg4 (a protease), Atg7 (an E1 like enzyme) and Atg3 (an E2 like enzyme) are required for lipidation of Atg8, these Atg proteins are required for micropexophagy as well [64]. Another important protein in micropexophagy is the vacuolar membrane protein Vac8. This is an armadillo repeat protein localized to the sequestering vacuolar membrane protrusions. A mutant lacking Vac8 is able to form the MIPA as well as the sequestering vacuolar membranes but the fusion step between these structures is impaired [67]. Recently, Subramani and colleagues [68] identified Atg30, a peroxisomal membrane protein important for recognition of the peroxisome to be degraded by micro- or macropexophagy. Atg30 interacts with the peroxisomal membrane proteins Pex14 and Pex3, two proteins known to be involved in macropexophagy in *H. polymorpha*. During

pexophagy, Atg30 transiently localizes to the PAS where it interacts with components of the autophagy machinery. Interestingly, Atg30 is required for both the formation of the MIPA during micropexophagy and for pexophagosome formation during macropexophagy.

Piecemeal autophagy

Roberts and coworkers [69] discovered that the nucleus belongs to the organelles which can be subjected to autophagic degradation. This process has been called piecemeal nucleus autophagy (PMN), a term that highlights the fact that only minor portions of nucleus are degraded. These are sequestered by the vacuolar membrane and subsequently degraded in the vacuole by a process that resembles microautophagy. However, the general microautophagy machinery seems not to be required for this process as PMN is not affected in early microautophagy mutants [69].

So far, few proteins involved in PMN have been identified. These include the nuclear envelop membrane protein Nvj1 and Vac8, which interact during PMN at nuclear vacuolar (NV) junctions [for a recent review see ref. 70]. These junctions increase in size when the level of Nvj1 is enhanced (e.g. at starvation conditions). Two other proteins that are localized at NV junctions and function in PMN are Tsc13 and Osh1. Osh1, which has been suggested to play a role in sterol lipid transport, is sequestered into NV junctions by direct interaction with Nvj1. Tsc13 physically interacts with Nvj1 and catalyzes the final step in very-long-chain fatty acid (VLCFAs) synthesis. VLCFAs are constituents of several complex lipids and influence the structure, function, and fluidity of membranes. In this way Tsc13 may contribute to the biogenesis of PMN structures [71].

It is not yet known how degradation of essential constituents of the nucleus are prevented to be degraded by PMN. Obviously, correct regulation is of vital importance as removal of the entire nucleus by autophagy would kill the cell.

Mitophagy

During recent years accumulating evidence has been obtained that mitochondria also may be subject to selective degradation (Fig. 6). Especially, mechanisms may exist that degrade organelles damaged by oxidative stress generated in the mitochondria. An important question is how the autophagy machinery can discriminate between vital and exhausted mitochondria. Recent data obtained in yeast suggest that mitochondrial fission and fusion processes are important to segregate functional from non-functional regions, followed by subsequent autophagic degradation of the non-functional parts [72].

The first protein identified to be involved in mitophagy in yeast is the outer mitochondrial membrane protein Uth1 [73]. Mammalian homologues of this protein have not been identified yet. When lactate grown *S. cerevisiae* cells are shifted to nitrogen limitation conditions, initially contacts are formed between vacuoles and mitochondria, followed by direct uptake of mitochondria by vacuoles by a process morphologically resembling microautophagy. In mutant cells lacking Uth1 the mitochondrial-vacuole contacts are not formed thus reducing autophagic degradation of mitochondria. Upon prolonged starvation mitochondria however are degraded in these cells. Detailed morphological analysis revealed that this was due to non-selective microautophagy, which apparently is independent of Uth1 [10].

Another protein involved in mitophagy in yeast is the protein phosphatase Aup1 [74]. Aup1 is a peripheral membrane protein that is localized to the mitochondrial intermembrane space and important for mitochondrial degradation that occurs in stationary phase yeast cultures. This process, however, probably occurs via a macroautophagic pathway as opposed to the microautophagic process described above. Possibly, Aup1 is involved in the signal transduction mechanism that marks mitochondria to be degraded for sequestration by the autophagosome [74].

A third component of the mitophagy machinery is the mitochondrial inner membrane protein Mdm38 [75]. Mdm38 is essential for the functional balance of the mitochondrial K^+/H^+ exchange system, which is known to be important for organelle morphology and

function. Upon artificial repression of *MDM38* mitochondria swell and fragment, followed by association of the fragments with vacuoles and subsequent degradation by a mechanism morphologically similar to selective microautophagy of mitochondria. Possibly, fission of the swollen organelle triggers autophagy. Indeed, in the double mutant *dnm1 mdm38*, in which mitochondrial fission is blocked, organelle fragmentation and selective mitophagy was suppressed.

3. Autophagy and disease

Like in all other eukaryotes, also in man autophagy is essential for survival under starvation conditions. This crucial function of autophagy is illustrated by the observations that knock out mice, in which autophagy is blocked in all tissues, die within hours after birth [76]. This early death is most likely related to the inability of these animals to survive during the neonatal starvation period.

In virtually all mammalian cells autophagy occurs at low constitutive levels, important for cellular housekeeping preventing the accumulation of damaged and mal-functional cell components. This basal level is enhanced during starvation and factors that increase the level of unwanted components in the cells (e.g. oxidative damage, protein aggregation). In mammals autophagy is also important in development, differentiation and tissue remodeling. Moreover, it can function as a defense system removing invading pathogens. Defects in autophagy in man are the cause of several serious diseases. Mutations in genes encoding proteins of the macroautophagy machinery (*ATG* genes) are known to abolish autophagy and cause disease. Also, deregulation of autophagy can seriously affect human health (e.g. mutations in mammalian TOR (mTOR) or Beclin 1 a human ortholog of yeast Atg6). An example of the latter is Carney Complex syndrome (CNC), a syndrome characterized by spotty skin pigmentation, cardiac and other myxomas and endocrine tumors, caused by a defect in the TOR signaling pathway, which leads to reduced autophagy [77]. Lysosomal storage diseases also fall within the class of autophagy related diseases. In these patients delivery of components to be degraded to the lysosome is

generally unaffected, but these components (e.g. proteins, lipids, glycogen) are not degraded due to the absence of the activity of a specific lysosomal hydrolase.

Autophagy sometimes may also contribute to disease. For instance, during tumor outgrowth autophagy promotes survival of tumor cells that experience nutrient starvation, which especially occurs in internal regions that are poorly vascularized.

From the above it is evident that the two major functions of autophagy, recycling of cell material during starvation and cellular housekeeping, are critically important for human health. Defects or deregulation is especially important in cancer, neurodegenerative diseases, lysosomal storage diseases, ageing and ageing related diseases. In addition autophagy combats invasion of cells by pathogens. Below these topics are discussed in more detail.

Cancer

Numerous observations suggest the existence of strong links between autophagy and cancer. Several tumor suppressor genes stimulate autophagy, whereas oncogenes are known that inhibit autophagy. The connection between both processes is probably related to the overlap that exists between the pathways involved in regulation of autophagy and tumorigenesis.

Mutations that affect the function of mTOR or Beclin 1 have been identified in human cancers [78, 79]. The function of mTOR overlaps with signaling pathways involved in tumorigenesis [for a recent review see ref. 19]. Several tumor suppressor genes have been shown being involved in the upstream inhibition of mTOR signaling and in this way stimulate autophagy. Moreover, oncogene proteins are known that activate mTOR.

The importance of Beclin 1 in human cancers is illustrated by the fact that mono-allelic deletions in Beclin 1 occur in a 40 – 75 % of cases of human breast, ovarian and prostate cancer. Whether this only relates to the function of Beclin 1 in autophagy or also to autophagy-independent functions of Beclin1 is not yet known [78, 79].

Although several observations indicate that enhanced autophagy may function as tumor suppressor and defects in autophagy can promote cancer, it is not yet understood how autophagy functions in causing or preventing cancer at the molecular level. Possible explanations include that autophagy may prevent cell death or alternatively plays a direct role in cell growth regulation [for a review see ref. 80].

Neurodegeneration

Autophagy is crucial for neuronal homeostasis, predominantly as housekeeping process to prevent accumulation of protein aggregates, which affect the function of neurons. Direct evidence for the vital role of autophagy comes from studies with mice which revealed that mice lacking Atg5 [81] or Atg7 [82] showed severe neurodegeneration in the central nervous system.

Soluble, misfolded proteins in the cytosol are normally degraded by the ubiquitin proteasome system. However, when the levels of such proteins overload the ubiquitin-proteasome system, they may form toxic oligomers or large aggregates that affect the function of the cell (and in brain cause neurodegeneration). Huntington's disease and several age-related diseases, like Alzheimer and Parkinson, are characterized by the accumulation of protein aggregates in the brain. The formation of these aggregates is stimulated by mutations in specific genes related to these diseases (e.g. polyglutamine-containing proteins in Huntington's disease and the amyloid precursor protein or the microtubule associated protein Tau in Alzheimer's disease [83]). Interestingly, in cells of patients suffering from these age-related diseases, autophagic vacuoles accumulate that contain ubiquitinated aggregates of the disease-related proteins. This was unexpected because it was anticipated for long that the cytosolic ubiquitin-proteasome degradation pathway and lysosome related autophagic process function independently from each other. That this is not the case is evident from the observations by Komatsu et al. [82], who showed that a conditional knockout of autophagy leads to neurodegeneration with ubiquitin-positive pathology. Moreover, Pandey and colleagues [84] recently showed that

autophagy acts as a compensatory degradation system when the ubiquitin-proteasome system is impaired in *Drosophila melanogaster*.

Consistent with these observations is the finding that ubiquitinated cytosolic protein aggregates can be selectively degraded by autophagy and interact with Atg8. This process involves the p62 protein, also called sequestosome 1 (SQSTM1) [85,86], which in neurodegenerative diseases is associated with ubiquitinated protein aggregates but in addition binds to human Atg8 paralogs [87].

Lysosomal storage diseases

Lysosomal storage diseases are generally caused by a defect in specific lysosomal hydrolases. For instance, in Pompe disease glycogen accumulates in multiple tissues, including skeletal and cardiac muscle, as a result of a deficiency of lysosomal acid alpha-glucosidase. As a secondary effect of these storage diseases, the malfunctioning lysosomes often become defective in fusion with autophagosomes and hence results in accumulation of autophagosomes as well.

An unusual type of lysosomal storage disease is Danon disease, which is caused by null mutations in the LAMP2 gene and not in a gene encoding a lysosomal hydrolase [88]. LAMP2 codes for integral lysosomal membrane proteins that also plays a role in CMA (LAMP-2A; see above).

Infectious disease

The molecular principles of macroautophagy have also been implicated in cellular defense against pathogens (bacteria, viruses, parasites). This process, also designated xenophagy [89], ultimately results in uptake of pathogens in the lysosome followed by their degradation [for recent reviews see 80,90].

Well studied examples of this are infections by Group A *Streptococcus* (GAS), *Salmonella*, *Shigella* or *Listeria*. These pathogens use the endocytic pathway for invading the host cell. After release from the endosome, the bacteria gain a replicative niche in the

cytosol until they are sequestered in autophagosomes and subsequently degraded in the lysosome. The importance of this pathway is underscored by the observation that *atg5*^{-/-} GAS cells show an increased survival [91].

Another example is infection by *Mycobacterium tuberculosis*, a pathogen that is taken up by phagocytosis. However, after uptake phagosome maturation is arrested and the bacteria multiply inside the phagosome. Upon induction of autophagy (e.g. by interferon gamma) the phagosome fuses with autophagosomes, which ultimately results in degradation of the pathogen in the lysosome [for a review see ref. 92].

Autophagy has also been suggested to be involved in the protection of cells against viruses. However, experimental evidence is still limited and confined to tobacco mosaic virus in plant [93] and Sindbis virus in mammalian cells [94].

On the other hand, autophagosomes may also play a role in the replication of some viruses. Distinct steps of the life cycle of viruses occur in association with membrane structures that are induced upon infection. These membranes generally originate from the ER or endosomes, but can also be formed from mitochondria or peroxisomes (for a recent review see [95]).

The double membrane vesicles induced upon poliovirus infection morphologically resemble autophagosomes and contain LAMP1 and LC3, the human homolog of *S. cerevisiae* Atg8. Moreover, stimulation of autophagy increases the release of polioviruses, whereas inhibition by 3-methyladenine or reducing the levels of Atg12 or LC3 by siRNA, results in a reduction in the release of infectious viruses [96], suggesting that autophagosomal membranes can also be involved in virus replication. In line with this role is the observation that replication of mouse hepatitis virus in embryonic stem cell lines from *atg5*^{-/-} cells was impaired [97]. In contrast, however, Zhao and colleagues [98] showed that ATG5 is not required for hepatitis virus replication in either bone marrow derived macrophages lacking ATG5 or primary low passage murine ATG5^(-/-) embryonic fibroblasts. Hence, further research is required to further establish whether or not autophagosomes can be involved in the life cycle of viruses [95, 99].

In addition to the defense against pathogens, autophagy is also implicated in immunity [80, 99] and chronic inflammation disease [80]. The latter illustrated by the recent finding that mutations in Atg16L1 may be related to Crohn disease, a chronic inflammatory bowel disease [100,101].

Autophagy, ageing and cell death

Dying cells often show morphological characteristics of autophagy (e.g. accumulation of autophagosomes) [102]. As mitochondria are important organelles in regulated cell death (apoptosis), autophagic degradation of mitochondria is likely to affect the onset of apoptosis. In most eukaryotic cells mitochondria are the main source of reactive oxygen species (ROS), which can damage membranes (lipids), proteins and DNA. Mitochondrial oxidative damage can lead to mitochondrial permeability transition (MPT), a result of opening of high conductance pores. Uncoupling of mitochondria results in ATP depletion, which may cause necrotic cell death. However, the organelles also release cytochrome c, which activates caspases and hence triggers apoptotic cell death [7,103].

The presence of healthy mitochondria is essential for vitality and survival. Autophagy of mitochondria and/or selective mitophagy most likely play an important role in removing damaged organelles. Although cell death is often accompanied by autophagy, it is still controversial whether autophagy promotes or prevents cell death. When damaged mitochondria are removed by autophagy, this will prevent cytochrome c release and activation of caspases, whereas a block in autophagy would promote caspase-dependent cell death. Because of the accumulation of harmful components in the cell, defects in autophagy may promote mitochondrial permeabilization and hence cell death [for recent reviews see refs. 4,18,80].

The sometimes conflicting data indicating that autophagy may also promote cell death may be related to the extent of mitochondrial damage and autophagy. At low levels of damage, autophagy may be sufficient to remove all dysfunctional organelles, whereas when damage enhances the autophagy capacity may be inadequate to degrade

mitochondria and the pro-apoptotic factors. Finally, autophagy could also cause cell death, when the autophagy machinery is overloaded and results in release of toxic proteins from the lysosome into the cytosol [104].

Non-functional mitochondria are important sources of ROS, which contribute to the accumulation of damaged and non-functional components in the cells. This accumulation is characteristic for ageing cells and ultimately results in loss in vitality. Hence, the timely autophagic removal of non-functional mitochondria has been suggested prevent ageing. Not only mitochondria are important ROS producing cells, but peroxisomes, which typically contain hydrogen peroxide producing oxidases and catalase, can also be major sites of ROS production in the eukaryotic cell [105]. Hence, peroxisomes and peroxisome degradation may also be important in ageing and cell vitality. Recent studies in yeast resulted in the first indications of a connection between peroxisomes, autophagy and cell death [106].

4. Perspectives

Although the process of autophagy is already known for over 50 years, only recently the crucial function of this process in a great variety of vital processes became evident. Important progress in the field was achieved by the identification of genes involved in autophagy and autophagy related processes (*ATG* genes) predominantly using yeast mutants defective in autophagy, the Cvt pathway or peroxisome degradation [44]. The analysis of (conditional) knock outs of lower and higher eukaryotes, including mice, uncovers more and more details of the processes in which autophagy is involved [80]. From the other side, genome-wide association studies result in the identification of SNPs in human *ATG* or *ATG* like genes, pointing to increasing numbers of diseases that might have a link with autophagic processes (e.g. Crohn disease). There are no indications yet that the fast increase in number of reports on autophagy and autophagy related process will soon decline.

So far, most research on autophagy focuses on protein degradation. Although a lot of detailed information is available now on the processes leading to delivery of proteinaceous material to the vacuolar lumen, much less is known on recycling of the degradation products (amino acids) for subsequent reuse by the cell.

Also, so far little is known on autophagic degradation and recycling of macromolecules other than proteins (e.g. nucleic acids, lipids, glycogen). Recent reports have presented data on selective degradation of ribosomes (ribophagy), indicating that massive RNA degradation and recycling may occur under certain growth conditions [107]. Even more rare are reports on vacuolar/lysosomal degradation and recycling of (phospho)lipids and glycogen, but data have been presented that suggest that these compounds are taken up in vacuoles as well [108, 109].

In yeast so far only one lipase, Atg15 [110], has been implicated in membrane degradation, whereas only one putative amino acid permease, Atg22, together with other amino acid transporters like Avt3 and Avt4, were shown to be involved in efflux of amino acids from the vacuole to the cytosol [3,111]. Such proteins have however still not been identified in higher eukaryotes, including man [17].

At present most attention has been paid to macroautophagy and macro-autophagy related processes, whereas microautophagy is much less studied. However, several data suggest that micro-autophagy and macro-autophagy may be interconnected processes, because macroautophagy can result in a large flow of membranes to the vacuolar membrane, which may be compensated by via microautophagy. In yeast micro-autophagic pathways seem to be very important for selective organelle degradation processes (mitophagy, piecemeal autophagy of the nucleus, micropexophagy). Very little is known on microautophagy in higher eukaryotes.

As in yeast micro- and macroautophagy may occur simultaneously [11], it is difficult to biochemically discriminate which of the two processes is responsible for degradation of a specific compound or organelle. Here only detailed high resolution microscopy analysis can provide an answer. Also, the use of *atg* mutants to determine whether a process

occurs via a micro- or macroautophagic process is not always feasible, because several Atg proteins play a role in both processes (e.g. in macro- and micropexophagy) [10,64]. In micropexophagy, the role of Atg proteins is most likely related to the involvement of the MIPA, which requires functional Atg8, a key protein in macroautophagy as well. In contrast, the formation of small micro-autophagic vesicles, which may be important for resizing the vacuolar membrane, apparently does not involve Atg genes [61].

Another highly intriguing question is how the autophagy machinery recognizes components to be degraded and how certain components are protected against degradation. The first proteins important for recognition of cargo have now been identified, but it is to be expected that other proteins, including those involved in regulation of recognition, are also involved.

A related question is how the vacuole protects its own bounding membrane against degradation. For instance, the outer and inner membranes of autophagosomes originally derive from the same vesicular structure. However, both membranes apparently are not equally sensitive towards degradation upon fusion of the autophagosome with the vacuole. The inner membrane, as part of the autophagic body (Fig. 1), is rapidly degraded upon entering the vacuole, whereas outer membrane becomes part of the vacuolar membrane and obviously is protected. Similarly, the vacuolar membrane is protected against the hostile vacuolar hydrolases, but portions of the same membrane are efficiently degraded upon entering the lumen by microautophagy.

In conclusion, various topics related to autophagy need urgent analysis, whereas undoubtedly investigators in other fields of research may become involved in autophagy studies as novel, additional cellular processes or diseases may have connections with this fascinating process of cellular cannibalism.

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Legends to Figures

Fig.1. Schematic representation of autophagy processes.

Cytoplasmic components may be degraded via different autophagy processes. Shown are chaperone mediated autophagy (CMA: degradation of specific cytosolic proteins); macroautophagy (bulk degradation of cytosol and organelles); microautophagy (engulfing portions of cytosolic material and organelles); macropexophagy (selective degradation of individual peroxisomes); micropexophagy (selective degradation of peroxisomal clusters) and the cytoplasm to vacuole (Cvt) pathway (a biosynthetic pathway involving selective transport and maturation of two vacuolar hydrolases). In macroautophagy, the Cvt pathway and macropexophagy, double (or multiple) sequestering membranes enwrap the cargo forming an autophagosome. The outer membrane of the autophagosome fuses with the vacuolar membrane delivering the cargo for degradation to the vacuole. In microautophagy and in micropexophagy, sequestration involves invagination and/or protrusion of the vacuolar membrane itself. In micropexophagy the tips of the vacuolar membrane protrusions are finally fused by an additional de novo formed membrane structure, called MIPA (micropexophagic membrane apparatus). The chaperone mediated autophagy does not involve membrane sequestration steps.

Fig. 2. Dissection of the macroautophagy pathway.

Schematic dissection of macroautophagy, together with the core proteins involved in each step in brackets. At nitrogen limitation conditions (1), macroautophagy is induced [Tor, Atg1 (ULK1), Atg13] leading to nucleation (2), expansion (3) and completion of membrane formation (4) for sequestration of cytoplasmic components [Atg2, Atg3, Atg4, Atg5, Atg6 (BECLIN-1), Atg7, Atg8 (LC3), Atg9, Atg10, Atg12, Atg13, Atg16 (Atg16L), Atg18, Vps15, Vps34]. The subsequent fusion step (5) and uptake in the vacuole (6) does not require Atg proteins. After degradation by vacuolar hydrolases (6) building blocks (e.g. amino acids) recycle to the cytosol mediated by specific effluxers (7; e.g. Atg22).

Fig. 3. Morphological characteristics of the Cvt and macro-pexophagy pathways.

Schematic representation of the morphologically similar Cvt and macropexophagy pathways.

In both cases, the cargo is selectively recognized and recruited to the pre-autophagosomal structure (PAS), followed by sequestration and delivery of the cargo to the vacuole lumen.

Fig. 4. Micro-pexophagy in *Candida boidinii*.

Fig. 4A,B. Electron micrographs of methanol-grown *Candida boidinii* cells shifted from methanol/ammonium (A) to methanol in the absence of ammonium (B). In the early stages of the process, the vacuole is fragmenting forming arm-like protrusions surrounding the peroxisomal cluster prior to uptake. (C) Selective uptake of peroxisome cluster in the vacuole after treatment of methanol/ammonium *C. boidinii* cells with the plant toxin T-514 [112], which selectively permeabilizes the peroxisomal membrane thereby rendering the organelles non-functional (bar = 1 μ m; M = mitochondria, V = vacuole, N = nucleus, P = peroxisome).

Fig. 5. Macropexophagy in *Hansenula polymorpha*.

Fig. 5A. Fluorescence microscopy demonstration of glucose-induced macropexophagy in *H. polymorpha*. Cells produce Green Fluorescent protein with a peroxisomal targeting signal (GFP-SKL) to mark peroxisomes, the vacuole membrane is stained by FM4-64 (red). Left (0 h) a methanol-grown control cell is shown that lacks vacuolar GFP, at the right hand a cell that has been exposed to glucose for 1h. In this cell vacuolar GFP, illustrative for the uptake of the organelle, is readily observed together with still intact peroxisomes. The cell wall is indicated in blue.

Fig. 5B. Electron micrograph showing the sequestration of a peroxisome by a phagophore in a methanol-grown *Hansenula polymorpha* cell exposed to glucose for 1h. Note the characteristic association of the sequestered organelle with a vacuole vesicle (bar = 1 μ m). V = vacuole, P = peroxisomes, M = mitochondria.

Fig. 6. Mitochondrial microautophagy in *H. polymorpha*.

(A) Electron micrographs showing a characteristic example of mitophagy in a methanol/ammonium sulphate grown cell of *H. polymorpha* (A). In the magnification (B) the invading vacuole membrane can be observed. M = mitochondria, V = vacuole, N = nucleus.

ACCEPTED MANUSCRIPT

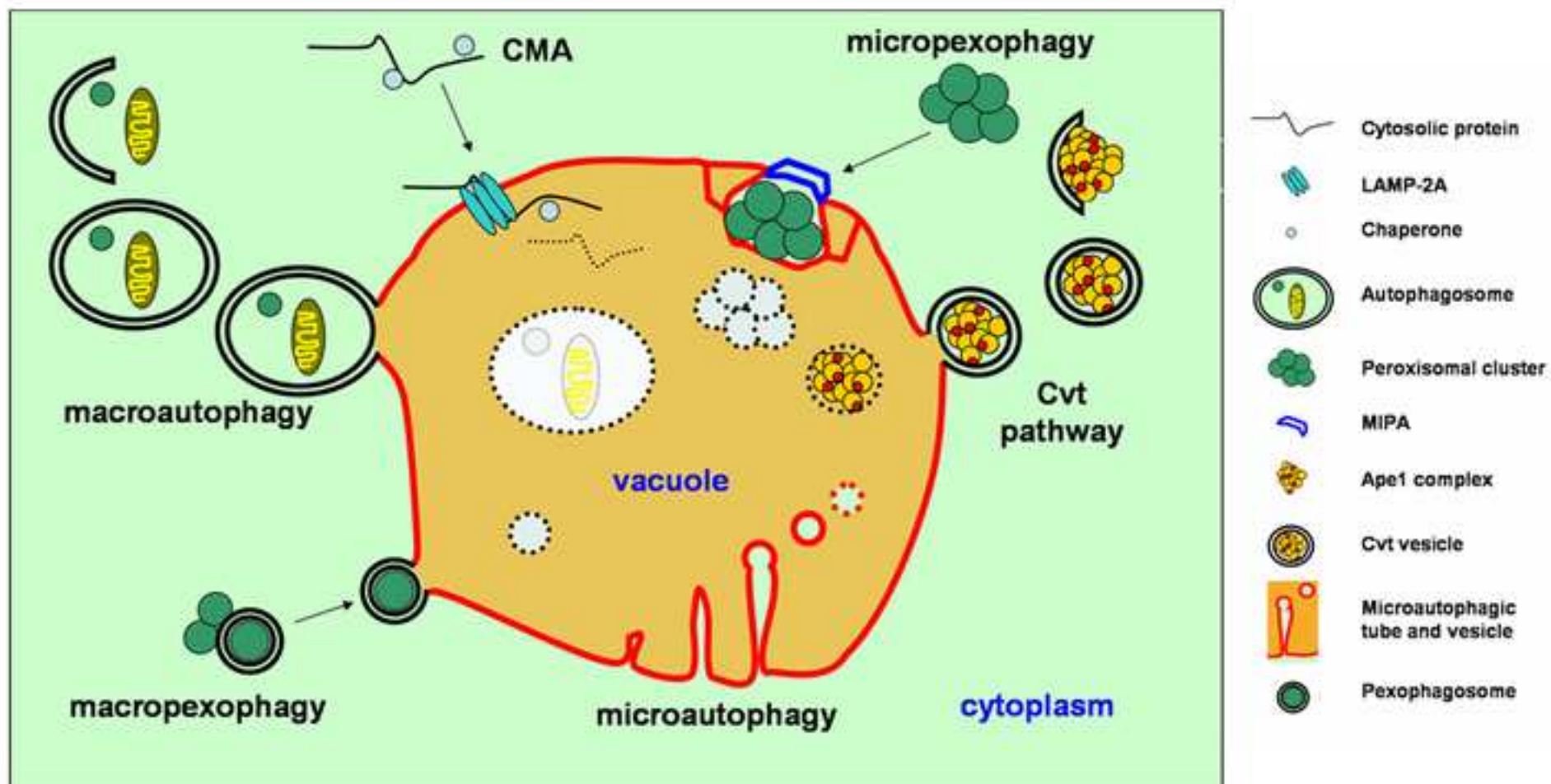


Fig.1

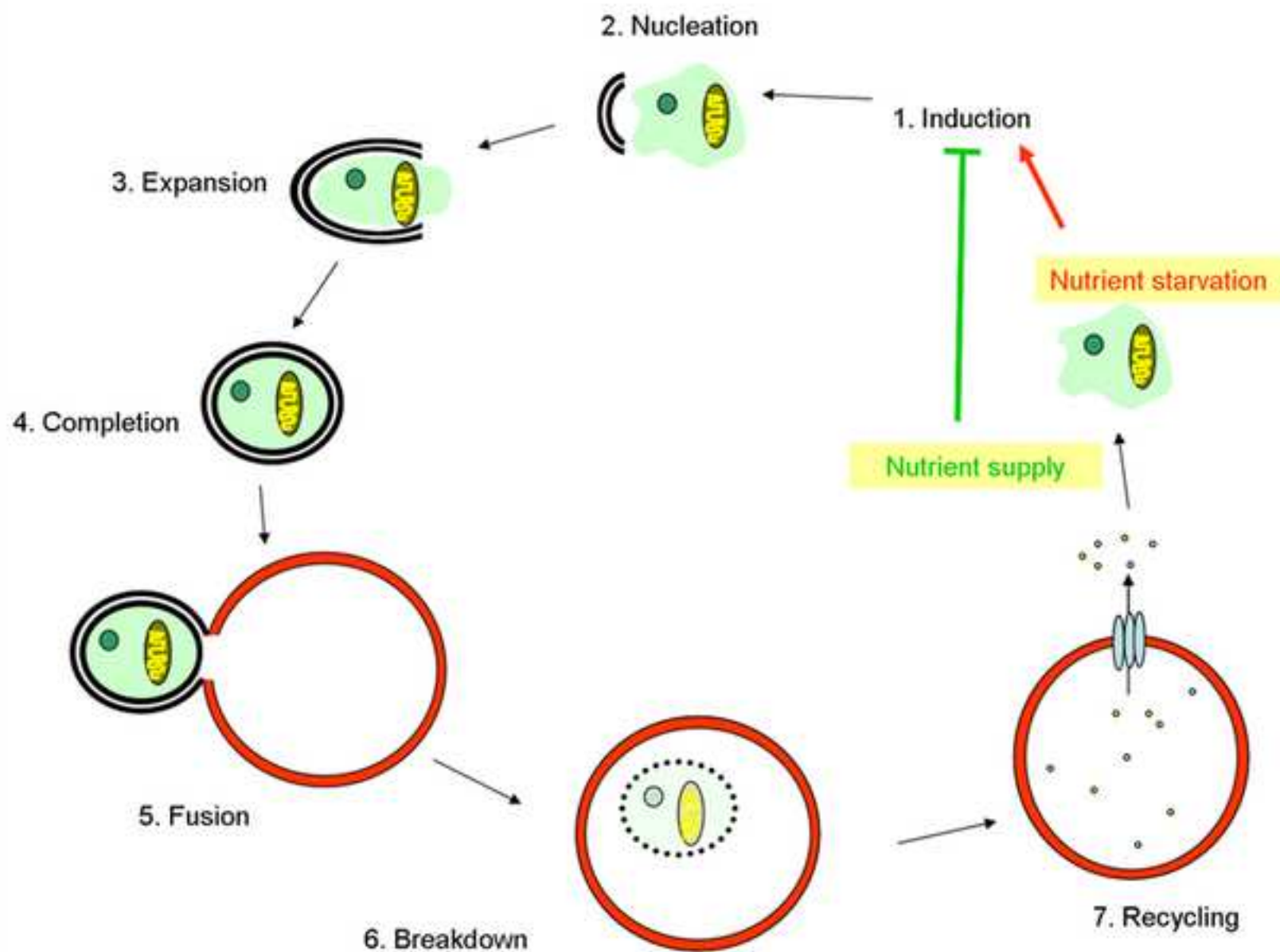


Fig.2

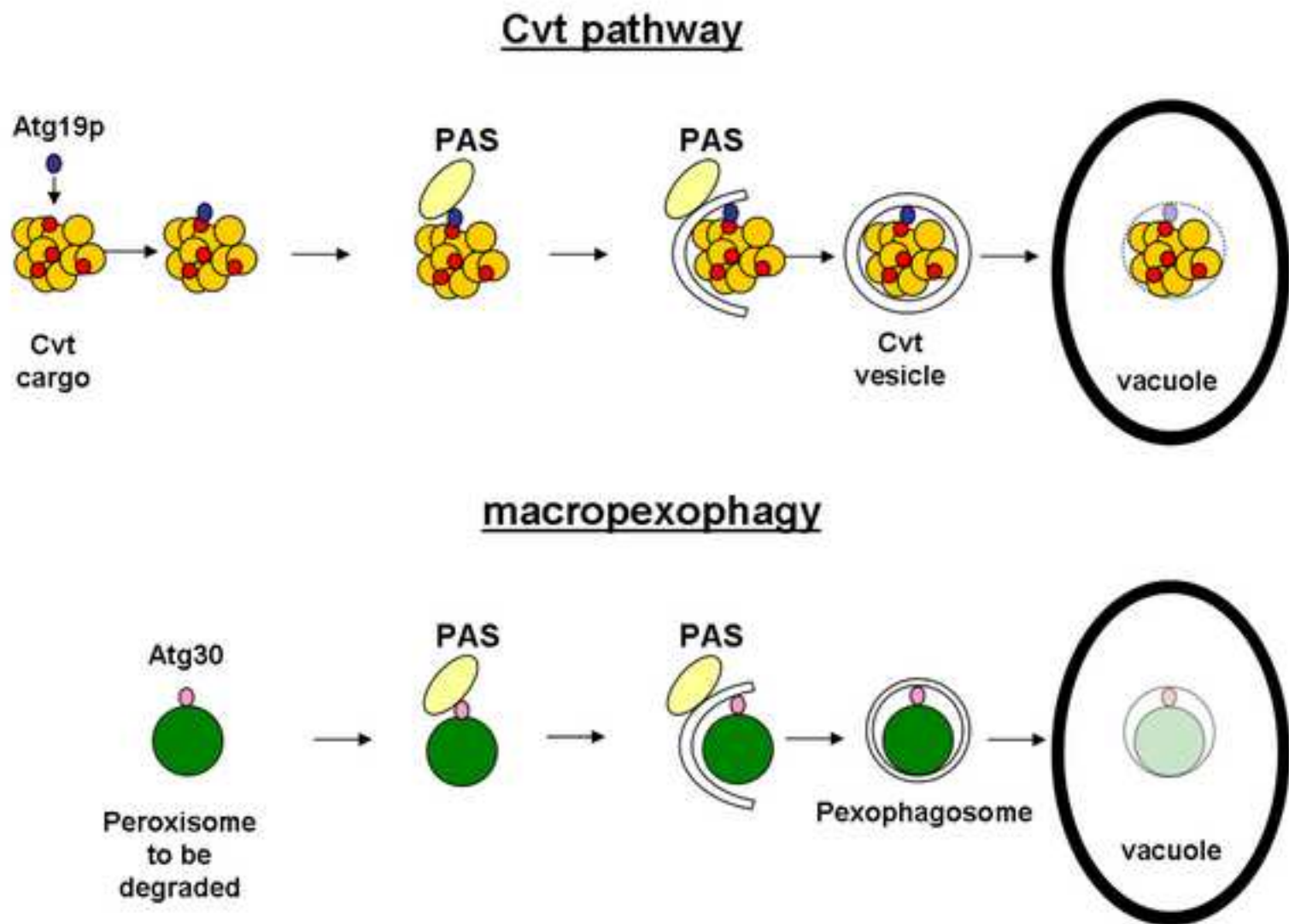


Fig.3

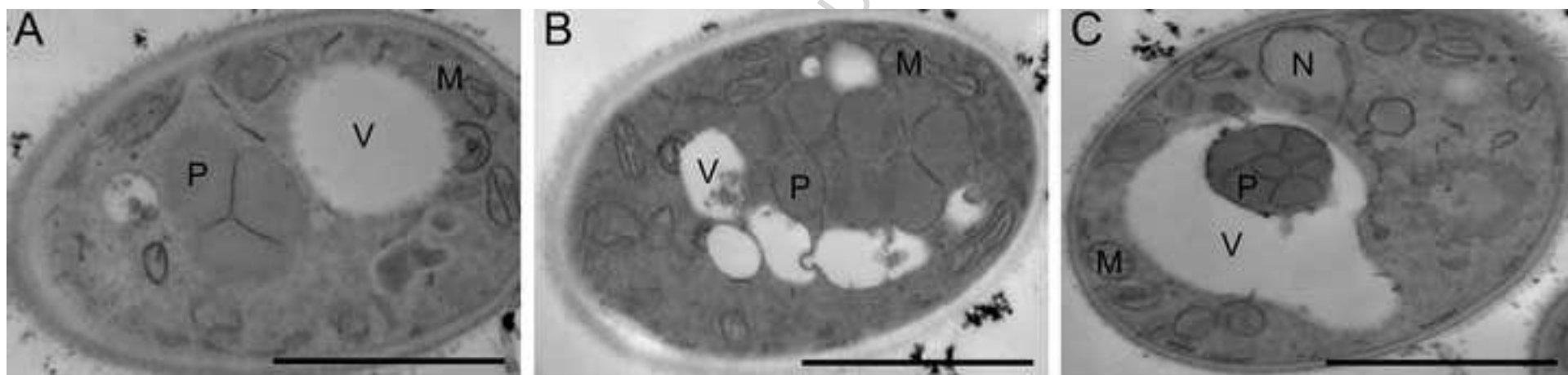


Fig.4

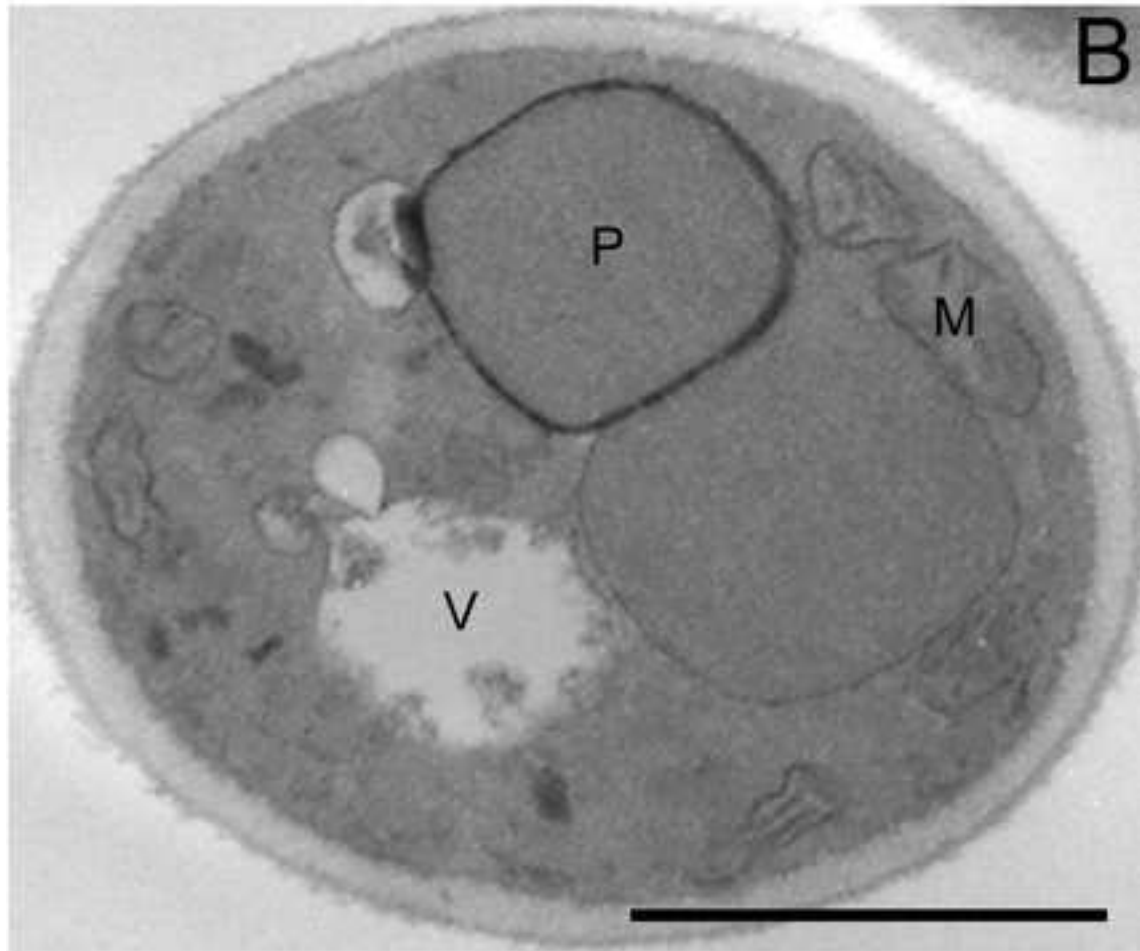
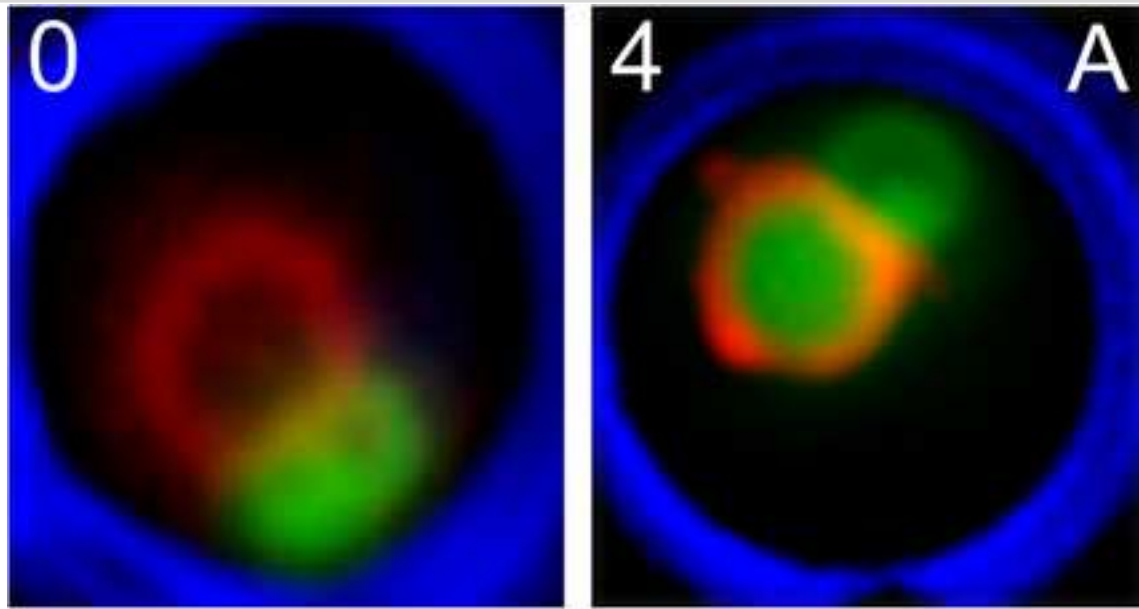


Fig.5

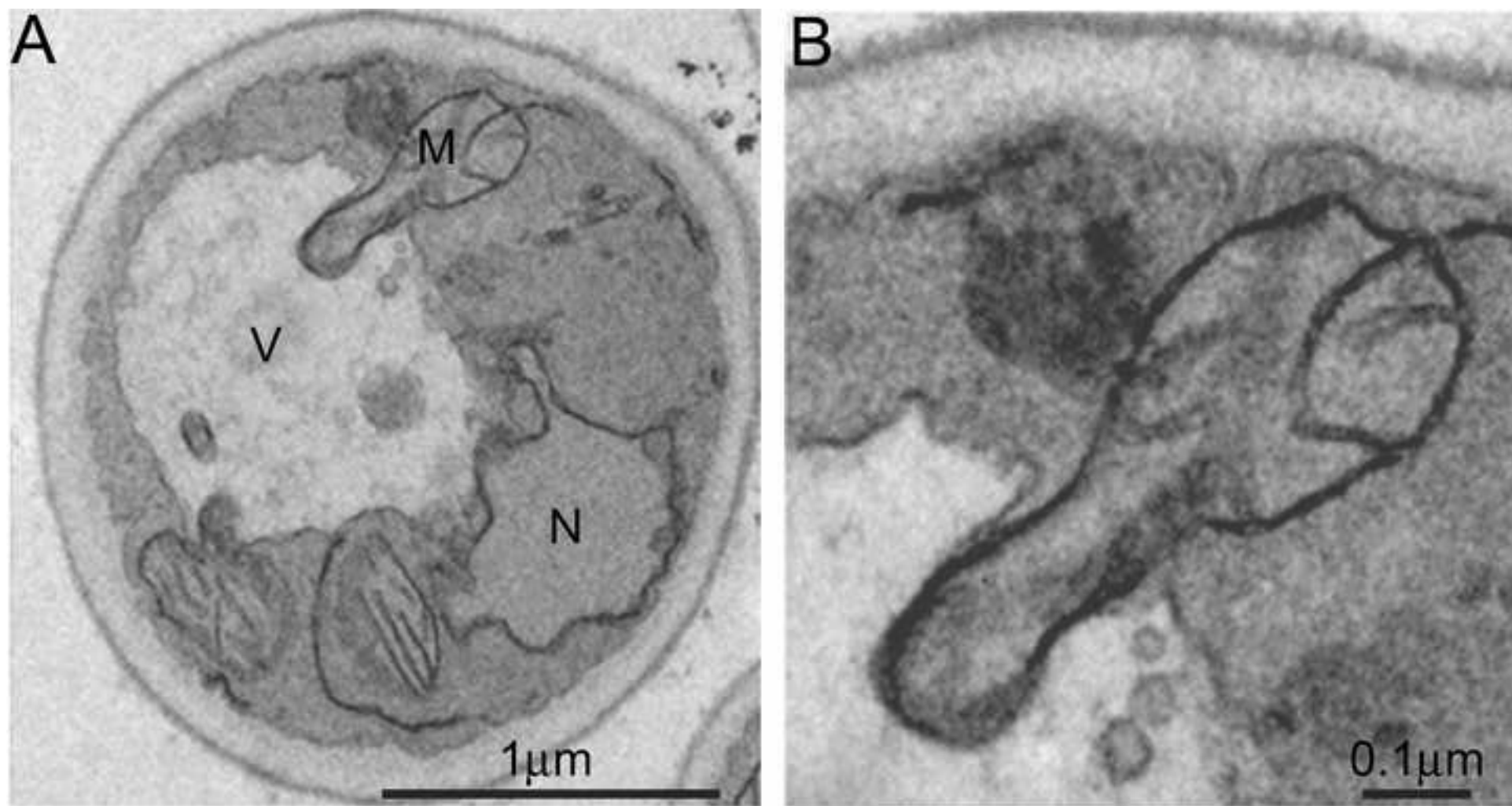


Fig.6