FEBS Letters 589 (2015) 1546–1551

FEBS Letters

journal homepage: www.FEBSLetters.org

Review

Function of human WIPI proteins in autophagosomal rejuvenation of endomembranes?

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ARTICLE INFO

Article history:
Received 30 March 2015
Revised 30 April 2015
Accepted 5 May 2015
Available online 14 May 2015

Edited by Wilhelm Just

Keywords:
Autophagy
Phosphatidylinositol 3-phosphate
WIPI
WIPI1
WIPI2

ABSTRACT

Despite the availability of a large pool of experimental approaches and hypothetical considerations, the hunt for the enigmatic membrane origin of autophagosomes is still on. In mammalian cells proposed scenarios for the formation of the autophagosomal membrane include both de novo assembly, and rearrangements plus maturation of pre-existing membrane sections from the endoplasmic reticulum (ER), plasma membrane, Golgi or mitochondria. Earlier, we identified the human WD-repeat protein interacting with phosphoinositides (WIPI) family and showed that WIPI proteins function as essential phosphatidylinositol 3-phosphate (PtdIns3*P*) effectors at the nascent autophagosome. Interestingly, WIPI proteins localize to both pre-existing endomembranes and nascent autophagosomes. In this context, and on the basis of historical records on the formation of autophagosomes, we discuss with appropriate modesty an alternative perspective on the membrane origin of autophagosomes.

1. Introduction

Conserved across eukaryotes, autophagy is an essential survival mechanism to compensate periods of starvation and to secure cellular homeostasis. Moreover, autophagy is considered a pivotal process in determining the lifespan of eukaryotic organisms and to critically counteract the onset of age-related human pathologies [1]. Given the excitement and importance to understand autophagy in health and disease, molecular details of the process of autophagy are increasingly available. However, comprehensive understanding of autophagy is hindered by one of the biggest missing piece of the puzzle, the membrane origin of autophagosomes. This important issue has been a matter of intense debate for decades [2,3].

The process of autophagy is defined as an ancient catabolic process for the degradation of cellular constituents, including organelles, proteins and lipids, with the three distinguishable forms macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) [1]. A hallmark for macroautophagy is the formation of double-membraned vesicles, termed autophagosomes, which deliver their sequestered material to the lysosomal compartment (Fig. 1A). In contrast, both microautophagy and CMA directly transfer cytoplasmic material and proteins into the lysosome without forming autophagosomes.

Macroautophagy, usually referred to as autophagy and also hereafter, occurs on a constitutive, tissue-specific basal level and is therefore considered to clear the cytoplasm by acting stochastically in bulk. Additionally, cellular stress such as nutrient starvation, induce the autophagic activity above basal level. In this context it has been recognized that a variety of different cellular targets are subjected to the specific degradation through autophagy, including protein aggregates and damaged organelles. Hence a variety of specific terms for selective autophagic processes have been implemented, such as mitophagy, ER-phagy, lipophagy, ribophagy and xenophagy [4]. Currently, experimental emphasis is concentrated on the further identification of cargo material of autophagosomes and the underlying mechanisms for targeting specific cytoplasmic components for autophagic destruction [5]. Also, attention is directed toward the identification of membrane sources and membrane forming mechanisms to build the autophagosomal vesicle [3].

2. The process of autophagy

The process of autophagosome formation is initiated upon ER-localized PtdIns3*P* production, positively regulated by energy-sensing AMP-activated kinase (AMPK) and negatively

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http://dx.doi.org/10.1016/j.febslet.2015.05.008

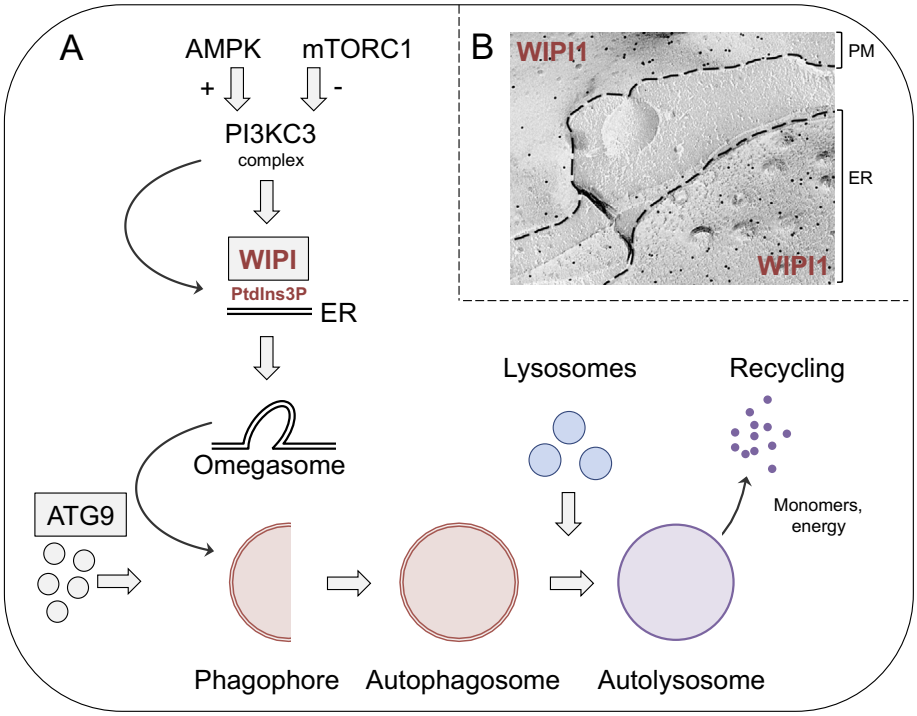

Fig. 1. The process of autophagy. (A) Autophagy is a lysosomal degradation mechanism that provides monomers and energy for anabolic processes, in particular in times of starvation. Under nutrient-rich conditions autophagy is inhibited by mTORC1 (minus symbol), because ULK1 (not shown) is phosphorylated at inhibitory sites. Under nutrient starvation, mTORC1 is not active and unable to inhibit autophagy via ULK1. Energy deficiencies are compensated by elevating autophagy through AMPK-mediated phosphorylation of ULK1 (not shown, indicated by the minus symbol). As a consequence, PtdIns3*P* is produced at the ER by the activity of PI3KC3 complex, composed by VPS34, VPS15, Beclin 1 and ATG14 (not shown). Rapidly, WIPI proteins (demonstrated for WIPI1 and WIPI2) accumulate at the ER, which arranges to dynamic omegasome structures that serve as cradle to form the autophagosomal precursor, the phagophore. The membrane origin for the phagophore is unknown, but endosomes carrying ATG9 are considered to feed phagophore formation. WIPI1 and WIPI2 become membrane proteins of both phagophore and autophagosome (not shown). At the phagophore, WIPI2 mediates the recruitment of the ATG16L complex for LC3 lipidation (not shown). LC3 lipidation drives cargo selection and the expansion of the phagophore to form the autophagosome with a double membrane. Autophagosomes fuse with lysosomes for cargo breakdown. Monomers are released to the cytoplasm for recycling purposes, energy is gained. (B) Upon starvation-induced autophagy WIPI1 localizes to both the ER, in particular the nuclear envelope, and also the plasma membrane (PM). Black dots within ER and PM indicate the labeling of GFP–WIPI1 in human U2OS cells by anti-GFP freeze fracture immuno-EM. The image is reproduced from Proikas-Cezanne and Robenek, 2011, under the terms of the Creative Commons Attribution License (© 2011 The Authors Journal compilation, © 2011 Foundation for Cellular and Molecular Medicine/Blackwell Publishing Ltd.). The schematic drawing of the omegasome was obtained from Motifolio.

controlled by nutrient-sensing mammalian target of rapamycin complex 1 (mTORC1) (Fig. 1A).

Energy-deficiency promotes AMPK to phosphorylate activatory sites of the serine/threonine-specific protein kinase ULK1 (UNC51-like kinase 1) that functions together with FIP200 (focal adhesion kinase family interacting protein of 200 kDa) in a higher order protein complex. AMPK activity is regulated by liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ). Nutrient-deficiency liberates the process of autophagy from being repressed by mTORC1, consisting of the serine/threonine-specific protein kinase mTOR (mammalian target of rapamycin), RAPTOR (regulatory-associated protein of mTOR), PRAS (40 kDa proline-rich AKT substrate), mLST8 (mammalian lethal with SEC13 protein 8) and DEPTOR (DEP domain containing mTOR interacting protein). Insulin/IGF (insulin-like growth factor) receptor signaling via AKT (also called protein kinase B), as well as amino acids activate mTORC1 to promote anabolic processes including protein synthesis. Thereby, catabolic processes including autophagy are inhibited. Following mTORC1 activation, mTOR phosphorylates ULK1 at inhibitory phosphorylation sites and represses PtdIns3*P* production for autophagosome formation [6].

The ULK1 complex probably promotes PtdIns3*P* production by phosphorylating Beclin 1, a regulatory subunit of the phosphatidylinositol 3-kinase class 3 (PI3KC3) complex, that also associates vacuolar protein sorting-associated protein 34 (VPS34), vacuolar protein sorting-associated protein 15 (VPS15) and autophagy-related 14 (ATG14). Through the ATG14 subunit, this PI3KC3 complex localizes to the cytoplasmic site of the ER. Subsequently, PI3KC3 produces a pool of PtdIns3*P* which becomes the entrance signal for autophagosome formation [3]. ER-accumulated PtdIns3*P* recruits WIPI proteins that specifically bind to PtdIns3*P* and decode the PtdIns3*P* signal to permit the formation of an autophagosomal precursor membrane [7], the origin of which is unknown. Subsequently, the ATG16L complex is recruited to permit the conjugation of microtubule-associated protein 1A/1B-light chain 3 (LC3) to phosphatidylethanolamine (PE), referred to as LC3 lipidation, for specific cargo selection and hemifusion events that elongate the autophagosomal precursor to a double-membraned autophagosome [5]. The autophagosome fuses with lysosomes and the sequestered cargo becomes degraded for subsequent recycling and storage purposes (Fig. 1A).

Essential for the formation of autophagosomal precursor membranes are Golgi-derived vesicles harboring the integral transmembrane protein ATG9 (Fig. 1A) [8]. ATG9-positive vesicles localize close to the Golgi and to endosomes and provide a membrane reservoir for the nascent autophagosome [9]. It is considered that ATG9-positive vesicles are utilized during phagophore expansion, or to form the autophagosomal precursor membrane upon homotypic fusion [10]. However, homotypic fusion of ATG9-positive vesicles would generate flat membranes and subsequent lipid- and protein-driven processes would be required to deform such flat membranes and stabilize membrane curvature.

Very little is know about the mechanism of membrane curvature during autophagosome formation, but newer studies correlate the function of some autophagy related (ATG) proteins with sensing membrane curvature. In this context, ATG14 was proposed to function as a membrane curvature sensor via its BATS (Barkor/ATG14 autophagosome targeting sequence) domain that is also responsible to position the PI3KC3 complex to the cytoplasmic face of the ER. The BATS domain in ATG14 preferentially binds highly curved liposomes enriched in PtdIns3*P* hence is considered to sense membrane curvature [11]. Recently, ATG3 was also proposed to function as a membrane curvature sensor, functioning through preferential binding to PE-enriched membranes via its amphiphatic α-helix [12]. ATG1/ULK1 was demonstrated not only to sense curved membranes but also to permit the tethering of incoming vesicles to the growing autophagosomal precursor membrane through its helical C-terminal EAT domain [13]. Moreover, induction and stabilization of membrane curvature was further suggested through LC3 lipidation [14]. From these results it can be assumed that membrane curvature sensing is mainly focused on the two critical phospholipids involved in the formation of the autophagosome, PtdIns3*P* that becomes specifically bound by WIPI proteins, and PE to which LC3 becomes conjugated.

3. In search for the membrane origin of autophagosomes

Christian de Duve developed the concept of cellular autophagy in 1963 [15,16]. De Duve based this concept of cellular self-digestion on morphological and biochemical results, including (i) the discovery of autophagosomes, then referred to as "round bodies of irregular density" in kidneys of newborn mice by Sam Clark in 1957 [17], (ii) the subsequent important observation that such vesicle formation is inducible by glycogen [18], and (iii) de Duve's seminal work of visualizing glycogen-induced autophagosomes shown to contain various cargo material, including mitochondria, ribosomes and membranes [19]. With the discovery of autophagosomes the debate on the nature and origin of the limiting membrane initiated.

Alex Novikoff proposed in 1964 that the limiting membrane derives from areas of the ER, which in fact provided the first concept on the membrane origin of autophagosomes [20]. By three-dimensional modeling of serial section electron microscopy (EM) displaying autophagosomes in rat hepatocytes, Novikoff later provided compelling evidence for interconnections between the ER and autophagosomes and, back in 1978, suggested that autophagosomes are formed from dynamic serpentine areas of the ER [21]. This hypothesis was supported by further early studies during the 1970s that referred to the ER as the main source for the autophagosomal membrane [22–25]. Meanwhile, other studies showed that the autophagosomal membrane could also derive from the Golgi [26,27], Golgi-derived endosomes [28] or the plasma membrane [29]. Hence, it was generally assumed that organelle membranes and also the plasma membrane provide an opportunity to produce autophagosomes, whereby the ER was early considered as the main source for autophagosomes [21,30,31].

Following up on this, Masahiro Sakai and colleagues provided evidence that pre-existing membranes undergo substantial membrane rearrangements prior to becoming the limiting autophagosomal membrane [32], which proposed autophagosomes to exhibit a distinct membrane composition, in line with studies by others in the 1980s [33,34]. Moreover, not only was the ER and the Golgi suggested to provide the limiting membrane but additionally ER and Golgi were also proposed to be the cargo of autophagosomes [19,35–37], as it was shown for mitochondria [19,38] and peroxisomes [39] to become specifically degraded through autophagy.

Studies in the early 1990s by William Dunn using immunological probes [40], and by Tahashi Ueno and colleagues using organelle markers [41], strongly supported the view that the ER represents the main pre-existing membrane source for the forming autophagosome in mammalian cells. However, alternative studies by Akitsugu Yamamoto and colleagues [42] with immunological probes also indicated that post-Golgi membranes should provide the limiting membrane of autophagosomes. However, in the late 1990s work by Per Seglen demonstrated that ER markers are not enriched in autophagosomal membrane precursors. These precursors displayed a unique membrane or even organelle feature, and were referred to as isolation membrane or phagophore [43]. This critical observation, however, did not disprove that the autophagosomal membrane initially derived from the ER [43], it underlined the earlier notion of substantial ER membrane rearrangement prior to autophagosome formation [32].

Subsequent considerations entertained a new perspective on the process of autophagosome formation, whereby the phagophore is formed de novo, albeit from unknown membrane sources [44–46]. Along this line, phagophore formation was demonstrated to be followed by membrane expansion to generate the autophagosome [44,47]. In some cases, autophagosome were shown to contact endosomes [48–50] and the chimeric endosome–autophagosome product was termed amphisome by Gordon and Seglen in 1988 [49]. The autophagosome, or amphisome, subsequently fuses with lysosomes for cargo degradation, the fusion step in fact first evidenced by Christian de Duve in the early 1960s [15].

Based on the notion that an autophagosomal precursor membrane expands to form the autophagosome, it was postulated that the autophagosomal membrane could not purely derive from pre-existing membranes only. Instead, a precursor membrane, the phagophore, should receive membrane input for expansion. However, the membrane origin remained mystical.

The landmark discovery of yeast autophagy-related (ATG) genes by Yoshinori Ohsumi in the early 1990s opened the door to thoroughly dissect the process of autophagy, and to subsequently identify ATG markers for autophagosomal membranes [44,51]. Seminal work by Noboru Mizushima and colleagues identified two essential ubiquitin-like conjugation systems specific for autophagy, the ATG12 and ATG8/LC3 systems, required for the formation of autophagosomes [52,53]. Subsequently, LC3 was demonstrated to localize at both the inner and outer membrane of autophagosomes [54]. Based on this, LC3 has become the most widely used marker to analyze the process of autophagy in mammalian cells.

In the mid 2000s mainly two alternative models for autophagosome formation were entertained, one model considering that the phagophore is derived from pre-existing membranes, such as the ER, and the other model suggested that the phagophore is formed de novo [2,55]. Both of the models, however, manifested that the phagophore expands to produce the autophagosome.

In the course of visualizing the process of autophagy more recently, dynamic ER structures, referred to as omegasomes that immediately form upon autophagy induction (Fig. 1A), were identified through live-cell microscopy by Nektarios Ktistakis in 2008 [56]. High resolution of such initial ER structures were achieved through 3D-tomographic reconstruction of serial section EM by Tamotsu Yoshimori [57] and Eeva-Liisa Eskelinen [58] in 2009. Interestingly, these results somehow resemble the early work of Alex Novikoff, who suggested that autophagosomes form from dynamic serpentine areas of the ER (see above) [21].

Based on the identification of a dynamic ER subdomain that serves as cradle for the forming phagophore, Tamotsu Yoshimori suggested a unifying model for the formation of the phagophore [57]. In this model, autophagy is initiated at the ER which subsequently undergoes dynamical alterations to build a cradle for the

de novo formation of the phagophore [3]. However, the membrane origin of the phagophore still remained an open question.

Moreover, recent studies also found evidence that apart from the ER, numerous other membrane sites and systems were able to contribute to the formation of the phagophore, including the ER-Golgi intermediate compartment (ERGIC) [59,60], Golgi-derived endosomes [61], plasma membrane [62], mitochondria [63] and mitochondria-ER contact sites [64]. This variety of possibilities resembles early results on the membrane origin of autophagosome that also suggested multiple membrane sources (see above).

Todays imaging techniques provide a more detailed look into the process of autophagosome formation, however, it is still obscure whether or not the phagophore originates from pre-existing membranes through rearrangements, or from various membrane sources that are assembled de novo.

4. WIPI proteins at the nascent autophagosome

Human WIPI proteins, that we identified earlier, belong to the ancient PROPPIN family of β-propeller proteins with essential PtdIns3*P* effector function at the nascent autophagosome [7,65]. Detailing the PtdIns3*P*-effector function of WIPI proteins is particularly interesting because WIPI proteins are rapidly recruited to endomembranes upon autophagy induction, and subsequently become membrane proteins of phagophores and autophagosomes [7]. Hence deciphering the functional localization of WIPI proteins may reveal the mechanism of phagophore formation in the proximity of pre-existing membranes.

By freeze fracture immuno-EM we found that starvation-induced autophagy promotes the specific localization of WIPI1 to the ER, prominently at the nuclear envelope (Fig. 1B) [66]. At the ER, in particular the omegasome, WIPI2 was also identified [67], and additionally in the Golgi region [66]. Both WIPI1 (Fig. 1B) and WIPI2 further accumulated at the plasma membrane (PM) upon starvation-induced autophagy [66]. Moreover, WIPI1 and WIPI2 were detected at the inner and outer membrane of autophagosomes as demonstrated by freeze fracture immuno-EM of unfixed cells [7,66]. By immuno-EM of fixed cells WIPI1 was also detected at membranes that resemble phagophore structures [68].

The characteristic localization of WIPI1 and WIPI2 at both endomembranes and nascent autophagosome can be attributed to their specific binding to PtdIns3*P* via evolutionarily conserved amino acids within the WIPI β-propeller [7]. With regard to the ER, localized production of PtdIns3*P* by the PI3KC3 complex has been demonstrated to trigger phagophore formation [3]. WIPI proteins not only specifically bind to PtdIns3*P*, they also fulfill an essential effector function at the nascent autophagosome, as demonstrated for WIPI2 which recruits the ATG16L complex to the phagophore for LC3 lipidation [69]. However, the PtdIns3*P*-effector contribution of WIPI1, WIPI3 and WIPI4 to phagophore formation is currently unknown [7].

As WIPI1 and WIPI2 specifically bind to the ER and subsequently to the phagophore and autophagosome, the following interpretations can be put forward:

1. Upon autophagy initiation, WIPI proteins accumulate at ER-produced PtdIns3*P* and independently also at the phagophore forming de novo. In this case, incoming membrane material for the de novo formation of the phagophore should display PtdIns3*P* to recruit and anchor WIPI proteins. Subsequently, PtdIns3*P*-bound WIPI in both ER cradle and incoming membranes for the de novo formation of the phagophore, may then connect them to one another. This scenario might suggest that different pools of WIPI proteins are recruited to different sites of PtdIns3*P* production upon autophagy induction. Further, such WIPI-decorated membranes may juxtapose one membrane site serving as cradle for phagophore formation and incoming membrane portions serving as material for phagophore expansion. Although appearing somewhat complicated, this model would explain why WIPI proteins locate at different endomembranes upon autophagy induction.
2. Alternatively, the accumulation of WIPI proteins at three membrane structures, namely at PtdIns3*P*-enriched sites of the ER, the phagophore and the autophagosome, reflects ER membrane rearrangements that give rise to the phagophore. In this scenario, the phagophore would not be formed de novo, rather it would form through regulated remodeling of endomembranes, e.g. the ER. This scenario in fact does not exclude the notion of phagophore expansion, because the initial phagophore would receive further vesicular membrane input to expand and build the autophagosome. This would explain why autophagosomes greatly differ in morphology, size and content. Such hypothetical scenario however, differs from the above in one most critical detail, being that the phagophore would not be formed de novo. The phagophore instead would be the product of ER membrane rearrangements with the opportunity to receive further membrane input due to tissue-specific or context-dependent needs.

Both of the above interpretations are based on the models considered for phagophore formation, namely maturation of endomembranes that become the phagophore, or the de novo assembly of vesicular membranes to form the phagophore. The latter is suggested to occur in the cradle of the ER. Of note, de novo formation of the phagophore was historically considered due to the lack of compelling detailed evidence that pre-existing membranes could become rearranged to give rise to the phagophore [2]. The specific PtdIns3*P*-dependent recruitment of WIPI proteins to endomembranes at the onset of autophagy and their subsequent localization at phagophores and autophagosomes, however, provide an attractive opportunity perhaps to understand the membrane formation of autophagosomes through more refined high resolution imaging.

5. An alternative view on the membrane origin of autophagosomes

The de novo assembly concept of the phagophore, within the ER cradle or in close proximity to other endomembranes or semiautonomous organelles, is extremely convincing, albeit experimentally unproven. As the membrane origin of autophagosomes has been searched for over 50 years without deriving a final conclusion, it may be relevant to reconsider the principle and ancestral function of autophagy.

In ancient eukaryotic cells the endomembrane system evolved autogenously [70], and along the process of autophagy as part of the developing membrane trafficking system. This can be assumed because essential ATG genes have been identified in the last common eukaryotic ancestor, and the process of autophagy is restricted to eukaryotic cells [71]. Early autophagy conferred fundamental advantages for ancient eukaryotic cells, including survival upon temporal nutrient starvation through intracellular digestion, releasing ancient eukaryotic cells from the requirement to exist close to nutrient sources and permitting the step into distant habitats [72]. By fighting and destroying invading pathogens ancestral autophagy further evolved as innate immune response [72,73].

As autophagy developed along with the endomembrane system, early autophagy, however, may have also retained the functionality of the emerging endomembrane system and internalized semiautonomous organelles. Hence the principle task of autophagy may

in fact be to segregate membrane sections in order to retain membrane function through regulated turnover, thereby beneficially gaining monomers and energy. In such view the autophagosomal membrane would not only become the membrane chamber for the sequestered cargo (Fig. 2A), but instead the major cargo itself and the further content of the autophagosomal vesicle the material that is consequently degraded with it (Fig. 2B).

Hence, early autophagy may be considered as ATG-driven segregation of particular membrane structures into autophagosomes, in order to rejuvenate the endomembrane system and semiautonomous organelle networks. In other words there would be no membrane origin of autophagosomes, but rather a constitutive petite attack of all membranes and compartments in eukaryotic cells. The inner content of autophagosomes sequestered like this would consequently include cytoplasmic bulk material, in line with the experimentally proven notion of stochastic degradation of cytoplasmic material through autophagy. Following, specific autophagy of both cargo and damaged membranes and organelles, may have evolved along with stochastic degradation to provide further functional advantages for cellular stress control. Finally, some forms of specific autophagy, such as CMA, only emerged in higher eukaryotes.

Considering that endomembranes and semiautonomous organelles present the primary target for autophagy may explain why over the past 50 years of hunting for the membrane origin of autophagosomes, recurring findings robustly demonstrate a great variety in membrane diversity as sources for both limiting membrane of autophagosomes and their sequestered content [74]. Further, this view may also explain why principle differences between autophagy in yeast and higher eukaryotic cells become increasingly apparent [75,76]. Moreover, phylogenetic considerations in fact suggest that mammalian autophagy displays more of the ancestral function of autophagy, and yeast, in particular *Saccharomyces cerevisiae*, more specialized forms of autophagy [76].

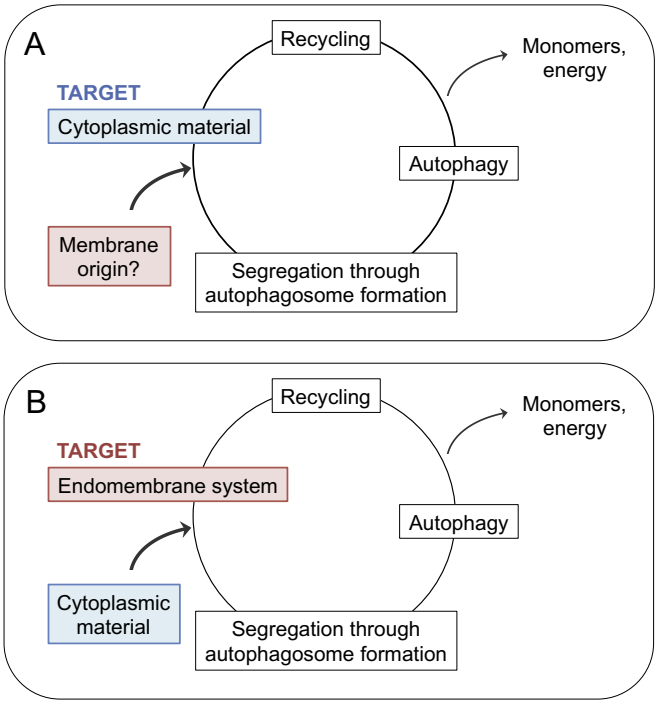

Fig. 2. Hypothetical considerations. (A) Autophagy as a catabolic process targeting cytoplasmic material for degradation and recycling. The autophagosomal membrane of unknown origin is formed de novo to chamber the sequestered cargo, including membranes, proteins and organelles. Degraded cargo material (monomers) is used for recycling purposes, energy is gained. (B) Autophagy as an endomembrane rejuvenating process for membrane turnover. Endomembrane sections are targeted by the ATG machinery to rearrange and build the autophagosomal membrane. The autophagosome sequesters further membranes, proteins and organelles. Degraded cargo material (monomers) is used for recycling purposes, energy is gained.

6. Outlook

In mammalian cells, it is of major interest to decipher (i) the mechanism of imposed ER dynamics upon autophagy induction prior to the formation of the phagophore, and (ii) homo- and heterotypic fusion events during phagophore formation and expansion. As WIPI proteins become recruited to ER-produced PtdIns3*P* at autophagy initiation, ER sections with accumulated WIPI–PtdIns3*P* may impose an ER membrane asymmetry to initiate membrane protrusions and curvature, respectively omegasomes. In this context it will be interesting to dissect how ATG proteins, shown to correlate with membrane curvature regulation, such as ATG14L [77] and ATG3 [12], communicate with WIPI protein complexes and ATG9-positive vesicles during phagophore formation. It is anticipated that such results may shed new light on the appearance of the very first phagophore section during autophagy initiation.

Acknowledgements

We apologize to researchers whose work we were unable to cite due to length constraints. We acknowledge financial support from the Ministry of Science, Research and the Arts Baden Wuerttemberg (MWK Stuttgart, Landesforschungsschwerpunkt), and the International Research Training Group (GRK 1302) of the Deutsche Forschungsgesellschaft (DFG). The International Max Planck Research School 'From Molecules to Organisms', Tuebingen, Germany, supports AJM with a pre-doctoral stipend.

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