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Multiple roles of the cytoskeleton in autophagy

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ABSTRACT

Autophagy is involved in a wide range of physiological processes including cellular remodeling during development, immuno-protection against heterologous invaders and elimination of aberrant or obsolete cellular structures. This conserved degradation pathway also plays a key role in maintaining intracellular nutritional homeostasis and during starvation, for example, it is involved in the recycling of unnecessary cellular components to compensate for the limitation of nutrients. Autophagy is characterized by specific membrane rearrangements that culminate with the formation of large cytosolic double-membrane vesicles called autophagosomes. Autophagosomes sequester cytoplasmic material that is destined for degradation. Once completed, these vesicles dock and fuse with endosomes and/or lysosomes to deliver their contents into the hydrolytically active lumen of the latter organelle where, together with their cargoes, they are broken down into their basic components. Specific structures destined for degradation *via* autophagy are in many cases selectively targeted and sequestered into autophagosomes.

A number of factors required for autophagy have been identified, but numerous questions about the molecular mechanism of this pathway remain unanswered. For instance, it is unclear how membranes are recruited and assembled into autophagosomes. In addition, once completed, these vesicles are transported to cellular locations where endosomes and lysosomes are concentrated. The mechanism employed for this directed movement is not well understood. The cellular cytoskeleton is a large, highly dynamic cellular scaffold that has a crucial role in multiple processes, several of which involve membrane rearrangements and vesicle-mediated events. Relatively little is known about the roles of the cytoskeleton network in autophagy. Nevertheless, some recent studies have revealed the importance of cytoskeletal elements such as actin microfilaments and microtubules in specific aspects of autophagy. In this review, we will highlight the results of this work and discuss their implications, providing possible working models. In particular, we will first describe the findings obtained with the yeast *Saccharomyces cerevisiae*, for long the leading organism for the study of autophagy, and, successively, those attained in mammalian cells, to emphasize possible differences between eukaryotic organisms.

Key words: autophagy, autophagosome, Cvt pathway, actin, microtubules, cytoskeleton.

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I. INTRODUCTION

Macroautophagy, here referred to simply as autophagy, is a degradative pathway mostly implicated in the recycling of portions of cytosol and in the removal of superfluous or damaged organelles. In addition to proteins, this transport route is uniquely able to catabolize other cellular constituents such as lipids, carbohydrates and nucleic acids. This process occurs at a basal level in most tissues and contributes to the routine turnover of cytoplasmic components. However, it can also be massively induced by a change in the environmental conditions or by cytokines and other signaling molecules to adapt and/or cope with various physiological and pathological situations (Table 1). As a result, autophagy is important for cellular remodeling and development, and is involved in preventing ageing and controlling cell growth (Levine & Klionsky, 2004). Moreover, it plays a protective role in several human diseases such as cancer, neurodegeneration (Huntington's, Parkinson's and Alzheimer's diseases) and muscular disorders (Huang & Klionsky, 2007; Levine, 2007; Levine & Kroemer, 2008; Mizushima *et al.*, 2008; Shintani & Klionsky, 2004*a*). Autophagy also defends cells from invasion by certain pathogenic bacteria such as *Mycobacterium tuberculosis*, group A *Streptococcus* and *Staphylococcus aureus*, viruses such as the herpes simplex virus and the tobacco mosaic virus, and intracellular parasites like *Toxoplasma gondii* (Amano, Nakagawa & Yoshimori, 2006; Gutierrez *et al.*, 2004; Huang & Klionsky, 2007; Kirkegaard, Taylor & Jackson, 2004; Levine & Deretic, 2007; Levine & Kroemer, 2008; Nakagawa *et al.*, 2004; Yap, Ling & Zhao, 2007). In opposition to these cytoprotective roles, autophagy can also be detrimental in specific circumstances. For example, some cancer cells use this pathway to recover from radiation therapy (Levine, 2007; Paglin *et al.*, 2005) and various bacteria and viruses such as *Listeria monocytogenes*, *Shigella flexneri* and the poliovirus have evolved mechanisms to subvert autophagy for their own purposes (Birmingham, Higgins & Brumell, 2008; Mizushima *et al.*, 2008; Ogawa *et al.*, 2005; Taylor & Kirkegaard, 2008). Finally, autophagy

Table 1. Some of the roles of autophagy in health and disease.

Cellular process	Positive roles	Negative roles
Cell homeostasis	Viability and adaptation to stress conditions (starvation, high population density and elevated temperatures)	
Anti-ageing	Turnover of damaged mitochondria and consequent decrease in the cellular damage caused by free radicals leads to life-span extension	
Development and cell differentiation	Mediates cellular architectural changes by controlling cell growth and type II programmed cell death	
Innate and adaptive immunity	Cellular defence against intracellular bacteria and viruses, and antigen cross-presentation	Subversion of the autophagy machinery to establish a replicative niche
Cancer	Tumour suppressor by controlling cell growth and type II programmed cell death	Permits tumours to survive nutrient-limiting and low-oxygen conditions; protects some cancer cells against ionizing radiation
Neurodegenerative disorders	Facilitates the removal of toxic neuropeptides and micro-aggregates	
Cardiomyopathy	Protects during ischemia and pressure overload	Harmful during reperfusion
Liver diseases	Allows removal of misfolded proteins accumulated in the Endoplasmic reticulum	Increased mortality due to excessive mitochondrial autophagy

may be the central player of type II programmed cell death and in some cases appears to be regulated in conjunction with apoptosis (Gorski et al., 2003; Maiuri *et al.*, 2007).

Autophagy is conserved among all eukaryotes. Although this process was described at the morphological level in mammalian cells in the 1950s, researchers only recently have begun to gain insight into its molecular mechanism. The intracellular endomembrane system, including the endoplasmic reticulum (ER), Golgi complex, endosomes, lysosomes/vacuoles and plasma membrane, is maintained by dynamic membrane flow among various compartments. In general, these transport events involve vesicular budding from an existing donor organelle followed by fusion with an acceptor compartment. By contrast, autophagy employs unique membrane rearrangements distinct from any other intracellular processes (Reggiori, 2006). Nevertheless, similar to other intracellular trafficking events, autophagosome movement in mammalian cells employs microtubule-dependent machinery (Fass *et al.*, 2006; Jahreiss, Menzies & Rubinsztein, 2008; Köchl *et al.*, 2006).

A unique feature of autophagy that has lately emerged is that this pathway is able to specifically eliminate unwanted structures. This has led to sub-grouping autophagy into selective and nonselective types (Reggiori & Klionsky, 2005; van der Vaart, Mari & Reggiori, 2008). This process is defined as selective when a precise structure is specifically and exclusively eliminated, whereas it is considered nonselective when multiple different components are eliminated through a mechanism that appears to be random. Interestingly, actin filaments have been implicated in selective types of autophagy in the yeast *S. cerevisiae*, but they are dispensable for the bulk process in the same organism (Hamasaki *et al.*, 2005; He *et al.*, 2006; Monastyrska *et al.*, 2006; Reggiori *et al.*, 2005*a*).

These recent observations have provided evidence for the relevance of the cytoskeleton to specific aspects of autophagy. Herein, we review the body of experimental work that has led to these findings and to the discovery of possible molecular connections between the machinery involved in autophagy and cytoskeletal elements.

II. THE MOLECULAR MECHANISM OF AUTOPHAGY

(1) Autophagosome biogenesis

Autophagy is induced when eukaryotic cells are starved or, for example, when mammalian cells bind glucagon or cytokines such as the interferon-γ (IFNγ) and the tumor necrosis factor-α (TNFα) (Kondomerkos *et al.*, 2005; Yap *et al.*, 2007). The result is the simultaneous nucleation and expansion of cytoplasmic cisternae of unknown origin, termed phagophores or isolation membranes (Reggiori, 2006; Reggiori & Klionsky, 2005) (Fig. 1). The expansion is probably mediated through the acquisition of lipid bilayers by fusion with vesicles, whereas the molecular basis of the nucleation is still almost completely mysterious (Reggiori, 2006; Reggiori & Klionsky, 2005). In yeast, autophagosome biogenesis occurs at the phagophore assembly site or pre-autophagosomal structure (PAS). This site probably includes all the autophagosomal intermediates that first lead to the formation of the phagophore and successively to that of the autophagosome. Therefore, the PAS may be the actual vesicle precursor but it cannot be excluded that it may just organize and donate membranes to the expanding vesicle. The growing phagophore ultimately closes to become a double-membrane autophagosome (Fig. 1), which is different from the conventional, single-membrane transport vesicles that bud from a pre-existing organelle. In yeast, the mature autophagosome directly docks and fuses with the vacuole, allowing the release of its inner vesicle, the autophagic body, into the lumen of this organelle where it is degraded together with its cargo material (Fig. 1). Finally, the components resulting from the degradation of the autophagic bodies and their contents, e.g. amino acids, lipids and sugars, are transported back into the cytosol for re-use. The nature of the sequestration process is another unique characteristic of autophagy: the sequestered material is removed from the cytosol to the equivalent of the extracellular space, the lysosome/vacuole lumen. By contrast with most vesicle transport pathways that specifically preserve the topology of the cargo, autophagy results in its degradation.

In mammalian cells, there is sometimes an additional maturation step before these events; complete autophagosomes may first fuse with endosome- and *trans*-Golgi-network (TGN)-derived vesicles but also endosomes, to become amphisomes (Reggiori, 2006). The process of degradation then begins in the amphisomes and is completed in the lysosomes. Another difference from yeast is that the smaller size of the lysosome relative to the vacuole prevents the release of the autophagic body into the lysosome lumen.

(2) Selective types of autophagy

Autophagy has long been considered a bulk process with cytoplasmic structures being randomly sequestered into autophagosomes. However, there is an increasing number of examples of selective types of autophagy where a specific cargo destined for destruction is exclusively incorporated into an autophagosome. (Reggiori & Klionsky, 2005; van der Vaart *et al.*, 2008) (Table 2). For example, superfluous organelles such as peroxisomes or mitochondria can be specifically targeted for degradation, and the autophagic elimination of invasive bacteria appears to involve a selective mechanism. There may even be mechanisms for selecting particular cytosolic proteins during bulk autophagy (Ohshiro *et al.*, 2008; Onodera & Ohsumi, 2004). Although different cargos for selective types of autophagy have been described (Table 2), remains unknown how they are accurately recognized by autophagosomes (Table 2). One of the best-characterized examples is the cytoplasm to vacuole targeting (Cvt) pathway in yeast *S. cerevisiae* (Fig. 1). The principal cargo of the Cvt pathway is the precursor form of the resident vacuolar hydrolase aminopeptidase I (prApe1). Following delivery *via* an autophagosome-like vesicle, prApe1 is processed in the vacuole lumen into the

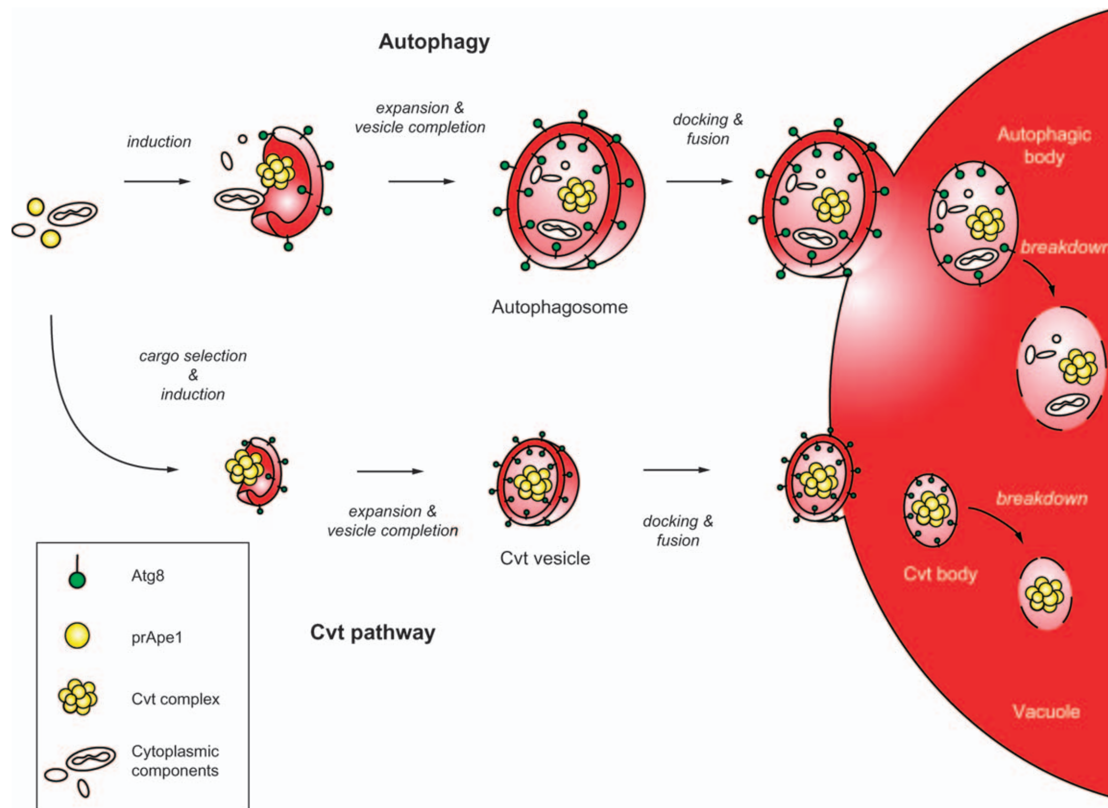

Fig. 1. The cytoplasm to vacuole targeting (Cvt) pathway and autophagy in yeast. Autophagy is induced upon starvation, and cytosolic components are randomly sequestered into autophagosomes. By contrast, the Cvt pathway, a selective type of autophagy, operates under vegetative conditions and is involved in delivering prApe1 oligomers into the vacuole using small double-membrane vesicles called Cvt vesicle. During starvation, the prApe1 oligomers are also selectively incorporated into autophagosomes. The biogenesis and subsequent clearance of the double-membrane vesicles can be divided into at least five discrete steps: induction, expansion, vesicle completion, docking and fusion, and breakdown. After fusion with the vacuole, the inner membrane vesicles referred to as an autophagic body or Cvt body, are released into the vacuole lumen where, together with their contents, they are degraded or processed by resident hydrolases.

active enzyme Ape1. This transport process can be divided into several steps similar to those occurring during autophagosome biogenesis (Fig. 1). After synthesis, prApe1 forms an oligomer in the cytosol, which then binds to the Atg19 receptor to form the Cvt complex. Subsequently, this complex associates with Atg11, and the latter protein mediates recruitment of the complex to the PAS. As a result, the Cvt complex is packed into double-membrane vesicles that are smaller than nonspecific autophagosomes and are termed Cvt vesicles. Cvt vesicles appear to exclude bulk cytoplasm, and instead are tightly apposed to the cargo. These vesicles then fuse with the vacuole and release prApe1 into the interior of this organelle where the zymogen is proteolytically processed into the mature active form of the enzyme (Yorimitsu & Klionsky, 2005*b*). The Cvt pathway seems to be present only in fungi and so far is the only reported example of a biosynthetic autophagy-related process. Nonetheless, the Cvt pathway shares most of the machinery utilized for bulk autophagy (see Section II.3), and morphologically and topologically these pathways also show great similarity (Shintani *et al.*, 2002; Shintani & Klionsky, 2004*b*). Another well-described example of a selective type of autophagy is pexophagy, the selective degradation of peroxisomes (Table 2). This process occurs in many organisms, ranging from unicellular eukaryotes to mammals, but it has been studied in most detail in methylotrophic yeasts such as *Pichia pastoris* and *Hansenula polymorpha* (Farre & Subramani, 2004). In these yeasts, peroxisome biogenesis is induced when cells are grown in the presence of methanol as a sole carbon source. When the cells are shifted to media containing preferred carbon sources such as glucose, peroxisomes become superfluous and are rapidly degraded *via* pexophagy (Farre *et al.*, 2008). Again, the machinery utilized by pexophagy overlaps to a great extent with that used for bulk autophagy (Dunn *et al.*, 2005; Hutchins, Veenhuis & Klionsky, 1999).

Importantly, and in contrast to the bulk process, selective types of autophagy possess an extra step during the formation of the double-membrane vesicle that allows the high fidelity selection of the cargo that has to be eliminated (Reggiori & Klionsky, 2005; van der Vaart *et al.*, 2008). This allows the exclusion of bulk cytosol from the interior of the double-membrane vesicles. For example, the selective import of the prApe1 oligomer *via* the Cvt pathway requires

Table 2. Different types of selective autophagy.

Name	Cargo	Organism	Reference
Cvt pathway	prApe1 and prAms1	yeast	Shintani *et al.* (2002)
pexophagy	peroxisomes	yeast and mammals	Farre *et al.* (2008)
mitophagy	mitochondria	yeast and mammals	Kim *et al.* (2007)
reticulophagy	ER	yeast and mammals	Bernales *et al.* (2006); Klionsky *et al.* (2007)
ribophagy	ribosomes	yeast	Kraft *et al.* (2008)
xenophagy	bacteria and viruses	mammals and plants	Levine (2005)
aggrephagy	protein aggregates	yeast, *D. melanogaster*, *C. elegans* and mammals	Overbye *et al.* (2007)

Cvt, cytoplasm to vacuole targeting; ER, endoplasmic reticulum; prApe1, precursor aminopeptidase 1; prAms1, precursor α-mannosidase.

Atg19, which serves as a receptor. The binding of Atg19 to prApe1 targets the Cvt complex to the PAS *via* its interaction with Atg11; the latter elicits the signal that triggers the PAS and Cvt vesicle formation (Shintani *et al.*, 2002; Shintani & Klionsky, 2004*b*). Recent studies in *Pichia pastoris* have unveiled a similar mechanism for pexophagy where Atg30 plays an equivalent role to Atg19 and, together with Atg11, mediates the recognition and selection of peroxisomes for elimination (Farre *et al.*, 2008). Another example of selective cargo recognition is in the disposal of cytoplasmic proteinacious aggregates by autophagosomes during aggrephagy. In several situations, this process involves p62, a protein that specifically interacts with ubiquitin and polyubiquitin chains attached to physiological and pathological aggregates and also to the pool of Atg8/microtubule-associated protein light chain 3 (LC3) present on the interior face of the forming autophagosome (Komatsu *et al.*, 2007; Pankiv *et al.*, 2007) (Section II.3). This dual binding capacity of p62 allows this protein to dictate specificity by effectively presenting ubiquitinated aggregates to double-membrane vesicles. Relatively little is known about the mechanism(s) involved in the recognition of invasive pathogens, but the overall process is presumably similar in nature, involving a surface epitope on the pathogen and one or more components of the autophagic machinery. For example, the VirG surface protein of *Shigella flexneri* appears to be recognized by Atg5 as a prelude to sequestration into autophagosomes (Ogawa *et al.*, 2005).

(3) The *AUTOPHAGY* genes

Genetic screens in *S. cerevisiae* and other fungi have led to the identification of a number of molecular factors essential for autophagy. There are currently over 30 genes that are primarily involved in bulk and selective types of autophagy, and they have been named autophagy-related genes (*ATG)* (Klionsky *et al.*, 2003). Fifteen of them compose the basic machinery required for the formation of double-membrane vesicles in all eukaryotes (Levine & Klionsky, 2004; Reggiori, 2006) (Table 3). The proteins they encode are recruited to the PAS in a temporal order and are involved in the formation and expansion of the PAS/phagophore (Cheong & Klionsky, 2008; Suzuki *et al.*, 2007). However, their specific function and the exact relationships among them are largely unknown. Here we will only very briefly mention what is known about the role of these fifteen key Atg proteins in double-membrane vesicle formation because numerous reviews are already available (Geng & Klionsky, 2008; Reggiori, 2006; Suzuki & Ohsumi, 2007; van der Vaart *et al.*, 2008; Xie & Klionsky, 2007; Yorimitsu & Klionsky, 2005*b*).

Some of the first Atg components to be found at the yeast PAS under autophagy-inducing conditions are the serine/threonine protein kinase Atg1 and its binding partners Atg13 and Atg17 (Cheong & Klionsky, 2008; Suzuki *et al.*, 2007). The Atg1-Atg13-Atg17 complex interacts with several proteins that are required exclusively for selective or nonselective types of autophagy, and therefore it is proposed that this complex governs the switch between the different modes of autophagy (Cheong & Klionsky, 2008; Kamada *et al.*, 2000; Reggiori *et al.*, 2004). A similar

Table 3. The 15 conserved autophagy-related gene (Atg) proteins involved in double-membrane vesicle formation (adapted from Reggiori, 2006).

Protein	Role	Interactions
Atg1	Serine/threonine protein kinase	Atg13, Atg11, Atg17
Atg2	Atg9 recycling	Atg9, Atg18
Atg3	Atg8 conjugation system (E2)	Atg7, Atg8, Atg12
Atg4	Cysteine protease	Atg8
Atg5	Atg12 conjugation system	Atg12, Atg16
Atg6	PtdIns-3-P synthesis	Atg14, Vps15, Vps34
Atg7	Atg8 and Atg12 conjugation systems (E1)	Atg3, Atg8, Atg10, Atg12
Atg8	Ubiquitin-like protein	Atg3, Atg4, Atg7, Atg19
Atg9	Transmembrane protein	Atg2, Atg18, Atg23, Atg27
Atg10	Atg12 conjugation system (E2)	Atg12
Atg12	Ubiquitin-like protein	Atg3, Atg5, Atg7, Atg10, Atg16
Atg13	Modulates Atg1 activity	Atg1, Atg17, Vac8
Atg14	PtsIns-3-P synthesis	Atg6, Vps15, Vps34
Atg16	Associates with the Atg12–Atg5 conjugate	Atg5, Atg12, Atg16
Atg18	PtdIns-3-P binding protein	Atg2, Atg9

PtdIns-3-P, phosphatidylinositol 3-phosphate; Vps, vacuolar protein sorting.

complex apparently exists in mammalian cells (Hara *et al.*, 2008). Atg9, the only transmembrane protein among the conserved basic machinery, is also one of the first factors localizing to the PAS (Suzuki *et al.*, 2007). In contrast to the rest of the Atg proteins that transiently localize to the forming autophagosomes, Atg9 shuttles between the PAS and several peripheral sites, some of which are in close proximity to the mitochondria (Reggiori *et al.*, 2005*b*). The Atg1-Atg13-Atg17 complex together with Atg18 and Atg2, are involved in the retrograde transport of Atg9 from the yeast PAS (Mari & Reggiori, 2007; Reggiori *et al.*, 2004). In mammals, Atg9 also cycles, but in this case between the TGN and endosomes; nonetheless this trafficking is regulated by the Atg1 orthologue unc-51-like kinase 1 (ULK1) (Young *et al.*, 2006). One of the functions of Atg9 appears to be the recruitment of the autophagy-specific phosphatidylinositol 3-kinase (AS-PI3K) complex to the PAS, which is composed of Atg14, Atg6, vacuolar protein sorting 15 (Vps15) and Vps34. The AS-PI3K complex generates the phosphatidylinositol 3-phosphate (PtdIns-3-P) crucial for the recruitment of additional Atg proteins to the PAS (Suzuki *et al.*, 2007). In yeast, PtdIns-3-P is also necessary for retrograde transport of Atg9 (Mari & Reggiori, 2007; Reggiori *et al.*, 2004). Because of the trafficking characteristics of Atg9 and its association with lipid bilayers, it is proposed that, in addition to initiating double-membrane vesicle biogenesis, Atg9 participates in the delivery of lipids necessary for the extension of the phagophore (Reggiori *et al.*, 2005*b*, 2004).

The two ubiquitin-like molecules Atg12 and Atg8 also seem to be involved in the recruitment of additional membranes to the PAS. Two highly conserved conjugation systems are important in this process (Geng & Klionsky, 2008; Ohsumi & Mizushima, 2004). In both yeast and mammals, Atg12 is covalently conjugated to Atg5 in a ubiquitin-like manner, which is mediated by the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg10. The newly formed Atg12–Atg5 conjugate then associates with Atg16 and this event appears crucial to trigger the expansion of the autophagosomal membrane and finally its fusion with the vacuole/lysosome (Mizushima *et al.*, 2001). Activation of the Atg12 conjugation system triggers the Atg8 conjugation system that directs the association of Atg8 to the PAS, after being conjugated to phosphatidylethanolamine (PE); Atg12–Atg5-Atg16 may function as an E3 ubiquitin ligase, and Atg16 appears to target the site of Atg8–PE formation (Fujita *et al.*, 2008; Hanada *et al.*, 2007). After synthesis, the C-terminus of Atg8 is cleaved by Atg4, a cysteine protease, exposing a C-terminal glycine residue. This cleaved form is conjugated to PE, mediated by the E1-like activating enzyme Atg7, and the E2-like conjugating enzyme Atg3. After double-membrane vesicle completion, the majority of the Atg proteins is released back into the cytoplasm and can be reused for additional rounds of vesicle formation. This includes the dissociation of the Atg8 bound to the external side of autophagosomes through a second cleavage by the Atg8-processing enzyme Atg4, which cleaves the lipid anchor, and the retrieval of Atg9. This uncoating event seems to be a prerequisite for fusion between autophagosomes and lysosomes/vacuoles. Importantly, a pool of Atg8 remains attached to the inner membrane of the autophagosome and is delivered into the lumen of the lysosome/vacuole, which makes it a reliable autophagic protein marker (Fig. 1). These two conjugation systems are highly conserved and are present in mammals (see Section IV).

III. THE CYTOSKELETON

The cytoskeleton is a network of elongated protein polymer fibres that support cell shape, compartmentalization and intracellular trafficking or even whole-cell movement. Microfilaments and microtubules are the two basic components that constitute the cytoskeletal system. Both are protein polymers that are constantly restructured in a tightly regulated manner in order to facilitate a dynamic spatial organization and rapid remodeling of the cytoskeleton (Pollard, 2003; Shih & Rothfield, 2006). Although there is a third distinct type of polymer fibres present in the cell known as intermediate filaments that are composed of many different cytoskeletal or nucleoskeletal proteins they are essentially static in structure and do not associate with molecular motors (Helfand, Chang & Goldman, 2004); in this review we focus mainly on microtubules and microfilaments.

(1) The microtubule network

Microtubules are a crucial cellular component because they are involved in cell division and differentiation, in the determination of cell shape, in chromosome segregation, in cytoplasm organization and in the positioning of organelles, and they are a structural element of flagella and cilia (Desai & Mitchison, 1997). Microtubules are tube-like structures composed of self-assembling $\alpha\beta$-tubulin heterodimers. To generate a microtubule, α- and β-tubulin monomers first heterodimerize and then assemble into protofilaments. Then, 12 to 15 of these linear protofilaments are joined to form a hollow cylinder structure with an approximate diameter of 25–30 nm (Fig. 2A). Successive polymerization of additional heterodimers onto this initial template structure leads to the assembling of the microtubule (Nogales, 1999). Because of the arrangement of the tubulin dimers within the microtubule, α-tubulins are exposed at one end while β-tubulins are exposed at the other. This ordered rearrangement gives the microtubule a structural polarity. The terminus exposing α-tubulins is termed the minus end and is anchored near the centre of a cell, whereas the edge exposing β-tubulins is the plus end and extends towards the cell surface (Fig. 2). Microtubule growth and disassembly occur at both ends. However, the plus end is the most dynamic extremity and therefore polymerizes and depolymerizes faster than the minus end.

Microtubules interconvert between periods of slow growth and fast shrinkage. In general though, a population of microtubules exhibits an overall bulk steady state, even if some of these structures are growing while others are

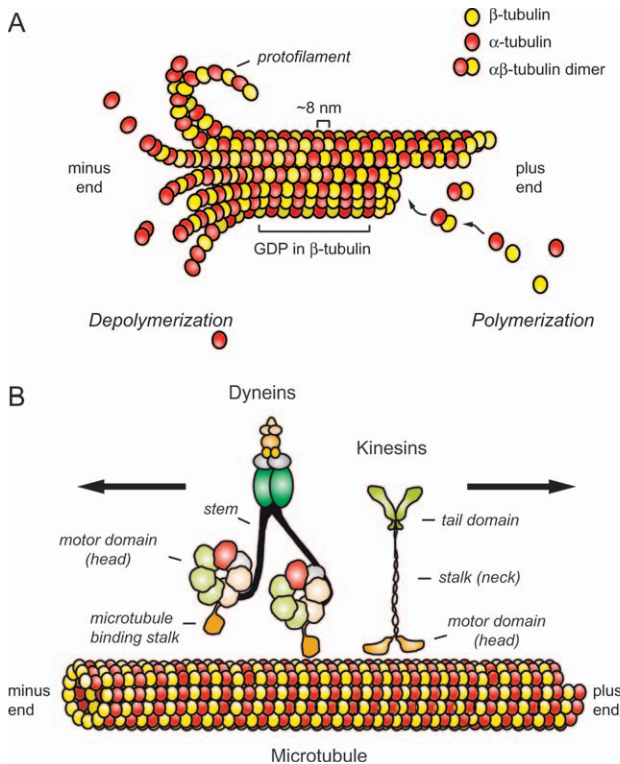

Fig. 2. Structure of microtubules, kinesins and dyneins. (A) Microtubules are tube-like structures composed of α- and β-tubulin heterodimers that are assembled into long protofilaments, which are assembled together to form a microtubule. One extremity of the microtubule is termed the minus end, the other is the plus end. During polymerization or growth, GTP-tubulin dimers are incorporated preferentially at the plus end. Subsequently, the GTP bound to β-tubulin is hydrolyzed into GDP, leading to a microtubule lattice that is principally composed of GDP-tubulin dimers. When GDP-tubulin becomes exposed at one of the microtubule extremities, the mechanical strain stored in the lattice is released and this triggers rapid depolymerization. (B) Kinesins and dyneins mediate movement along microtubules and both often form homodimers. In general, kinesins move the cargo toward the plus end whereas dyneins transport the cargo in the opposite direction. Kinesin and dynein are structurally very different. Kinesins contain a motor and a tail domain linked by a stalk region. Cytoplasmic dyneins are multi-subunit complexes with a motor domain consisting of six or seven AAA (ATPase associated with diverse cellular activities) domains arranged in a ring. Two lever arms protrude from this ring-shaped head; the cargo-engaging stem and the microtubule-binding stalk (modified from Mallik & Gross, 2004).

shrinking. A single microtubule never reaches a steady-state length, but persists in prolonged states of polymerization and depolymerization that interconvert infrequently. This phenomenon is referred to as dynamic instability and allows microtubules to adopt spatial arrangements that can change rapidly in response to cellular cues. The principal factor governing the rate of microtubule growth is the concentration of free GTP- and GDP-bound tubulin dimers floating in the surroundings of the microtubule extremities. Because GTP-tubulin dimers are more favorably incorporated, the newly formed microtubules initially consist of GTP-tubulin. The incorporation of GTP-tubulin dimers at the end of microtubules stimulates the GTPase activity of β-tubulin to hydrolyze the GTP bound to β-tubulin into GDP (Weisenberg & Deery, 1976). The α-tubulin also binds GTP, but it is bound in a non-exchangeable manner and is not hydrolyzed during polymerization. The conversion of GTP into GDP leads to a microtubule lattice that is predominantly composed of GDP-bound tubulin dimers (Fig. 2A). Importantly, the hydrolysis of GTP drives the conformational change of 'straight' GTP-bound tubulin dimers into 'curved' GDP-bound tubulin dimers. Because the GDP-bound tubulins are prevented from adopting the fully curved conformation while in the lattice, the energy generated from GTP hydrolysis is stored in the lattice as a mechanical strain. This strain is released only when GDP-tubulin is exposed at the microtubule ends and provides the driving force for rapid depolymerization or shrinkage of this structure (Amos, 2004; Muller-Reichert *et al.*, 1998) (Fig. 2A).

Cells possess a large variety of proteins that can modulate microtubule dynamics and they can be sub-grouped into microtubule-associated proteins (MAPs), destabilizing factors and nucleating factors (Amos & Schlieper, 2005). MAPs are proteins that bind, stabilize and promote the assembly of microtubules. Most MAPs are negatively regulated by kinases. Phosphorylation reduces their affinity for the microtubule lattice inhibiting their ability to stabilize them. Microtubule destabilizing factors, by contrast, have an opposite function; they destabilize microtubules by simultaneously reducing their assembly rate and accelerating their turnover. The precise mechanism by which these factors accomplish these results poorly understood. Nucleating factors are a third class of proteins that play a role in microtubule dynamics. In most eukaryotic cells, microtubules primarily nucleate in close proximity to the centrosome, whereas in fungi they do this adjacent to the spindle poles. The centrosome consists of a pair of centrioles surrounded by a complex collection of proteins known as the pericentriolar material (PCM). In higher eukaryotes, γ-tubulin, a third type of tubulin, localizes to the PCM and is part of a ring-shaped structure containing several other proteins known as the γ-Tubulin Ring Complex (γ-TuRC) (Goldstein & Philp, 1999). This complex is a nucleating factor that serves as a template for the microtubule lattice and stimulates microtubule nucleation (Amos, 2004; Desai & Mitchison, 1997).

Microtubules form a complex, interconnected network, which often serves as tracks for intracellular movement powered by specific motor proteins that are part of either the kinesin or dynein protein families (Brown, 1999). Most utilize the energy generated by ATP hydrolysis to translocate in a stepwise manner along the surface of the microtubules. In general, kinesins move cargo towards the plus end of microtubules, whereas dyneins are involved in movement towards the minus end (Gross, Vershinin & Shubeita, 2007; Wang, Khan & Sheetz, 1995) (Fig. 2B).

Kinesins moving along microtubules convey from the centre of the cell to its periphery a variety of cargos including vesicles, organelles and RNA. They also play an important role in the movement of chromosomes during

mitosis and meiosis. Next to their role in transport, some types of kinesins control microtubule polymerization and stability, whereas others are important for organizing the microtubular network by zippering, cross-linking and moving microtubules (Goldstein & Philp, 1999; Hunter & Wordeman, 2000). Most kinesins contain an N-terminal catalytic motor domain or head that directly interacts with the microtubule and hydrolyzes ATP, and a globular tail domain that provides the binding specificity for different cargoes, adaptor proteins and other motor proteins (Fig. 2B). The tail domain sometimes is also non-covalently associated with so-called kinesin light chains. The head and the tail are connected by a coiled-coil stalk or neck domain important for movement and control of direction (Fig. 2B). Kinesins often form dimeric units that are connected by the stalk region (Fig. 2B). It remains poorly understood how kinesins recognize the correct cargo and how this is delivered to the correct destination (Brown, 1999; Goldstein & Philp, 1999; Vale, 2003).

Dyneins, are structurally unrelated to kinesins and belong to the class of AAA (ATPase associated with diverse cellular activities) proteins. They can be classified into two subfamilies: cytoplasmic and axonemal dyneins (Mallik & Gross, 2004). In addition to the transport of intracellular cargos, cytoplasmic dyneins display a diverse range of functions: they play a key role in the orientation of the cell spindle during mitosis, nuclear migration and neuronal transport (Gibbons, 1996; Wang *et al.*, 1995). By contrast, axonemal dyneins are immobilized. They are not required to be progressive since they function as a large linear array of motors. In cilia and flagella, for example, adjacent microtubules slide over each other by the acting of opposite rows of axonemal dyneins positioned on their surface (Mallik & Gross, 2004). This movement generates the bending motion of cilia and flagella. Despite the difference in their cellular functions, cytoplasmic and axonemal dyneins have quite similar structures. They are multisubunit complexes composed of heavy, intermediate, light intermediate and light chains, and therefore are much larger than kinesins (Cross, 2004; Mallik & Gross, 2004). The dynein heavy chains possess motor domains that are much more complex than those of kinesins and consist of six or seven structurally related sub-domains, called the AAA domains, which are arranged in a ring (Fig. 2B). Two lever arms protrude from this ring-shaped head. One is called the 'stem' and in addition to engaging the cargo, it provides most of the force for the movement, while the other arm interacts with the microtubule track through a long microtubule-binding stalk (Gee, Heuser & Vallee, 1997) (Fig. 2B). The dynein motor domain contains multiple ATP binding sites that hydrolyze this nucleotide to generate the energy necessary for movement. Like kinesins, dyneins form homodimers (Fig. 2B) and multiple dynein homodimers can act together in the transport of a single cargo (McGrath, 2005).

(2) Actin filaments

Microfilaments, also known as actin filaments or filamentous actin (F-actin), are tube-like structures composed of long filamentous polymers. They consist of two coiled strands of chains of actin subunits also called globular actin (G-actin) (Winder & Ayscough, 2005) (Fig. 3A). The diameter of actin filaments at approximately 5 nm is much smaller than that of microtubules. In addition, they are significantly shorter than microtubules and their orientation throughout the cell is more random. Like microtubules, actin filaments are polar structures with two different extremities termed the barbed end and the pointed end (Winder & Ayscough, 2005). In general, microfilaments form *de novo* either from the side or the severing end of an existing filament. Under appropriate conditions, however, actin filaments can self-assemble. This event starts with a nucleation process consisting of three actin monomers assembling into an initial core. Further elongation of this

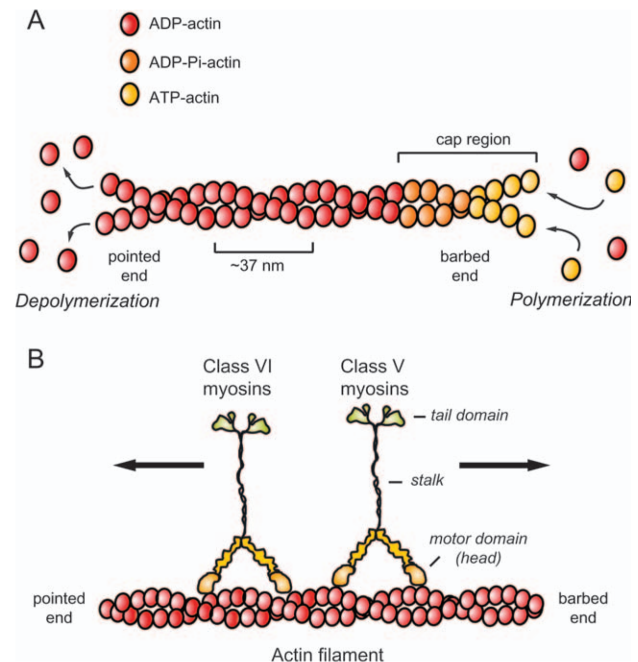

Fig. 3. The structure of actin filaments and myosins. (A) Actin filaments or microfilaments are composed of two coiled chains of actin monomers; one end is called the barbed or plus end, and the other is the pointed or minus end. Microfilament polymerization or growth mostly takes place at the barbed end by the addition of ATP- actin subunits. Hydrolysis of ATP bound to these monomers and the subsequent release of phosphate (Pi), results in actin filaments primarily composed of ADP-actin with a cap-region enriched in ATP-actin and ADP-Pi-actin. Disassembly or depolymerization of actin filaments primarily occurs at the pointed ends and releases ADP-actin subunits. (B) Motor proteins of the myosin superfamily mediate movement along microfilaments. Class V and VI myosins are the most well-studied members of this protein superfamily; they form homodimers and play a central role in vesicular transport. Class V myosins move towards the barbed end whereas class VI myosins traffic in the opposite direction. Myosins possess a motor domain important for displacement along microfilaments and a tail domain involved in cargo recognition. These two domains are connected by a coiled-coil stalk region (modified from Mallik & Gross, 2004).

nucleus by the addition of a multitude of actin subunits gives rise to a new microfilament.

Although actin filaments do not exhibit dynamic instability like microtubules, they are assembled and disassembled in a highly dynamic manner as well and the regulation of their rearrangements is important for processes such as intracellular trafficking, contractility, cell locomotion and cell division (Winder & Ayscough, 2005). Microfilament assembly and disassembly involves the addition and loss of actin subunits at both ends. Actin monomers can either bind ATP or ADP, but the ATP-bound monomers are preferentially added to the growing end of actin filaments (Fig. 3A). Incorporation into the microfilaments stimulates rapid hydrolysis of ATP, and the resulting ADP and phosphate (Pi) remain bound to the actin unit generating an ADP-Pi-actin intermediate form. In a second event, Pi is released resulting in long filaments primarily composed of ADP-actin with cap-regions composed of ATP- and ADP-Pi-actin (Fig. 3A). The hydrolysis of ATP is not required for actin assembly but it is a pre-requisite for actin dissociation from filaments and consequently it is important for the disassembly of these structures. Not much is known about the mechanism of Pi dissociation except that it causes a conformational change in the actin subunits that causes destabilization of the filament (Belmont *et al.*, 1999).

Polymerization mostly occurs at the barbed ends of the microfilaments, whereas disassembly principally takes place at the pointed ends (Winder & Ayscough, 2005) (Fig. 3A). The assembly of actin filaments depends on a critical concentration of free ATP-actin. The level of this critical concentration at the fast-growing barbed ends differs from that at the slow-growing pointed ends due to the difference in the ATP-actin and ADP-Pi-actin composition of the cap regions at these two extremities (Stukalin & Kolomeisky, 2006; Vavylonis, Yang & O'Shaughnessy, 2005) (Fig. 3A). Control of filament growth is necessary for polymerization to occur at specific times and places.

A wide range of actin binding and remodeling proteins including nucleation factors, monomer binding proteins, capping proteins, and stabilizing and destabilizing factors, govern the balance between assembly and disassembly that determines the filament growth rate (Cooper & Schafer, 2000; Winder & Ayscough, 2005). Nucleation factors such as formins and the actin-related protein 2/3 (Arp2/3) complex are crucial to initiate the formation of new filaments, which is otherwise energetically unfavorable. The Arp2/3 complex consists of seven subunits: Arp2, Arp3, Arc15/p15, Arc18/p18/p21, Arc19/p19, Arc35/p35 and Arc40/p40, which are all highly conserved among eukaryotes (Mahaffy & Pollard, 2006; Mullins & Pollard, 1999). This complex has multiple roles in the regulation of the actin cytoskeleton. It branches existing actin filaments by binding to their side and thus initiating the outgrowth of new filaments. In addition, it interacts with the barbed ends of microfilaments to initiate branching at this location and it is involved in the cross-linking of actin filaments. As mentioned above, the concentration of free actin monomers is crucial for filaments assembly. Certain monomer-binding proteins inhibit polymerization by sequestering away free actin subunits, whereas others stimulate the same process by facilitating the exchange of ADP for ATP. Capping proteins can modulate the assembly and disassembly of microfilaments as well. These factors, such as gelsolin, that bind to the barbed ends can stop filament growth by blocking the addition of new monomers, whereas those associating with the pointed ends reduce the loss of subunits and consequently control the rapid extension of filaments. Actin depolymerizing factors such as cofilin, actophorin, depactin and destrin mediate depolymerization in two ways. First, they can create more ends that disassemble by severing the microfilaments. Second, they can increase the rate of subunit loss from the filament termini by inducing the dissociation of the capping proteins present at pointed ends (Maciver & Hussey, 2002). Finally, actin stabilizing proteins carry out their function by binding along the side of actin filaments and protect them against spontaneous depolymerization and severing (Winder & Ayscough, 2005). In addition to all these regulatory factors, there are actin-bundling and cross-linking proteins that participate in the organization of the actin network but also proteins that are involved in interconnections between actin filaments and either membranes, membrane proteins or other cytoskeletal elements.

Microfilaments can serve as tracks for directed intracellular movement of various cargos and also entire organelles (Winder & Ayscough, 2005). Motor proteins of the myosin superfamily travel along microfilaments (Fig. 3B). All the members of this superfamily share a similar motor domain and a tail portion involved in cargo binding, which are connected to each other by a coiled-coil stalk region (Fig. 3B). Myosins are sub-grouped into approximately 15 classes based on the amino acid sequence of their motor domains. This domain is considerably larger than that of kinesins and it can contain one or more ATP-binding sites. Myosins are structurally related to kinesins and similarly, they also often form homodimers (Brown, 1999; Krendel & Mooseker, 2005) (Fig. 3B). Class V and VI myosins are among the most well-characterized classes and they have been shown to play a central role in vesicular transport along actin filaments. Class V myosins are responsible for movement towards the plus or barbed ends whereas class VI myosins transport cargos in the opposite direction (Brown, 1999; De La Cruz *et al.*, 1999; Wells *et al.*, 1999). Besides cargo transport, myosins can also have other cellular functions. For example, class II myosins and actin are the key components responsible for the contraction of muscles. Class I myosins, on the other hand, participate in motility functions such as endocytosis, polarized morphogenesis and cell migration. The class I myosin Myo5 for instance, facilitates the pinching of endocytic vesicles off the plasma membrane (Evangelista *et al.*, 2000; Girao, Geli & Idrissi, 2008).

IV. THE ROLE OF MICROTUBULES IN AUTOPHAGY

(1) Microtubules are unnecessary for yeast autophagy

The first possible connection between autophagy and microtubules emerged with the discovery that one of the genes specifically involved in autophagy and isolated

through genetic screens in yeast, *ATG8*, is homologous to the mammalian microtubule-associated protein 1 light chain 3, MAP1-LC3 or simply LC3 (28% identity to rat MAP1-LC3) (Lang *et al.*, 1998; Reggiori & Klionsky, 2002). MAP1-LC3 belongs to the protein family of MAPs and interacts with MAP1A or 1B to form a complex that binds and modulates the shape of microtubules (Mann & Hammarback, 1994; Pedrotti *et al.*, 1996). It has now been shown that identically to yeast Atg8 (see Section II.3), LC3 is immediately cleaved after synthesis by an Atg4 cysteine protease. This cleaved cytosolic LC3-I form is then conjugated to PE to form LC3–PE through the actions of E1- and E2-like enzymes. The lipidated form of LC3, called LC3-II, is tightly associated with the autophagosomal membrane and is involved in the expansion of the phagophore. Therefore, LC3 functions as an Atg8 orthologue. In humans, in addition to three LC3 isoforms (LC3A, LC3B, and LC3C), four additional Atg8 homologues have been identified: GABARAP, GEC1/GABARAPL1, GATE16/GABARAPL2, and GABARAPL3. It is unclear if these GABARAP proteins have a completely redundant function with the LC3 isoforms or a peculiar role in autophagy, but at least the lipidated forms of GABARAP and GATE16, co-localize with autophagosomes (Kabeya *et al.*, 2000, 2004; Tanida, Ueno & Kominami, 2004).

The first published work about *ATG8* showed that this gene is essential for autophagy because in its absence, cells are unable to accumulate autophagic bodies in the vacuole when starved in the presence of protease inhibitors (Lang *et al.*, 1998). Instead the same mutant amassed structures in the cytosol that were proposed to be autophagosome-like. Together with impaired maturation of prApe1, this observation suggested that the *atg8*Δ deletant is unable to deliver autophagosomes and prApe1 to the vacuole (Lang *et al.*, 1998). Based on these results and the fact that Atg8 interacts *in vitro* and by yeast two-hybrid assay with the tubulins Tub1 and Tub2 *via* Atg4, Lang *et al.* (1998) proposed that Atg8 and Atg4 form a complex that binds to microtubules. Moreover, they also hypothesized that this complex could function in the attachment of autophagosomes to microtubules mediating their targeting to the vacuole.

Successive reports have challenged the initial idea about the molecular function of Atg8 (Huang *et al.*, 2000; Kirisako *et al.*, 1999). In particular, *atg8*Δ strains are severely impaired in autophagy but they do not accumulate complete autophagosomes in the cytoplasm (Kirisako *et al.*, 1999). Instead, these cells are blocked in autophagosome formation. This finding is in agreement with a recent report showing that Atg8 is required for autophagosome formation because it is involved in membrane tethering and hemifusion (Nakatogawa, Ichimura & Ohsumi, 2007) and/or in phagophore expansion (Xie, Nair & Klionsky, 2008). Crucially, Kirisako *et al.* (1999) also revealed that treatment of cells with nocodazole, a chemical that disrupts microtubules, does not affect autophagy, demonstrating that microtubules are not required for bulk autophagy in yeast. This result is also supported by evidence that autophagy proceeds normally in the *tub2*Δ mutant (Kirisako *et al.*, 1999). The reason for this difference between the results described in the early report and the more recent ones is unclear but it cannot be excluded *a priori* that Atg8 could also have functions connected with microtubules that are distinct from its role in autophagy (Cali *et al.*, 2008; Sagiv *et al.*, 2000).

(2) Microtubule-dependent movement of autophagosomes in mammalian cells

More than a decade ago, pioneering studies indicated that in rat hepatocytes and kidney epithelial cells, disruption of the microtubule network using agents such as nocodazole and vinblastine that interfere with microtubule polymerization, blocks fusion of autophagosomes with late endosomes and lysosomes but not the biogenesis of these double-membrane vesicles (Aplin *et al.*, 1992; Seglen *et al.*, 1996). However, a number of more recent investigations have shown that in mammalian cells, the disruption of the microtubule network provokes a delay in autophagy rather than a complete block in this process (Fass *et al.*, 2006; Jahreiss *et al.*, 2008; Köchl *et al.*, 2006).

Data from two of these recent publications have made it evident that in addition to a role in fusion, microtubules also regulate and facilitate autophagosome formation (Fass *et al.*, 2006; Köchl *et al.*, 2006). In one of these studies, primary rat hepatocytes expressing green fluorescent protein (GFP)-LC3 were pre-treated with nocodazole and vinblastine before inducing autophagy by nitrogen starvation (Köchl *et al.*, 2006). The rate and magnitude of autophagosome biogenesis was quantified by measuring the lipidation of GFP-LC3 but also by the translocation of this fluorescent chimera into punctate structures representing autophagosomes. The results indicated that the formation of autophagosomes is facilitated by microtubules, but does not require them. Moreover, analysis of LC3-II turnover and of the overlap of GFP-LC3-positive vesicles with LysoTracker Red-positive late endosomes/lysosomes confirmed that intact microtubules contribute to the fusion of autophagosomes with late endosomes/lysosomes (Köchl *et al.*, 2006).

Fass *et al.* (2006) proposed that once completed, autophagosomes are linked to and transported along microtubules. They established a Chinese hamster ovary (CHO) cell line stably expressing GFP-LC3, and newly formed autophagosomes labeled with this fluorescent probe were imaged in living cells in the presence or absence of nocodazole. GFP-LC3-positive autophagosomes were concentrated at the minus ends of microtubules in a microtubule-dependent manner under all growth conditions. In addition, time-lapse video microscopy revealed that only mature autophagosomes but not phagophores associate with microtubules and move along these tracks (Fass *et al.*, 2006). These authors also investigated the dynamics of autophagosome formation and degradation in the same cells in the absence of intact microtubules. In contrast to the data published by Köchl *et al.* (2006), they showed that this component of the cytoskeleton is not essential for the targeting and fusion of autophagosomes with late endosomes/lysosomes. The discrepancy in these results

could be due to the different cell lines used in the two studies. Nevertheless, Fass *et al.* (2006) also found that microtubules facilitate autophagosome biogenesis because the formation of these large vesicles occurs to a significantly lower extent in the absence of intact microtubules.

A further study on the same issue concluded that microtubule dissolution simply delays the arrival of autophagosomes in the proximity of late endosomes and lysosomes preventing their efficient fusion with these organelles (Jahreiss *et al.*, 2008). Using fluorescence microscopy and live-cell imaging they found that in mammalian normal rat kidney (NRK) cells, the majority of late endosomes/lysosomes are concentrated at the perinuclear region around the microtubule-organizing centre (MTOC), while the autophagosomes are formed randomly at the periphery of the cell (Jahreiss *et al.*, 2008). Obviously, to be able to fuse with late endosomes/lysosomes, autophagosomes must be transported into their proximity. Jahreiss *et al.* (2006) determined that newly formed autophagosomes move bidirectionally along microtubules in live NRK cells but they finally concentrate in a similar way as late endosomes/lysosomes. The MTOC-directed movement of autophagosomes depends on microtubules; the disruption of the latter using nocodazole abolishes this centripetal conveyance (Jahreiss *et al.*, 2008). Similar results obtained using time-lapse microscopy, showed that autophagosomes are formed throughout the cytoplasm in cervical cancer HeLa cells and move to the cell centre in a microtubule-dependent manner (Kimura, Noda & Yoshimori, 2008).

Despite the different hypotheses about the exact role(s) of microtubules in autophagy, all the published studies agree that microtubules facilitate autophagosome trafficking. An obvious question, however, is how are microtubules connected to autophagosomes? An interesting hint comes from another study that revealed that autophagosomes are moved by dyneins along microtubule tracks *en route* to the lysosomes located near the MTOC (Ravikumar *et al.*, 2005). Interestingly, the functional loss of dynein has been linked to certain neurodegenerative disorders. *In vitro* studies have demonstrated that the loss of dynein leads to an impairment of the clearance of aggregate-prone proteins by autophagy and to increased levels of LC3-II, reflecting a defect in the fusion between autophagosomes and lysosomes (Ravikumar *et al.*, 2005). These data perfectly complement a previous investigation showing that although microtubule disruption by nocodazole inhibits aggregate formation, this treatment leads to an overall increase in aggregate formation due to an impairment of autophagosome-late endosome/lysosome fusion (Webb, Ravikumar & Rubinsztein, 2004).

These data have recently also been confirmed using live-cell imaging analyses that revealed that dynein is required for autophagosome trafficking along microtubules and this centripetal movement discontinues once the autophagosome reaches the microtubule-organizing centre (Jahreiss *et al.*, 2008). In particular, treatment of GFP-LC3-expressing NRK cells with the dynein ATPase adenosine deaminase inhibitor or with RNAi targeting the same molecule, caused an impairment of the trafficking of GFP-LC3-positive vesicles and decreased the fusion of these structures with late endosomes/lysosomes (Jahreiss *et al.*, 2008). The latter phenomenon is almost certainly a consequence of the role of dynein on the centripetal movement of autophagosomes as this event is probably the rate-limiting factor for the eventual fusion with perinuclearly located lysosomes (Jahreiss *et al.*, 2008). Kimura *et al.* (2008) analyzed the involvement of dynein in autophagosome trafficking using a different approach. HeLa cells stably expressing GFP-LC3 were microinjected with anti-dynein intermediate chain antibodies, which are known to impair dynein activity, before monitoring autophagosome trafficking using time-lapse microscopy. The rapid movements of GFP-LC3-positive autophagosomes were almost completely blocked (Kimura *et al.*, 2008).

How dynein interacts with autophagosomes is still unknown. One attractive possibility is that this protein directly or indirectly binds to LC3 (Fig. 4A). This hypothesis is supported by the observation that the trafficking of autophagosomes was abolished when HeLa cells were microinjected with antibodies against the LC3 N-terminus (Kimura *et al.*, 2008). In addition to an indirect interaction with microtubules *via* dynein (Fig. 4A), LC3 could bind to these structures in other ways, tightening the association of autophagosomes to them and resulting in facilitated movement. LC3 could directly associate with microtubules through its N-terminal domain or indirectly *via* MAP1A and MAP1B (Kouno *et al.*, 2005; Mann & Hammarback, 1994) (Fig. 4B). All these scenarios are not mutually exclusive. As suggested by Kimura *et al.* (2008), for example, the N-terminus of LC3 could play a dual role by both recruiting dynein to the autophagosomes and by acting as an adaptor protein between microtubules and these double-membrane vesicles (Fig. 4B).

V. THE ACTIN CYTOSKELETON AND AUTOPHAGY

(1) Actin is required for cargo selection during selective types of autophagy in yeast

Two different studies have revealed that actin filaments are not necessary for bulk autophagy in yeast. In particular, treatment of cells with latrunculin A (LatA), a chemical that blocks actin polymerization, does not affect the autophagy-mediated delivery into the vacuole of either the cytosolic protein marker Pho8Δ60 nor GFP-Atg8 (Hamasaki *et al.*, 2005; Reggiori *et al.*, 2005*a*). Analysis of the same process in *act1* mutants (*ACT1* is the gene that encodes for actin) has led to the same conclusion (Reggiori *et al.*, 2005*a*).

By contrast, accumulating evidence suggests that microfilaments are essential for selective types of autophagy in this unicellular eukaryote (Hamasaki *et al.*, 2005; He *et al.*, 2006; Monastyrska *et al.*, 2006; Reggiori *et al.*, 2005*a*). A substantial amount of progress has been made by studying the molecular mechanisms of the Cvt pathway (Yorimitsu & Klionsky, 2005*a*). As discussed previously (Section II, Fig. 1), by this transport route oligomers formed by prApe1 are delivered into the vacuole by Cvt vesicles. In addition to Atg19 and Atg11, actin filaments appear to be a crucial component of the machinery that guarantees that the

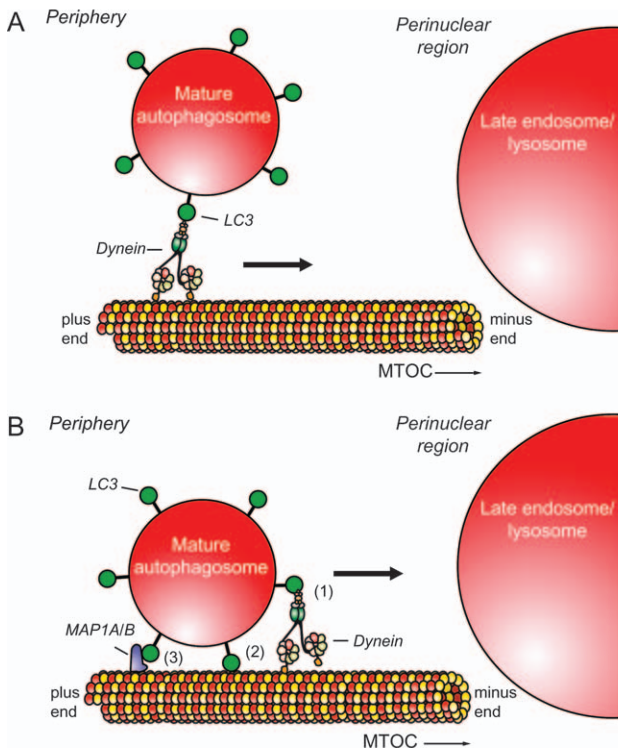

Fig. 4. Models for dynein–mediated trafficking of mammalian autophagosomes along microtubules. Microtubules and dynein play a crucial role in the centripetal movement of autophagosomes from peripheral locations in the cell to the microtubule-organizing centre (MTOC) where lysosomes are concentrated. (A) Microtubule-associated protein 1 light chain 3 (LC3) could provide the direct or indirect structural link that anchors autophagosomes to dynein, which then will carry these large vesicles along the microtubule tracks. (B) LC3 could bind directly to microtubules. Therefore, in addition to its function in binding dynein (1), the protein could also play a role as a direct (2) or indirect (3) adaptor between microtubules and autophagosomes, and, by increasing the affinity between these two structures, it could facilitate autophagosome trafficking. MAP, microtubule-associated protein.

prApe1 oligomers are specifically recognized and selectively packed into Cvt vesicles. Analyses of prApe1 processing by pulse-chase radiolabeling experiments in yeast cells grown in the presence of LatA or in mutant strains such as *act1-159* carrying specific point mutations in *ACT1*, showed a severe impairment in the Cvt pathway (Reggiori *et al.*, 2005*a*). This defect is caused by an inability to recruit the Cvt complex to the PAS in the absence of actin cables as revealed by either co-localization studies between cyan fluorescent protein (CFP)-Ape1 and yellow fluorescent protein (YFP)-Atg8 in LatA-treated cells, or protease-protection assays in the *act1-159* mutant (Reggiori *et al.*, 2005*a*). Importantly, this block is identical to that observed in the *atg11*Δ knockout (Kim *et al.*, 2001*b*). In the absence of Atg11, most of the Atg proteins fail to be recruited to the PAS, suggesting that this factor plays a crucial role in the organization of this specialized site under vegetative conditions (Shintani & Klionsky, 2004*a*). Atg8 is also not recruited to the PAS in the *act1-159* strain emphasizing further that microfilaments and Atg11 mediate the same step of the Cvt pathway (Reggiori *et al.*, 2005*a*).

Atg11 is a coiled-coil domain protein that interacts with several other Atg proteins, including Atg1, Atg9 and Atg19. Thus, it appears that Atg11 acts in part as a scaffold that dictates the recruitment of Atg proteins at the PAS, possibly coordinating the cargo with the vesicle-forming machinery (He *et al.*, 2006; Kim *et al.*, 2001*b*; Shintani *et al.*, 2002; Yorimitsu & Klionsky, 2005*a*). As noted in Section II, Atg9 is an integral membrane protein required for autophagy. Atg9 binds to Atg11 independently from Atg19 (He *et al.*, 2006). Atg9 has a quite distinctive intracellular distribution; unlike most Atg proteins that, when associated with membranes, localize primarily at the PAS, this protein localizes to this site plus several other cytoplasmic punctate structures. Atg9 shuttles between these peripheral sites and the PAS (Reggiori *et al.*, 2005*b*) (Section II). Interestingly, Atg9 delivery to the PAS is blocked in the absence of Atg11 as well as in the presence of LatA or the *act1-159* mutation (Reggiori *et al.*, 2005*a*) indicating that transport of Atg9 and the Cvt complex to the PAS is coordinated.

An interesting question is how Atg11 and actin filaments interact at a molecular level in order to mediate this coordinated movement. What is known is that in the *act1-159* mutant Atg11 is no longer detected on the PAS, underlying a possible connection between the movement of this protein and actin filaments (He *et al.*, 2006). An intriguing speculation arising from a structural comparison between Atg11 and Myo2, one of the two yeast myosin V proteins, highlighted that the third coiled-coil domain of Atg11 displays some similarity with that of Myo2 (Monastyrska *et al.*, 2006). It is still unknown, however, if Atg11 can bind actin filaments. This protein does not possess a motor domain and consequently it cannot move the Cvt complex along the actin cable by itself. One possibility could be that it associates with myosins or an unknown protein that possesses a similar motor activity. An alternative hypothesis emerged from a recent study in which it was shown that the Arp2/3 complex also plays an essential role in the Cvt pathway (Monastyrska *et al.*, 2008). Strains carrying temperature-sensitive mutations in genes encoding for Arp2/3 complex subunits display a strong defect in prApe1 transport (Monastyrska *et al.*, 2008).This study also revealed that Atg9 transport to the PAS is defective in the *arp2-1* mutant and that Arp2 briefly co-localizes with Atg9 at the peripheral sites. Importantly, using the yeast two-hybrid-based assay and co-immunoprecipitation experiments, they demonstrated that Atg9 interacts with the Arp2/3 complex *via* Atg11. This result provides a possible molecular link between actin filaments, Atg11, Atg9 and the Cvt complex, but also suggests potential models for the microfilament-dependent movement of these factors.

One attractive hypothesis could be that binding of the Arp2/3 complex to the Cvt complex and/or Atg9-containing structures induces actin nucleation leading to the synthesis of new actin filaments (Fig. 5A). The adjacent growth of these actin filaments could provide the force required for the directional transport of the Cvt complex and Atg9 to the PAS. This model has already been proposed

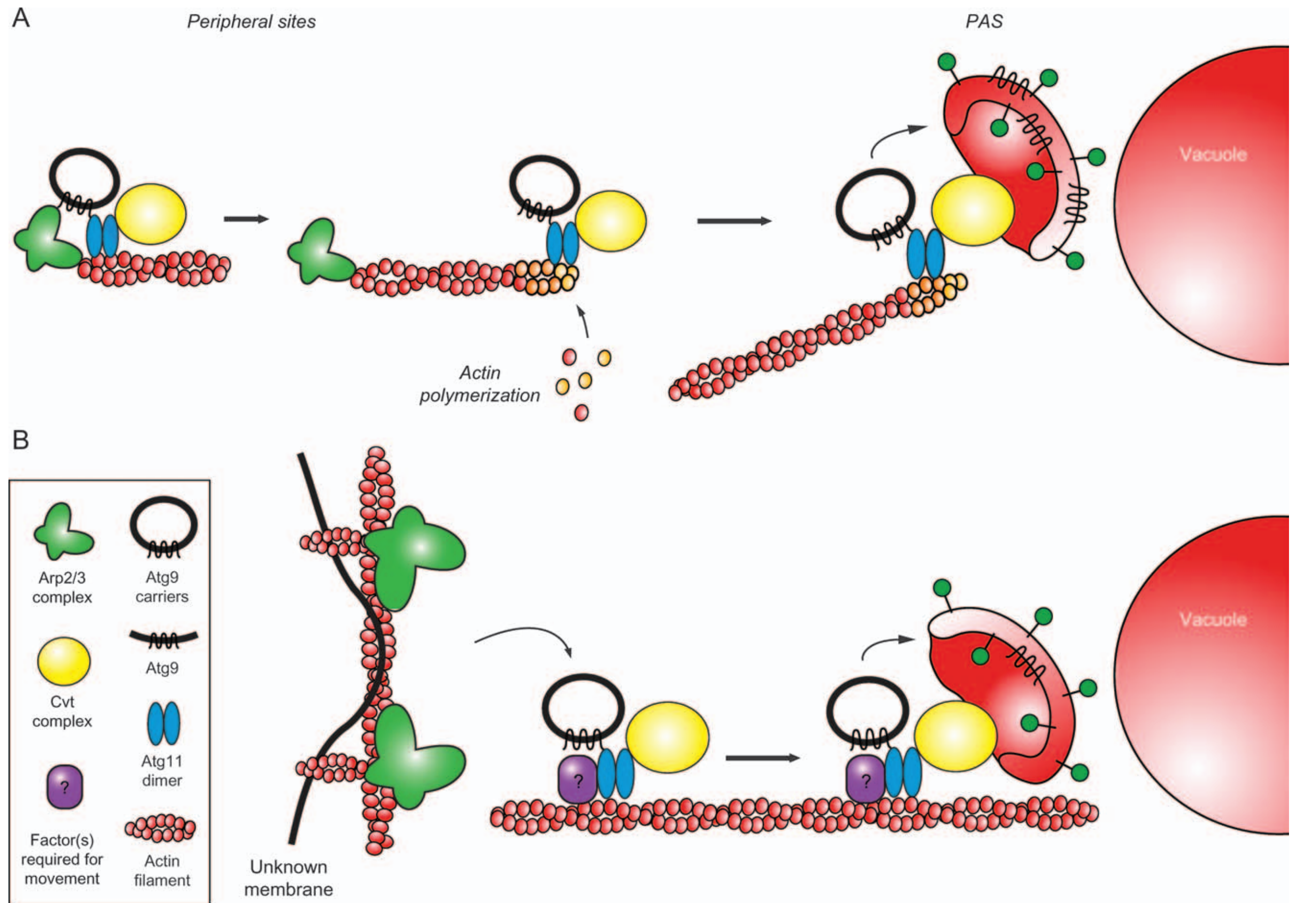

Fig. 5. Models for the role of actin filaments and the actin-related protein 2/3 (Arp2/3) complex in the cytoplasm to vacuole (Cvt) pathway. (A) Atg11 recruits and brings together the Cvt complex, Atg9 carriers and the Arp2/3 complex at a peripheral site in the cells. This event activates the Arp2/3 complex, which in turn induces actin nucleation. The polymerization of several actin cables and the consequent formation of new adjacent microfilaments drive the movement of Atg9 and the Cvt complex to the phagophore assembly site (PAS). In this model, Atg11 could also be involved in binding to the actin cable. (B) The Arp2/3 complex and actin filaments are essential for the formation of Atg9-containing carriers from a yet unknown membrane source. Next, Atg11 together with some unknown factor(s) takes over and transports these Atg9-containing structures and the Cvt complex to the PAS along the actin microfilaments. The models illustrated in A and B could co-exist in which case, the Arp2/3 complex would mediate the biogenesis of Atg9 carriers and their delivery plus that of the Cvt complex to the PAS.

for the Arp2/3 complex- and actin-dependent motility of yeast mitochondria or certain intracellular pathogens (Boldogh *et al.*, 2001; Gouin, Welch & Cossart, 2005). In this model, in addition to assembling all the different travelling partners, Atg11 could play a role in their stable association with actin cables (Fig. 5A). It cannot be excluded, however, that the Arp2/3 complex has a different function in the Cvt pathway. Its presence at the peripheral sites could initiate the formation of Atg9-containing carriers from an unknown membrane source by inducing actin polymerization, before Atg11 takes over and transports the Atg9 carriers together with the Cvt complex to the PAS along the actin cables (Fig. 5B). A similar function has been assigned to actin and to the Arp2/3 complex during membrane invagination occurring at the plasma membrane, which is required for the formation of endocytic vesicles (Kaksonen, Sun & Drubin, 2003). In this model, another unknown factor would then be required to act as a motor to push the Cvt complex and Atg9 toward the PAS. It is also possible that aspects of the two models coexist (Fig. 5) and the Arp2/3 complex mediates both the biogenesis of the Atg9 carriers and transport along microfilaments.

Importantly, actin filaments also seem to play a crucial role in other selective types of autophagy in yeast, in particular during the specific removal of superfluous organelles such as peroxisomes and ER. When yeast cells are grown in conditions that require peroxisome functions, these organelles proliferate. Once peroxisomes become unnecessary, they are selectively eliminated *via* a process called pexophagy (Hutchins *et al.*, 1999) (Section II and Table 2). In analogy to the Cvt pathway, when pexophagy is induced, peroxisomes presumably have to be specifically recruited to the PAS in order to be efficiently and selectively enwrapped by the emerging double-membrane vesicles. Importantly, after disruption of actin with LatA or in the

actin point mutant *act1-159*, peroxisome degradation is blocked, possibly due to an inability to target it specifically to the PAS. It is important to note that both Atg11 and the Arp2/3 complex are also essential for pexophagy (Kim, Huang & Klionsky, 2001*a*; Monastyrska *et al.*, 2008).

Interestingly and in contrast to delivery of prApe1 to the vacuole, the selective uptake of peroxisomes required intact actin filaments even in starvation conditions when autophagy is active (Reggiori *et al.*, 2005*a*). This observation could indicate that if specific structures are preferentially degraded during bulk autophagy, their selective elimination would also need the presence of actin cables. This hypothesis is sustained by an investigation that has shown that the uptake of ER fragments into autophagosomes during starvation is microfilament-dependent (Hamasaki *et al.*, 2005). When autophagy is induced in yeast cells by rapamycin or upon starvation, part of the ER fragments and the resulting mini-cisternae are transported together with other cytoplasmic components into the vacuole lumen by autophagosomes. When autophagy is triggered in cells pre-treated with LatA, however, delivery of ER fragments into the vacuole is perturbed, whereas that of the autophagosomal protein marker GFP-Atg8 is not. Consequently, this result confirms that bulk autophagy is not blocked upon disruption of the actin cytoskeleton but ER fragments escape engulfment by autophagosomes. An attractive hypothesis then is that disruption of the actin network interferes directly with the recognition and/or the sequestration of ER fragments by autophagosomes. Although the morphology of the ER network was almost the same in LatA-treated cells as in untreated cells, a possibility that cannot be excluded yet is that LatA affects the proper dynamics of this organelle and thus alters the fragmentation of this compartment essential for its incorporation into double-membrane vesicles (Hamasaki *et al.*, 2005; Prinz *et al.*, 2000).

(2) The role of the actin cytoskeleton in mammalian cells

Very little is known about the relationship between the actin cytoskeleton and autophagy in higher eukaryotes. In contrast to the findings that have shown that actin cables are dispensable for bulk autophagy in yeast (Reggiori *et al.*, 2005*a*), an electron microscopy study performed almost 20 years ago in rat kidney epithelial cells has shown that microfilament depolymerizing agents such as cytochalasins B and D block the formation of autophagosomes (Aplin *et al.*, 1992). Their data, however, have to be carefully interpreted. Rat kidney cells were incubated for 5 h in the presence of cytochalasins which provoked dramatic morphological changes as well as other effects. Therefore, it cannot be excluded that the detected block in autophagy is caused indirectly by the impairment of one or more other pathways.

VI. CONCLUSIONS

(1) The involvement of microtubules in the formation and fusion of autophagosomes with late endosomes/lysosomes in mammals has been under considerable debate. Microtubules appeared to be required for the fusion of autophagosomes with late endosomes/lysosomes, but not for the biogenesis of these double-membrane vesicles. However, some recent studies suggest that these cytoskeletal structures also play a role in autophagosome biogenesis while another report showed that microtubules are not essential for targeting and fusion events in CHO cells. Such discrepancies could be due to the use of different cell lines; autophagy could proceed at least partially in a tissue-specific way or perhaps microtubule-dependent biogenesis and trafficking of autophagosomes is more critical in some cell types. Another explanation for the reported differences could be variation in experimental conditions.

(2) Despite the different hypotheses about the exact role of microtubules in autophagy, it is clear that they facilitate autophagosome trafficking. Autophagosomes are formed at the periphery of the cell and move along microtubule tracks toward the lysosomes concentrated near the MTOC. Dynein is involved in this movement, and although the precise mechanism is not known, LC3 could form a link between these two structures; this protein is also important for the trafficking of autophagosomes (Fig. 4A). In addition, LC3 could increase the affinity of autophagosomes for microtubules *via* its ability to bind directly or indirectly to them (Fig. 4B).

(3) In contrast to mammalian cells, in yeast microtubules appear to be unnecessary for both the formation of autophagosomes and their fusion with the vacuole. The yeast PAS is adjacent to the vacuole and therefore, once complete, an autophagosome does not need to travel far to reach and fuse with this large hydrolytic compartment. A difference in the function or properties of Atg8 and LC3 could also underlie this distinction between eukaryotes. Even though Atg8 shows 28% homology to the mammalian MAP1-LC3A, Atg8 does not interact directly with tubulin, whereas LC3 can bind directly to microtubules. Therefore, LC3 may have additional functions in mammalian autophagy that are absent for Atg8 in yeast. Surprisingly, the role of microtubules in selective types of yeast autophagy has not been investigated.

(4) Studies in yeast have revealed that microfilaments are not required for bulk autophagy but are essential for selective types of autophagy such as the Cvt pathway, pexophagy and possibly reticulophagy. Actin filaments and the Arp2/3 complex together with Atg11 are crucial for the coordinated movement of the Cvt complex and Atg9-containing membranes to the PAS, an event essential to trigger autophagosome biogenesis . It is largely unknown how these three factors interact at a molecular level, but it is possible that Atg11 binds both the Cvt complex and Atg9, and also the Arp2/3 complex, which in turn is able to associate with microfilaments. How this putative complex move toward the PAS is a complete mystery due to the apparent lack of involvement of a motor protein.

(5) Little is known about the relationship between the actin cytoskeleton and autophagy in mammalian cells. Actin filaments have been considered essential for the initial formation of autophagosomes in starved cells. However, more recent works reported no association of GFP-LC3-labeled

autophagosomes with actin filaments. To date, there are no published studies investigating the role of actin filaments or microtubules in selective types of autophagy in mammals, due to the fact that simple assays to measure quantitatively selective types of autophagy in mammalian cells do not exist.

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