Chapter 3

Regulation of Autophagy by Protein Phosphorylation

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1. Introduction

Macroautophagy (hereafter called autophagy) is an intracellular lysosomal degradation process. Long-lived cytosolic proteins and entire organelles are enveloped by a double membrane. These vesicles are called autophagosomes and are transferred to lysosomes. The enclosed cargo is degraded by lysosomal hydrolases, and the resulting components are transported back to the cytosol and re-used for anabolic and catabolic processes. In recent years it became evident that autophagy is central for cellular homeostasis. Autophagy occurs at basal levels essentially in any cell type, and is actively induced under stress conditions, e.g. nutrient deprivation or infection. Additionally, the involvement of autophagy in human pathologies such as cancerogenesis and neurodegeneration is well-documented and current therapeutic approaches hence target autophagy signaling pathways.

During the past decade the molecular mechanisms for the induction and execution of autophagy have been partially resolved. The discovery of the yeast autophagy-related genes (Atgs) was central for this understanding. To date, 35 yeast Atgs have been identified, many of which have mammalian counterparts. However, autophagy induction is additionally controlled by a complex network of other cellular signaling pathways.

The process of autophagy is mainly regulated by six functional units: 1) the Ulk1-Atg13-FIP200 kinase complex, 2) the PI3K class III complex containing the core proteins Vps34, p150 and Beclin 1, 3) the PI3P-binding Atg2/Atg18 complex, 4) the multi-spanning transmembrane protein Atg9, 5) the ubiquitin-like Atg5/Atg12 system and 6) the ubiquitin-like LC3 conjugation system (reviewed in [1]). These six modules participate in different steps of autophagy execution, i.e. vesicle nucleation, elongation and autophagosome completion. In the following we will summarize the current knowledge about regulatory phosphorylation events occurring during autophagy induction upstream and downstream

of the Ulk1-Atg13-FIP200 and PI3K class III "initiator complexes", and during vesicle elongation, e.g. LC3 phosphorylation.

2. The Ulk1-Atg13-FIP200 protein kinase complex

Interestingly, Atg1/Unc-51-like kinase 1 is the only protein kinase among the Atg proteins. In 1993, Tsukada and Ohsumi reported the isolation of different autophagy-defective mutants of *S. cerevisiae* [2]. The apg1 strain was the first identified strain, and subsequently it was discovered that the corresponding gene encodes a serine/threonine protein kinase [3], which was subsequently termed Atg1. During the induction of canonical autophagy in yeast, Atg1 interacts with Atg13, Atg17, Atg29 and Atg31 [4-8]. Apparently a constitutive trimeric complex of Atg17, Atg29 and Atg31 serves as scaffold for the subsequent recruitment of other Atg proteins to the pre-autophagosomal structure (PAS) [9, 10]. In contrast, the interaction between Atg1 and Atg17 is dynamic and mediated by Atg13 [5, 11]. In 2000, Ohsumi and colleagues could nicely demonstrate that the association between Atg1 and Atg13 is negatively regulated by target of rapamycin (TOR) signaling [7]. In their model, nutrient-rich conditions lead to a TOR-mediated hyperphosphorylation of Atg13, which prevents its association with Atg1. In turn, starvation conditions or rapamycin treatment result in an inactivated TOR and a dephosphorylated Atg13. Dephosphorylated Atg13 exhibits a high affinity for Atg1 and induces its kinase activity, ultimately leading to the induction of autophagy [7]. Recently the group could confirm this model by showing that the expression of an Atg13 mutant protein which cannot be phosphorylated by TOR anymore leads to the induction of autophagy under nutrient-rich conditions [12]. Apparently Atg13 mediates the self-association of Atg1, and the appearance of Atg1-Atg1-dimers is correlated with the induction of autophagy [13].

In vertebrates, the situation is a little bit more complex. There exist at least five Atg1 homologs, designated as Unc-51-like kinases 1-4 (Ulk1-4) and STK36 (reviewed in [14-17]). Unc-51 (uncoordinated-51) is the single Atg1 homolog in *C. elegans* [3, 15]. Especially Ulk1 and Ulk2 have been well characterized as the functional homologs of Atg1/Unc-51, and only these two Unc-51-like kinases exclusively exhibit high similarity in both the N-terminal catalytic domain and the rest of the protein, including a central proline/serine-rich (PS) and the C-terminal domain (CTD) [16, 17]. Although overexpression of Ulk3 was able to induce autophagy in the human fibroblast cell line IMR90 [18], only Ulk1 and Ulk2 are capable of interacting with mammalian Atg13 via their conserved CTD [19-22]. Based on an siRNA screen in HEK293 cells, Ulk1 has been proposed as the primary autophagy regulator, since the knockdown of Ulk1 but not that of Ulk2 inhibited the autophagic response upon starvation or rapamycin treatment [23]. Both Ulk1 and Ulk2 are ubiquitously expressed in most adult mammalian tissues [24-26]. Interestingly, in red blood cells only *ulk1* mRNA is significantly up-regulated during terminal erythrocyte differentiation [27]. Accordingly, *ulk1*$^{-/-}$ mice reveal defects in the clearance of mitochondria and RNA-containing ribosomes in reticulocytes during erythrocyte maturation [27]. However, *ulk1*$^{-/-}$ mice are born viable and show normal autophagy induction upon starvation [27], which is in clear contrast to other

mouse models deficient for specific Atgs such as Atg5 or Atg7 [28, 29]. Furthermore, *ulk2*$^{-/-}$ mice are likewise viable and autophagy-competent [30, 31]. These observations suggest that Ulk1 and Ulk2 have partially redundant functions and that loss of one kinase can be compensated by the other during non-selective autophagy. However, the ability of Ulk2 to compensate Ulk1-deficiency appears to be cell type-specific and to depend on the type of autophagy. The selective role of Ulk1 for mitophagy described above has recently been confirmed by the observation that Ulk1-mediated phosphorylation of Atg13 at S318 is essential for mitophagy, but not for basal or starvation-induced autophagy [32]. Additionally, only Ulk1 is required for low potassium-induced autophagy in cerebellar granule neurons [31]. Finally, in Ulk1-silenced *ulk2*$^{-/-}$ MEFs or in *ulk1*$^{-/-}$ *ulk2*$^{-/-}$ MEFs autophagy induced by amino acid deprivation is blocked, further supporting the redundant functions of both kinases [30, 31]. Of note, starvation-induced autophagy was not inhibited in *ulk1*$^{-/-}$*ulk2*$^{-/-}$ DT40 B lymphocytes generated in our laboratory, and *ulk1*$^{-/-}$*ulk2*$^{-/-}$ MEFs display autophagy induction upon glucose deprivation [30, 33]. The molecular details of these Ulk1/Ulk2-independent autophagy pathways have to be deciphered in the future.

As described above, the CTD of Ulk1 and Ulk2 can interact with mammalian Atg13. In 2007, mammalian Atg13 has been identified by an *in silico* protein and DNA database screen [34]. Subsequently, the essential role of Atg13 for autophagy has been confirmed in HEK293 cells [19]. Atg17, the other essential component of the yeast Atg1 complex, is conserved in different yeast strains and most filamentous fungi, but primary sequence homologs could not be identified in higher eukaryotes [34]. However, Mizushima and colleagues demonstrated that the focal adhesion kinase (FAK) family interacting protein of 200 kDa (FIP200) is an Ulk1-interacting protein and essential for autophagy induction [35]. FIP200 is a large coiled-coil domain containing protein and regulates diverse cellular functions including cell size, proliferation and migration [35]. FIP200 was initially identified as gene inducing retinoblastoma 1 (RB1) expression in different human cell lines [36] and accordingly termed RB1CC1. Mizushima and colleagues speculated in the original manuscript that FIP200 might represent the functional homolog of yeast Atg17 [35]. Both proteins have multiple coiled-coil domains, enhance the catalytic activity of Atg1/Ulk1, are essential for Atg1/Ulk1 puncta formation, have multiple binding partners and thus scaffolding properties, and are mutually exclusively present in different species [35]. Indeed, several almost simultaneously published articles describe the existence of a mammalian Ulk-Atg13-FIP200 kinase complex, which is directly regulated by the mammalian target of rapamycin complex 1 (mTORC1) [20-22]. The interaction between Ulk1/2 and FIP200 is mediated by Atg13. However, one group described the direct interaction of FIP200 with Ulk [20]. In contrast to the yeast Atg1-Atg13-Atg17 complex, the components of the mammalian Ulk-Atg13-FIP200 complex are constitutively associated, especially independently of nutrient supply [20-22]. However, apparently the phosphorylation status within the Ulk-Atg13-FIP200 complex is considerably altered during autophagy induction (see below).

A fourth component of the Ulk-Atg13-FIP200 complex has recently been characterized. Since it has no obvious homolog in yeast, it was termed Atg101 [37, 38]. Atg101 stably associates

with the kinase complex, most likely via direct interaction with Atg13. It appears that Atg101 protects Atg13 from proteasomal degradation [37, 38].

2.1. The Ulk1-Atg13-FIP200 protein kinase complex and protein phosphorylation

In yeast, target of rapamycin complex 1 (TORC1) phosphorylates Atg13 at several serine residues, thus preventing the association of Atg13 with Atg1. In turn, TORC1 inactivation by rapamycin treatment or nutrient deprivation causes Atg13 dephosphorylation, resulting in Atg1-Atg13-Atg17 complex assembly and enhanced Atg1 kinase activity [7, 12]. Regarding TORC1-dependent phospho-sites in Atg13, four serine residues have been clearly identified, i.e. S437, S438, S646, and S649 [12]. Four additional sites were not precisely mapped, but could be deduced from known TORC1 sites or the conservation among *Saccharomyces* species (S348, S496, S535, and S541). As described above, the expression of the corresponding Atg13-8SA mutant was able to induce autophagy through activation of Atg1 even under nutrient-rich conditions [12]. Recently it has been reported that the Ksp1 kinase negatively regulates autophagy in yeast via the TORC1-Atg13 axis [39].

Atg1 itself is phosphorylated at multiple sites [40, 41]. Phosphorylation of T226 and S230 is important for Atg1 kinase activity and its function in autophagy. Both residues are located within the activation loop of the N-terminal kinase domain and apparently become phosphorylated by autophosphorylation [40, 41]. Additional phosphorylation sites have been reported. In one of these studies, a total of 29 constitutive or rapamycin-regulated phospho-sites were identified [40].

As mentioned above, the phosphorylation status within the mammalian Ulk1/2-Atg13-FIP200 complex varies dependent on the cellular energy and nutrient supply. Under normal growth conditions, mTORC1 associates with the complex via the direct interaction between the mTORC1 component raptor and Ulk1/2 [21]. Active mTOR phosphorylates Ulk1/2 and Atg13 and thus suppresses Ulk1/2 kinase activity [20-22]. In a first mass spectrometric approach, Dorsey et al. identified 16 phospho-acceptor sites in Ulk1 under fed conditions [42]. The authors suggested that S341 located in the PS region and S1047 in the CTD represent Ulk1 autophosphorylation sites which are required for protein stability. Upon starvation or rapamycin treatment, mTORC1 dissociates from the Ulk1/2-Atg13-FIP200 complex and the mTOR-dependent phospho-sites of Ulk1 become dephosphorylated. In a SILAC-based approach, Wang and colleagues recently identified 13 serine or threonine residues in Ulk1 phosphorylated under nutrient-rich conditions. Of these residues, S638 and S758 revealed a more than 10-fold decrease of phosphorylation upon starvation [43]. In another study, the mTOR-dependent phosphorylation of Ulk1 at S758 was confirmed [44], and the same phospho-site was also detected in the screen of Dorsey et al. [42]. Interestingly, the total phosphorylation levels of Atg13 were low under nutrient-rich conditions and remained largely unaltered upon nutrient depletion, suggesting that rather Ulk1/2 than Atg13 is the major target of mTOR-dependent autophagy regulation [43]. The dephosphorylated and thus activated Ulk1/2 autophosphorylates and phosphorylates both Atg13 and FIP200, which ultimately leads to the translocation of the complex to the pre-autophagosomal membrane and to autophagy induction [19-23, 35, 45, 46]. Next to the above described autophosphorylation

sites mapped for Ulk1 under nutrient-rich conditions, T180 in the activation loop of the kinase domain has been identified as Ulk1 autophosphorylation site [47]. This site is homologous to the site described for yeast Atg1 autophosphorylation [40, 41]. Notably, the functional relevance of Ulk1/2-mediated phosphorylation of both Atg13 and FIP200 remains elusive, and the relevant phospho-sites have not been verified yet. Our group was able to identify five Ulk1-dependent *in vitro* phosphorylation sites in human Atg13, i.e. S48, T170, T331, T428 and T478 [33]. However, expression of the corresponding pan-serine/threonine-to-alanine mutant of Atg13 in *atg13*$^{-/-}$ DT40 B cells did not block autophagy induction upon starvation [33]. Since Ulk1 and Ulk2 are dispensable for autophagy induction in this cellular system, potentially other kinases and Atg13 phospho-acceptor sites might play a role in the regulation of autophagy. As stated above, Ulk1-mediated phosphorylation of S318 in Atg13 solely influences mitophagy and has no impact on starvation-induced autophagy [32]. One might speculate that the Ulk-catalyzed phosphorylation of Atg13 and FIP200 controls the translocation of the kinase complex to pre-autophagosomal structures.

In addition to mTOR, the Ulk1/2-Atg13-FIP200 complex is regulated by other kinases such as PKA, Akt and AMPK, respectively (reviewed in [1, 14, 17]). The cAMP-dependent protein kinase A (PKA) inhibits yeast autophagy by phosphorylation of Atg1 and Atg13. In Atg1, PKA-dependent phosphorylation presumably takes place at S508 and S515, and in Atg13 at S344, S437, and S581 [48, 49]. In mammalian cells, depletion of the type IA regulatory subunit of PKA has been shown to activate mTOR and thus to inhibit autophagy [50]. A direct phosphorylation of Ulk1 at S1043 by PKA has been suggested by Dorsey et al., causing a rather closed and inactive conformation of Ulk1 [42]. A PKA-mediated phosphorylation of mammalian Atg13, in contrast, has not been confirmed yet.

Recently, S774 has been identified as a high-stringency Akt site in Ulk1 [47]. Phosphorylation of Ulk1 at this site was increased by insulin treatment. Notably, this insulin-mediated repression of autophagy was also observed in the presence of rapamycin, suggesting that this inhibition occurs independently of mTOR inhibition. Indeed, synergistic effects of rapamycin and Akt inhibitors on autophagy have been reported [47, 51]. Interestingly, the yeast kinase Sch9, which is a homolog of Akt or p70S6K, has been implicated in the regulation of autophagy. The authors proposed that PKA and Sch9 cooperatively regulate autophagy, and that this is partially independent of TORC1 [52].

Whereas mTOR activity is regulated by a diverse array of positive signals such as high energy levels, normoxia, amino acids and growth factors, the AMP-regulated protein kinase (AMPK) is activated under low energy levels and thus represents the classical energy-sensor of the cell. AMPK is a heterotrimeric kinase, consisting of a catalytic α-subunit and two regulatory β- and γ-subunits, respectively (reviewed in [53]). Additionally, multiple isoforms of each subunit exist, i.e. α1-2, β1-2, and γ1-3 [53]. Phosphorylation of T172 in the catalytic α-subunit is a prerequisite for AMPK activity. In turn, this phosphorylation can be carried out by the ubiquitously expressed and constitutively active LKB1, the Ca^{2+}-activated Ca^{2+}/calmodulin-dependent kinase kinase β (CaMKKβ), or the cytokine-activated TAK1 (transforming growth factor b-activated kinase-1) [14]. An additional level of regulation is mediated by the regulatory β- and γ-subunits. The γ-subunit can bind ATP, ADP and AMP

and thus appropriately senses the cellular energy status [53]. The β-subunit contains carbohydrate-binding modules, whose exact functions remain rather elusive. However, it has been speculated that these domains contribute to the subcellular localization of AMPK or targeting the kinase to glycogen-associated substrates [53]. In 2001, it was shown that the yeast AMPK ortholog SNF1 acts as a positive regulator of autophagy, and it has been speculated that this is mediated via Atg1 and Atg13 [71]. Although an initial report described the inhibition of autophagy by the AMPK activator AICAR [54], a positive regulatory role of AMPK for mammalian autophagy has subsequently been confirmed by several groups [55-58]. However, next to energy depletion other stimuli such as the increase of cytosolic Ca^{2+} concentrations, TRAIL-mediated activation of TAK1, and genotoxic stress-induced sestrin1 and sestrin2 expression have been implicated in the AMPK-dependent regulation of autophagy (reviewed in [14, 59-62]). Historically, the AMPK-dependent regulation of autophagy has been proposed to mainly function via the inhibition of mTOR. AMPK regulates the mTORC1 via the direct phosphorylation of two effectors: 1) the upstream regulator tuberous sclerosis complex 2 (TSC2) and 2) the mTORC1-subunit raptor [63-65]. However, in 2010 Behrends et al. first discovered the interaction between AMPK and Ulk1/2 in a global proteomic analysis of the human autophagy network [66]. Subsequently, different groups confirmed the direct interaction between these two kinases [43, 44, 65, 67, 68]. Four of these groups additionally reported the AMPK-mediated phosphorylation of Ulk1. The common theme of these reports is the starvation-induced activation of AMPK, the subsequent phosphorylation and activation of Ulk1, and in turn the induction of autophagy (reviewed in [14, 69]). In contrast, our group was able to identify Ulk1-dependent phosphorylation sites in all three AMPK subunits, suggesting a rather complex level of regulation by mutual phosphorylation [68]. The direct activation of Ulk1 by AMPK represents a valuable model for mTOR-independent autophagy pathways [69, 70]. Furthermore, due to the data obtained in yeast (see above), it has been proposed that this direct autophagy induction pathway most likely arose even earlier in evolution than the mTORC1-mediated regulation [53, 71]. Notably, the direct regulation of Ulk1 by AMPK appears to be rather complex, since the different groups identified different phospho-acceptor sites for AMPK (reviewed in [14, 69]). Kim et al. reported the mTORC1-mediated phosphorylation of S758 in Ulk1 under nutrient-rich conditions, which leads to the inhibition of the AMPK-Ulk1 interaction. Upon glucose starvation, AMPK is activated and 1) inhibits mTORC1 and 2) activates Ulk1 by the phosphorylation at S317 and S778 [44]. Egan et al. characterized Ulk1 both as an AMPK substrate and as 14-3-3-binding protein. However, this group identified four completely different AMPK-sites in Ulk1: S467, S556, T575 and S638 [67]. One of these sites (S556) has later been confirmed as AMPK- and 14-3-3-binding site in another study [47]. Lee et al. earlier showed that the association between Ulk1 and AMPK is important for the induction of autophagy, and mentioned the unpublished observation that purified AMPK could phosphorylate recombinant Ulk1 *in vitro* [65]. Finally and as described above, Shang et al. could identify 13 phospho-acceptor sites in Ulk1 using a SILAC-based approach. In this approach they confirmed the mTOR-site S758 identified by Kim et al., but not their AMPK-sites. However, in contrast to the report by Kim et al., phosphorylation of S758 leads to the association of AMPK and Ulk1 [43]. Notably, the AMPK-sites S556 and S638 in Ulk1 described by Egan et al. were confirmed by Shang et al.. As

described above, they observed significant dephosphorylation at S638 and S758 upon starvation. When cells are replenished with growth media, mTOR-mediated phosphorylation of S758 leads to the re-association of Ulk1 and AMPK, and subsequently mTOR and the Ulk1-associated AMPK function to maintain phosphorylation at S638 [43]. In summary, it appears that further studies are necessary to decipher this rather complex "Ulk1 phosphorylation barcode" as well as the exact kinetics of its mTOR- and AMPK-dependent generation. Furthermore, it has to be considered that this barcode might depend on both the type of autophagic stimulus and the type of (organelle-specific) autophagy.

Adding an additional level of complexity, recent reports describe the Ulk1-dependent phosphorylation of AMPK- and mTOR-subunits, respectively. Our group could show that all three AMPK subunits are phosphorylated by Ulk1 and Ulk2, and that thereby AMPK activity is negatively regulated [68]. The phospho-sites include S360/T368, S397, S486/T488 in the α1-subunit, S38, T39, S68 and S173 in the β2-subunit, and S260/T262 and S269 in the γ1-subunit (for some peptides the exact phosphorylation-site could not be narrowed down further). Thus, we propose that Ulk1 is not only necessary for the induction of autophagy, but also is involved in its containment [68]. Finally, two reports describe the Ulk1-dependent phosphorylation of raptor [72, 73]. The common theme here is that this phosphorylation leads to the inhibition of mTORC1 thus inducing autophagy. Dunlop et al. reported that Ulk1 promotes phosphorylation of raptor at S696, T706, S792, S855, S859, S863 and weakly S877 *in vivo*. The direct phosphorylation of the last five residues was confirmed by an *in vitro* kinase assay, with the strongest phosphorylation at S855 and S859, respectively. Collectively, these multiple phosphorylations inhibit mTORC1 activity through hindrance of substrate docking to raptor [72]. These results are in line with the findings by Jung et al. [73]. Collectively, the Ulk1-mediated phosphorylation of AMPK and raptor represent regulatory feedback loops and contribute to the positive and negative regulation of autophagy.

Next to the associated Atg13 and FIP200 and the AMPK and mTORC1 kinase complexes, two additional Ulk1 substrates related to the regulation of autophagy have been identified, i.e. the activating molecule in Beclin1-regulated autophagy 1 (Ambra1; see below) and the myosin light chain kinase (MLCK)-like protein Spaghetti squash activator (Sqa) in *Drosophila*. Regarding Ambra1, Fimia and colleagues showed that Ambra1 tethers the Vps34-Beclin 1-complex (see below) to the cytoskeletal dynein light chains. Upon autophagy induction, Ulk1 phosphorylates Ambra1 and thereby releases the Vps34-Beclin 1 core complex from dynein [74, 75]. Subsequently, this complex translocates to the ER where it contributes to autophagosome formation (see below). The second Ulk1 substrate is the *Drosophila* MLCK-like protein Sqa, whose mammalian ortholog is the zipper interacting protein kinase (ZIPK), which is also termed death-associated protein kinase 3 (DAPK3) [76]. Through the combination of observations obtained in *Drosophila* and mammalian cells, the authors propose a model in which starvation leads to the Atg1/Ulk1-mediated phosphorylation of Sqa/ZIPK at T279/T265 (Sqa/ZIPK sequence). This in turn leads to the phosphorylation of the myosin II regulatory light chain (Spaghetti-Squash, Sqh, in *Drosophila*, and MLC in human). Subsequently, myosin II regulates starvation-induced trafficking of mAtg9 from the trans-Golgi network (TGN) to autophagosomes [76-78]. The role of mAtg9 will be discussed below. Collectively, both models support the view that the

cytoskeleton plays a central role for the spatial organization of autophagy-inducing complexes. This is further supported by data showing that the exocyst, a hetero-octameric protein complex normally involved in tethering vesicles to the plasma membrane, serves as an assembly and activation scaffold for components of the autophagic machinery [79, 80]. It could be demonstrated that the small G protein RalB and an Exo84-dependent subcomplex of the exocyst are critical for the recruitment of the Ulk1-Atg13-FIP200 and the Vps34-Beclin 1 initiation complexes as well as the two ubiquitin-like conjugation systems and thus for autophagosome formation [79, 80].

The mutual regulation of AMPK, mTOR and Ulk1/2 and the phosphorylation events upstream and downstream of the Ulk1/2-Atg13-FIP200 kinase complex are depicted in figure 1A and B.

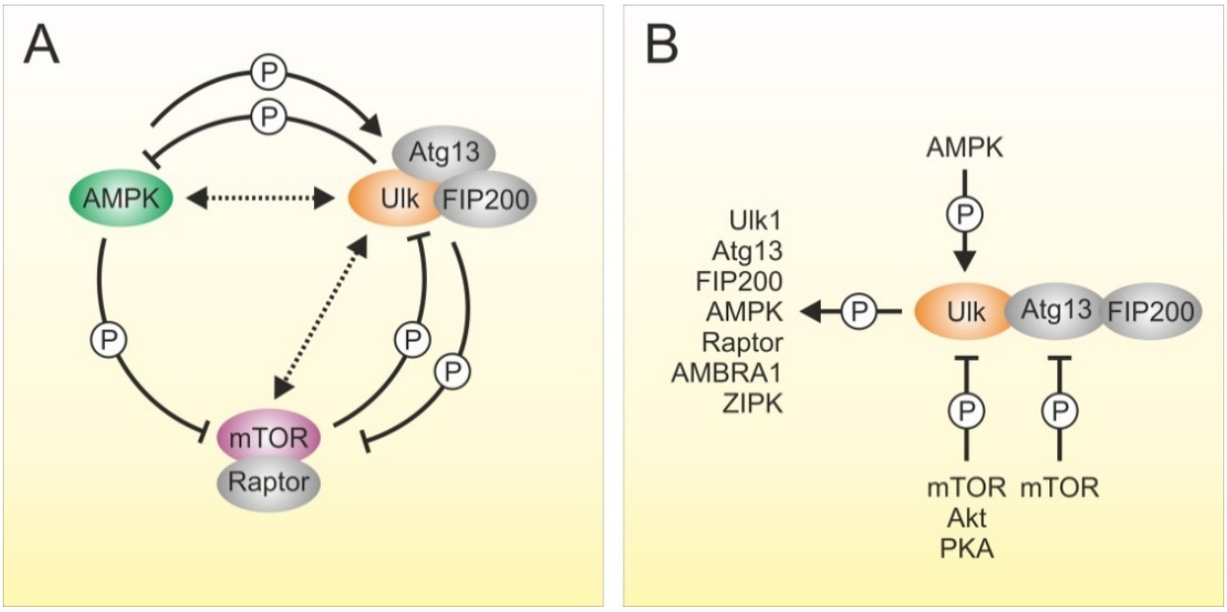

Figure 1. Mutual regulatory phosphorylations of AMPK, mTOR and Ulk1/2 (A) and phosphorylations downstream and upstream of the Ulk1/2-Atg13-FIP200 kinase complex (B). AMBRA1, activating molecule in Beclin1-regulated autophagy 1; AMPK, AMP-regulated protein kinase; Atg, autophagy-related gene; FIP200, focal adhesion kinase family interacting protein of 200 kDa; mTOR, mammalian target of rapamycin; PKA, protein kinase A; Ulk, Unc-51-like kinase; ZIPK, zipper interacting protein kinase. Panel A was modified from supplemental material to reference [68].

3. The PI3K class III complex

In yeast, the class III phosphatidylinositol 3-kinase (PI3K class III) Vps34 participates in both autophagy and vacuolar protein sorting (Vps). Accordingly, two distinct Vps34-containing complexes have been identified [81]. Both complexes share the core components Vps34, Vps15, and Atg6 (Vps30). The first complex (complex I) additionally contains Atg14 and is required for autophagy, while the second complex (complex II) contains Vps38 instead of Atg14 and is important for vacuolar protein sorting via endosomes. It has been shown that the destination-determining factors Atg14 and Vps38 target the Vps34-complexes to either the pre-autophagosomal structure (PAS) or to endosomal membranes, respectively [82]. In

mammals, different Vps34-complexes could be identified and characterized (reviewed in [83]). The mammalian PI3K class III core complex contains hVps34, hVps15 (p150), and Beclin 1 (Atg6). This core complex can in turn associate with different regulatory and function-determining proteins. It appears that there are three major sub-complexes, containing either Atg14L or UVRAG (UV-irradiation resistance-associated gene) or a dimer of UVRAG and Rubicon (RUN domain protein as Beclin 1 interacting and cysteine-rich containing), respectively [83, 84].

Atg14L is the putative mammalian homolog of yeast Atg14. Atg14L was identified by a sequence homology screen and has also been termed as Atg14 or Barkor [85-88]. Atg14L contains two coiled-coil domains (CCDs) that are essential for the interaction with the CCDs of Beclin 1 and hVps34, respectively [83]. The interaction between Vps34 and Beclin 1 is not affected by Atg14L. However, Beclin 1 is required for the association of Atg14L with Vps34 [87]. The Atg14L-containing Vps34-Vps15-Beclin 1 complex is essential for the early steps of autophagosome formation and likely represents the equivalent of yeast complex I. Atg14L co-localizes with Atg5 and Atg16L1 on the isolation membrane during autophagy induction [85]. In *atg14*$^{-/-}$ ES cells, starvation does not induce the formation of Atg16L and LC3 puncta or the lipidation of LC3 (see below). Additionally, the bulk degradation of long-lived proteins is suppressed in these cells [86].

UVRAG has been initially identified as Beclin 1-binding protein, and – like Beclin 1 – has been described as tumor suppressor [89]. UVRAG has been suggested as mammalian Vps38 homolog [85, 90], and indeed the binding of Atg14L or UVRAG to Beclin 1 is mutually exclusive [86-88, 90]. The exact role of UVRAG for autophagy induction, however, remains rather controversial. It has been reported that UVRAG expression increases Vps34 activity and that UVRAG and Beclin 1 act together to induce autophagosome formation [89]. However, Mizushima and colleagues could not detect any inhibitory effect of siRNA-mediated UVRAG knockdown on autophagy flux and GFP-LC3 puncta formation [85]. Interestingly, it was recently demonstrated that UVRAG mutations associated with microsatellite unstable colon cancer do not affect autophagy [91]. Notably, UVRAG exhibits a positive regulatory role at later stages of autophagosome maturation independently of Beclin 1. It has been reported that UVRAG interacts with the class C Vps (C-Vps), which is a key component of the endosomal fusion machinery [92]. This UVRAG-C-Vps interaction apparently stimulates Rab7 GTPase activity, which is important for complete autophagosome maturation and autophagsome fusion with late endosomes/lysosomes [83, 92]. In parallel, the UVRAG-C-Vps complex accelerates endosome-endosome fusion, thereby promoting the rapid degradation of endocytic cargo [92].

In 2009, two groups reported the identification and characterization of an additional Beclin 1-associated protein, termed RUN domain protein as Beclin 1 interacting and cysteine-rich containing (Rubicon) [86, 88]. Rubicon is composed of an N-terminal RUN domain, a central CCD and a C-terminal cysteine-rich region. Both reports demonstrated that Rubion-containing Vps34-Beclin 1-complexes are devoid of Atg14L. Since UVRAG is able to bind the core complex independently of Rubicon but Rubicon in turn can only bind to the core

complex when UVRAG is bound, it appears that UVRAG mediates the interaction between Rubicon and Beclin 1 [86, 88]. Interestingly, there is no apparent Rubicon homolog in yeast, and there is no sequence homology between Rubicon and Atg14 or Vps38, respectively [86, 88]. Current data show that Rubicon suppresses autophagy, presumably at later stages such as autophagosome maturation. Additionally, Rubicon was shown to decrease Vps34 catalytic activity [88]. However, it has been suggested that the negative regulation of autophagy occurs independently of Beclin 1 and of Rubicon's association with the core complex [83]. In fact, the Rubicon-mediated inhibition of Vps34 kinase activity does not require Beclin 1, and the formation of Rubicon-associated late endosomal/lysosomal structures, which apparently inhibit autophagosome maturation, takes place independently of Beclin 1 [88]. Accordingly, it has been proposed that binding of Rubicon to the Vps34-Beclin 1 core complex rather neutralizes the inhibitory effect of Rubicon [83].

In one of the studies mentioned above, Beclin 1 and interacting proteins were purified from different tissues of Beclin 1-EGFP-transgenic mice [88]. Notably, the protein levels of Beclin 1-EGFP, Vps34, Vps15 and UVRAG were similar and reproducibly higher than those of Atg14L and Rubicon suggesting the existence of a UVRAG-containing core complex. These results could be confirmed for Beclin 1 complexes purified from cultured cells [83, 86, 87]. Interestingly, many previously reported Beclin 1-interacting proteins were not detected in these purifications, indicating that those interactions are rather unstable, transient or specific for certain conditions [88]. These associated proteins include Bcl-2 homologs [93-97], Ambra1 [98], nPIST [99], VMP1 [100], Rab5 [101], FYVE-CENT [102], estrogen receptor [103], MyD88/TRIF [104], SLAM [105], Survivin [106], PINK1 [107], and HMGB1 [108-110]. Additionally Bif1 and IP3Rs have been reported to indirectly bind Beclin 1 via UVRAG and Bcl-2, respectively [111, 112]. Finally, several viral proteins have been reported to bind Beclin 1, such as HSV-1 ICP34.5 [113], γ-herpesvirus viral Bcl-2 [97, 114-117], HIV Nef [118] and the M2 protein of influenza virus [119].

The association between Beclin 1 and anti-apoptotic members of the Bcl-2 family such as Bcl-2, Bcl-x_L and viral Bcl-2 is especially interesting, since it represents an important node between apoptosis and autophagy signaling pathways [93-97, 114-117]. Actually, Beclin 1 was initially identified as Bcl-2 interacting protein by a yeast-two-hybrid screen [94], and Beclin 1 contains a BH3-domain which mediates the binding to Bcl-2 family proteins [95, 96]. Independently, several groups subsequently showed that anti-apoptotic Bcl-2, Bcl-x_L or viral Bcl-2 proteins can inhibit Beclin 1-dependent autophagy [95, 97, 115]. Especially viral Bcl-2 homologs bind Beclin 1 with high affinity, resulting in an ever stronger inhibition of autophagy compared to cellular Bcl-2 family proteins [115]. Surprisingly, although Beclin 1 represents a BH3-only protein, it does not alter the anti-apoptotic capacity of Bcl-2 [120, 121]. It has been shown that Bcl-2 and Beclin 1 co-localize at both the endoplasmic reticulum (ER) and mitochondria [97]. However, it has been proposed that Bcl-2 exerts its anti-autophagic effect on Beclin 1 especially when targeted to the ER, and not when localized at mitochondria, which suggests an organelle-specific regulation of the autophagic machinery [95, 97]. Recently, the nutrient-deprivation autophagy factor-1 (NAF-1) has been identified as a co-factor that specifically targets Bcl-2 to antagonize the autophagic pathway at the ER

[122]. Nevertheless, an important role of mitochondria-targeted Bcl-2 for the regulation of autophagy has been deciphered by Cecconi and colleagues. They showed that a pool of the positive autophagy regulator Ambra1 can bind to mitochondria- but not to ER-localized Bcl-2. Upon starvation, mitochondria-resident Ambra1 is released from Bcl-2 and can associate with both mitochondrial and ER-localized Beclin 1 [123]. Beclin 1-dependent autophagic processes are thus presumably initiated at both organelles and Bcl-2 proteins accordingly regulate autophagy in two ways, directly by binding Beclin 1 and indirectly by sequestering the positive regulator Ambra1 [123, 124].

Two different models have been proposed to explain the Bcl-2/Bcl-xL-mediated inhibition of Beclin 1-dependent autophagy [97, 125, 126]. First, it could be shown that Bcl-2 interferes with the formation of the autophagy-promoting Vps34/Beclin 1-complex and that Bcl-2 overexpression decreases Vps34 catalytic activity [97]. Second, it has been demonstrated that Beclin 1 forms a dimer and that the Beclin 1 dimer interface is disrupted by UVRAG [125]. Apparently Beclin 1 monomerization activates the lipid kinase activity of Vps34. Bcl-2-like proteins in turn reduce the affinity of UVRAG for Beclin 1 approximately 4-fold and accordingly stabilize the Beclin 1 dimer [125]. Bcl-2 and Bcl-xL obviously bind Beclin 1 with relatively low affinity since several groups could show that cellular Bcl-2 homologs cannot be detected in stable Vps34-Beclin 1-complexes [83, 85, 86, 88, 127]. This in turn suggests that the transient interaction between Bcl-2-homologs and Beclin 1 enables a flexible and dynamic regulation of autophagy induction. Meanwhile, several mechanisms have been proposed how this interaction can be negatively regulated, including the phosphorylation of either Bcl-2 proteins or Beclin 1 (see below), the competitive displacement of Beclin 1 by BH3-only proteins or BH3 mimetics, or the disruption of the Bcl-2/Beclin 1 interaction by membrane-anchored receptors and adaptors such as IP3Rs or MyD88/TRIF, respectively (reviewed in [127]).

3.1. Downstream effectors of the PI3K class III complex

Different downstream effectors of phosphatidylinositol 3-phosphate (PI3P), which is the product of PI3K class III catalytic activity, have been implicated in autophagy regulation. In 2008, Ktistakis and colleagues showed that upon starvation double FYVE domain-containing protein 1 (DFCP1) translocates to a punctate compartment which partially co-localizes with autophagosomal proteins and which is in dynamic equilibrium with the ER [128]. The authors termed these structures omegasomes, since the ring-like structures associated with the underlying ER forming an Ω-like shape [128]. Using electron microscopic tomography two reports could meanwhile confirm the tight connection between the ER and the isolation/phagophore membrane [129, 130]. DFCP1-translocation to omegasomes depends on Vps34 and Beclin 1 function [128]. Interestingly, it appears that there is no yeast homolog of DFCP1 [128]. Live imaging experiments revealed that omegasomes form near Vps34-positive vesicles and provide membrane platforms for the accumulation of autophagy-related proteins, expansion of autophagosomal membranes, and emergence of completed autophagosomes [128]. The omegasome model was recently complemented by data from the labs of Yoshimori and Mizushima [131, 132]. Their results explain how the ER acquires PI3P, which is usually absent from this organelle. They

demonstrate that Atg14L localizes close to the omegasome-marker DFCP1 and that knockdown of Atg14L blocks the formation of these structures, indicating that Atg14L is upstream of omegasome formation [131, 132]. Accordingly, the Atg14L-dependent alteration of the ER membrane composition via the recruitment of the PI3K class III complex and the subsequent generation of PI3P represent the basis for isolation membrane formation [1, 132].

The second important PI3P effectors belong to the Atg18 family [1]. In yeast, three members have been identified so far, i.e. Atg18, Atg21 and Ygr223c [1, 133]. These proteins are WD40-repeat containing proteins and bind PI3P and $PI3,5P_2$ through the FRRG-motif [1]. It could be shown that Atg18 is important for autophagy, whereas Atg21 and Ygr223c rather contribute to the Cvt pathway and to micronucleophagy [1, 134-136]. Recently, it could be demonstrated that Atg18 supports the efficient completion of the sequestering autophagic vesicle and facilitates the recruitment of Atg8-PE (see below) to the autophagosome formation site [137]. In mammals, four Atg18 homologs have been identified, i.e. WD-repeat protein interacting with phosphoinositides (WIPI)1-4 [1, 133, 138]. It appears that WIPI1 and WIPI2 share close ancestry with Atg18, and WIPI3 and WIPI4 with Ygr223c [133].

Regarding the hierarchical recruitment of Atgs to the ER subdomain, it has been shown that Atg14L-dependent recruitment of the PI3K class III complex requires the kinase activity of Ulk1, placing the Ulk1-Atg13-FIP200 complex most upstream [131, 132]. Following the association of Ulk1-Atg13-FIP200- and Vps34-Vps15-Beclin 1-Atg14L-complexes to these ER-puncta, the two PI3P-binding proteins DCFP1 and WIPI1 are recruited. Finally, Atg5-Atg12-Atg16L1 complexes and LC3 are recruited and accordingly represent the most downstream components [131]. The Beclin 1-interacting VMP1 also localizes to this site of autophagosome formation, independently of any other known Atg proteins [131]. Interestingly, almost all mammalian Atg proteins except DFCP1 accumulate at the same compartments upon induction of autophagy. However, DFCP1 puncta are always localized adjacently to these Atg-positive structures [131].

Considering all the observations described above, a specialized subdomain of the ER appears to represent the most likely membrane source or the scaffold for autophagosome formation [1]. However, other organelles have been implicated in autophagosomal membrane generation (reviewed in [1, 139]). As described above, Ambra1 was found in complex with mitochondria-resident Beclin 1, where it potentially enhances Beclin 1-dependent autophagy [123]. In 2010, it has been reported that mitochondria supply membranes for autophagosome biogenesis during starvation [140]. The authors observed that the autophagosomal markers Atg5 and LC3 (see below) transiently localize to puncta on mitochondria. In their model, mitochondrial-derived membranes are utilized during autophagy, and autophagosome formation is dependent on ER/mitochondria connections [140]. Apparently these connections are necessary to transfer phosphatidylserine (PS) to the mitochondria, where PS is converted to phosphatidylethanolamine (PE). PE in turn is the target of Atg8-conjugation (see below). One might speculate that these mitochondria-ER connections are identical to DFCP1-positive omegasomes [140]. Alternatively, the mitochondria-ER connections might enable the transport of mito-lipids to the ER, where then autophagosome formation takes place [1]. Finally, it should be noted that other organelles than mitochondria or the ER have been implicated in

autophagosome biogenesis, including Golgi complex and endosomes, the nuclear envelope and the plasma membrane (reviewed in [1, 139])

3.2. The PI3K class III complex and protein phosphorylation

The catalytic Vps34 subunit of the PI3K class III complex itself is regulated by protein phosphorylation. It could be shown that cyclin-dependent kinase 1 (Cdk1) and Cdk5 can phosphorylate Vps34 at T159. This phosphorylation interferes with the Vps34-Beclin 1 interaction and thereby reduces Vps34 activity. Additionally, Cdk5 phosphorylates Vps34 at T668. This phosphorylation apparently has a direct negative effect on Vps34 activity, since this residue is located in the catalytic domain of the enzyme. Collectively, these two phosphorylations lead to a reduced PI3P generation and accordingly to a down-regulation of autophagosome formation [141]. Cdk1 plays an important role during mitosis, and Cdk1-mediated phosphorylation of Vps34 explains the observation that autophagy is under strict mitotic control [141-143]. By this means, the input to the autophagic compartment might be reduced during mitosis. In contrast, Cdk5 is a neuronal Cdk and has been shown to play a role in Alzheimer's disease. Abnormal activation of Cdk5 potentially contributes to neurodegeneration by negatively regulating autophagy [141].

Recently, a positively regulating phosphorylation of Vps34 has been reported. It could be demonstrated that protein kinase D (PKD) phosphorylates Vps34 at multiple sites, including T677 in the catalytic domain [144]. These phosphorylations lead to increased Vps34 activity, PI3P generation, and autophagosome formation. In line with this observation, PKD co-localized with LC3-positive structures. In addition, the authors could show that PKD acts downstream of DAPK, and that both DAPK and PKD are required for autophagy induction by oxidative stress [144].

As stated above, one way to abrogate the interaction between Bcl-2 family proteins and Beclin 1 is protein phosphorylation. Interestingly, the association between these two proteins can be disturbed by phosphorylation of either one of the two partners. Zalckvar et al. showed that DAPK phosphorylates Beclin 1 at T119, which is a crucial position within the BH3 domain of Beclin 1 [145, 146]. The authors demonstrated that this phosphorylation promotes the dissociation of Bcl-x_L from Beclin 1 and thus the induction of autophagy. Thus, it appears that DAPK regulates the Vps34-Beclin 1 complex by two independent mechanisms: 1) indirectly through phosphorylation of PKD, which phosphorylates and activates Vps34, and 2) directly through the phosphorylation of Beclin 1 [144-146]. In turn, an earlier report described the starvation-induced phosphorylation of Bcl-2 at T69, S70 and S87 by c-Jun N-terminal kinase 1 (Jnk1) [117]. Again these phosphorylations lead to the dissociation of Bcl-2 from Beclin 1 and to the subsequent induction of autophagy. The three residues are located within a non-structured loop between the BH3 and BH4 domain of Bcl-2. Interestingly, this loop is not present in viral Bcl-2 from Kaposi's sarcoma-associated herpesvirus, and accordingly viral Bcl-2 escapes this mode of regulation. Furthermore, the authors could show that Bcl-2 phosphorylation upon starvation is mediated by Jnk1 but not by Jnk2 [117]. The high mobility group box 1 (HMGB1) has also been shown to negatively

regulate the association between Bcl-2 family proteins and Beclin 1. Interestingly, two mechanistic pathways have been proposed to explain the HMGB1-mediated regulation [108-110]. First, HMGB1 itself associates with Beclin 1. Starvation-induced production of reactive oxygen species leads to the translocation of HMGB1 to the cytosol. There HMGB1 competes with Bcl-2 for Beclin 1. Apparently, an intramolecular disulfide bridge between C23 and C45 of HMGB1 is central for its interaction with Beclin 1 and its function to sustain autophagy [108-110]. Second, HMGB1 promotes activation of the Erk1/2 pathway, which results in the Erk1/2-mediated phosphorylation of Bcl-2 and the subsequent dissociation from Beclin 1 [108-110].

As discussed above, the Ulk1/2-Atg13-FIP200 kinase complex also (indirectly) regulates the function of the PI3K class III complex. Under starvation conditions, the Ulk1/2-Atg13-FIP200 complex is the most upstream unit and essential for the recruitment of the Atg14L-containing PI3K class III complex [131]. The identification of Ambra1 as a direct Ulk1 substrate might explain this observation. Ambra1 phosphorylation leads to the dissociation of the Vps34-Beclin 1 complex from the dynein motor complex and to its translocation to the ER, where autophagosome nucleation takes place (see above) [74, 75].

Regarding the downstream PI3P effectors, the lipid-binding motif of Atg18 has been identified as potential PKA phosphorylation site [48]. Interestingly, in this evolutionary proteomics approach the autophagy-related proteins Atg1 and Atg13 have additionally been detected as potential PKA substrates. The authors could confirm Atg1 as an *in vivo* PKA substrate (see above), which supports the general validity of this comparative approach [48]. It has been speculated that Atg18 phosphorylation might alter the lipid binding capacity [147].

The above described phosphorylation events regulating the PI3K class III complex are schematically depicted in figure 2.

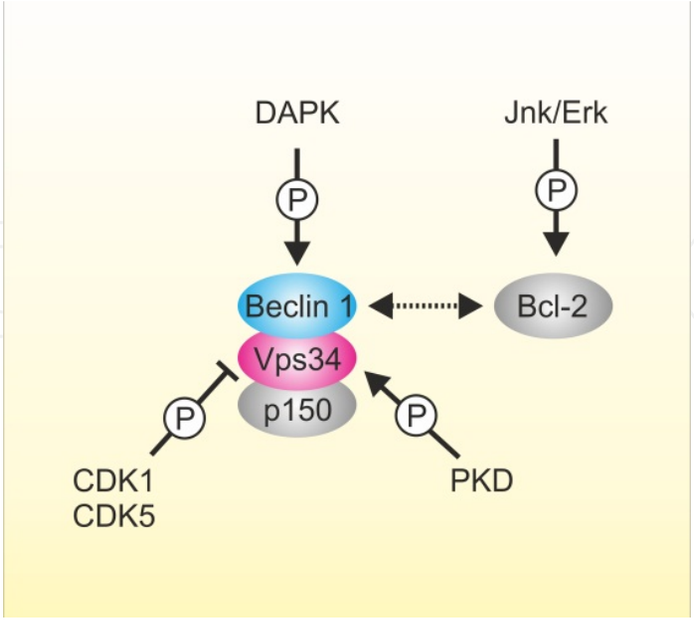

Figure 2. Phosphorylations of the PI3K class III complex. Bcl-2, B-cell CLL/lymphoma 2; CDK, cyclin-dependent kinase; DAPK, death-associated protein kinase; Erk, extracellular signal-regulated kinase; Jnk, c-Jun N-terminal kinase; PKD, protein kinase D; Vps34, vacuolar protein sorting 34.

4. Atg9

Atg9 is a multi-spanning membrane protein which is required for autophagy in several eukaryotic cells (reviewed in [148, 149]). In yeast, Atg9 localizes to the pre-autophagosomal structure (PAS) and to dispersed punctate structures in the yeast cytoplasm [149-151]. There exist two functional Atg9 orthologs in mammals, i.e. Atg9L1 and Atg9L2. Atg9L1 is ubiquitously expressed and is also termed mAtg9 or Atg9A [148]. In mammalian cells, mAtg9 localizes to the TGN, endocytic compartments, and autophagic membranes [78, 149]. Although the dynamic shuttling of Atg9/mAtg9 between these organelles has been well documented, its exact function has not been clarified yet. However, it has been speculated that Atg9 contributes to the regulation of autophagosome size or that it functions as a carrier of lipids for the forming autophagosomes [149].

4.1. Atg9 and protein phosphorylation

Under nutrient-rich conditions, yeast Atg9 localization to the PAS is mediated via its interaction with the peripheral membrane protein Atg11, which is a specific factor for the cytoplasm to vacuole targeting (Cvt) pathway [149, 152]. During autophagy, Atg9 localization to the PAS is independent of Atg11 but requires the physical interaction with Atg17 [149, 153]. This Atg17-dependent PAS localization of Atg9 requires Atg1, but not its kinase activity. This is in accordance with reports stating that Atg1 kinase activity is dispensable for the PAS organization under autophagy-inducing conditions [153-155]. However, Atg1 kinase activity is required for the regulation of the equilibrium between the assembly and disassembly of Atg9 at the PAS [153]. Furthermore, two additional components of the Atg9 cycling complex, i.e. the peripheral membrane protein Atg23 and the type I membrane protein Atg27, are required for efficient Atg9 trafficking during autophagy, but they are not essential [149]. The retrograde transport of Atg9 from the PAS to the peripheral pool involves the Atg1-Atg13 complex, the Atg9-interacting proteins Atg2 and Atg18, and the PI3K class III complex [149, 151].

In mammals, mAtg9 localizes to the TGN and a peripheral endosomal pool under nutrient-rich conditions [78, 149]. During starvation, Atg9 localizes to the peripheral pool and co-localizes with the autophagosomal membrane marker GFP-LC3 (see below) [78, 149]. Additionally, it has been reported that starvation-induced trafficking requires Ulk1 and Atg13 [19, 78]. It has been shown that siRNA-mediated Ulk1 knockdown leads to an Atg9 distribution similar to that of unstarved cells, i.e. localization to the TGN [78]. The Ulk1-mediated phosphorylation of ZIPK/DAPK3 and its contribution to mAtg9 trafficking from TGN to autophagosomes has been described above [76-78]. However, recently Itakura et al. reported that mAtg9 and the Ulk1 complex independently localize to the autophagosome formation site during starvation-induced autophagy [156]. The authors could show that recruitment of mAtg9 to the autophagosome formation site is independent of FIP200, but that mAtg9 recycling requires FIP200 [156]. FIP200-independent localization of mAtg9 to the autophagosome formation site was not only observed for canonical starvation-induced autophagy, but also for mitophagy and *Salmonella* xenophagy [156, 157].

Notably, it could be shown that mAtg9 interacts with the p38-interacting protein (p38IP) and that p38IP is required for starvation-induced mAtg9 trafficking and autophagosome formation [158]. Additionally, the authors could confirm that the MAPK p38 regulates the interaction between mAtg9 and p38IP. The following model has been suggested: in full growth medium, p38IP is found in the mAtg9 pool at peripheral endosomes and is associated with phosphorylated p38, which inhibits autophagy. Upon starvation, p38 is dephosphorylated and binds p38IP with a reduced affinity. Thus, p38 cannot longer block the autophagic process and released p38IP supports mAtg9 trafficking and autophagy [158].

5. Atg12-Atg5 and LC3(Atg8)-PE conjugation systems

In 2007, Ohsumi's group published a hierarchy map of Atgs involved in the yeast PAS organization. The two ubiquitin-like conjugation systems play a role at a late step of autophagosome formation, i.e. expansion and closure of the membrane [1, 10]. Atg12 and Atg8 represent the ubiquitin-like (Ubl) proteins, and E1-, E2- and E3-like enzymatic activities participate in the conjugation of Atg12 to Atg5 and Atg8 to PE, respectively (reviewed in [159]). Within the Atg12-Atg5 conjugation system, Atg12 is activated by the E1-like enzyme Atg7. Following activation, Atg12 is transferred to the E2-like Atg10 and then conjugated to the target protein Atg5. There is no E3-like enzyme involved in the Atg12-Atg5 conjugation system. However, Atg5 interacts with Atg16, and this Atg12-Atg5/Atg16 complex assembles to a multimeric complex consisting of four Atg12-Atg5/Atg16 units [159, 160]. Orthologs of each component of the Atg12-Atg5 conjugation system have been found in mice and humans, and their functions are similar to the yeast counterparts [159]. So far, Atg5 appears the sole target of Atg12 conjugation, and this conjugation is irreversible [159].

Within the Atg8 conjugation system, Atg7 possesses the E1-like activity and Atg3 the E2-like activity. Interestingly, the Atg12-Atg5 conjugate has been proposed to possess E3-like activity, since it accelerates the transfer of Atg8 from Atg3 to PE [159, 161]. Prior to its activation by Atg7, the C-terminal residue R117 of Atg8 is removed by the protease Atg4, leading to the exposure of G116 [162]. In contrast to the Atg12-Atg5 conjugate, the Atg8-conjugation to PE is reversible, and the cleavage is likewise catalyzed by Atg4 [162].

In mammals, there exist at least eight Atg8 orthologs, which can be subdividied into two families: 1) the LC3 subfamily consisting of microtubule-associated proteins 1A/1B light chain 3A (MAP1LC3A), LC3B and LC3C (LC3A exists in two variants generated by alternative splicing), and 2) the GABARAP-GATE16 subfamily consisting of the gamma-aminobutyric acid receptor associated protein (GABARAP), GABARAPL1, Golgi-associated ATPase enhancer of 16 kDa (GATE16, also termed GABARAPL2) and GABARAPL3 [163]. Of these, LC3B is probably the best characterized mammalian Atg8 ortholog. According to Atg8 processing, LC3B is cleaved N-terminally of G120 within six minutes after synthesis, generating a cytosolic LC3-I fragment of 18 kDa which lacks the C-terminal 22 amino acids [164, 165]. Interestingly, there exist four Atg4 orthologs in mammals (Atg4A-D, also termed autophagin-1-4), and it has been demonstrated that these Atg4 homologs have selective

preferences toward the different LC3/GABARAP family members [165, 166]. It has been shown that Atg4B (autophagin-1) has the broadest specificity for mammalian Atg8 orthologs [166, 167]. Subsequently, LC3-I is converted to LC-II by conjugation to PE. In SDS-PAGE, LC3-II migrates faster than the I-form and can be detected at an apparent molecular weight of 16 kDa [164, 165]. *In vitro*, the Atg8-like modifiers can also be conjugated to phosphatidylserine (PS). However, *in vivo* PE appears to be the predominant target, indicating that additional factors within this conjugation system ensure selectivity [168]. Again paralleling the process in yeast, LC3-II can be deconjugated by the activity of Atg4 homologs. In 2009, Satoo et al. reported the structure of the Atg4-LC3 complex. It appears that large conformational changes of Atg4 within the regulatory loop and the N-terminal tail are necessary for both the processing of the LC3-proform and the delipidation of LC3-II [169].

Both conjugates, i.e. Atg12-Atg5 and Atg8/LC3-PE, localize at membranes during the autophagic process. Whereas the Atg12-Atg5 conjugate rather localizes to the isolation membrane and is excluded from mature autophagosomes, Atg8/LC3-PE can be detected throughout "the whole life" of an autophagosome, i.e. from biogenesis to fusion with lysosomes/vacuoles [1]. Accordingly, both conjugates are commonly used for the detection of autophagic processes in different assays, including immunoblotting, fluorescence microscopy, fluorescence-activated cell sorting, and immunohistochemistry (reviewed in [170, 171]). It has been confirmed that also the other mammalian Atg8 orthologs localize to the autophagosomal membrane in its lipidated form [165].

Atg8 and its orthologs have at least two central functions during autophagy: biogenesis of the autophagosomal membrane and recognition of target cargo. Ohsumi's group could demonstrate that Atg8 mediates tethering and hemifusion of membranes, and that these two functions contribute to the expansion of autophagosomal membranes [172]. Notably, it could be demonstrated that the size of autophagosomes is directly determined by the amount of Atg8 [173]. Recently, it could be demonstrated that LC3- and GABARAP/GATE16 subfamilies are essential for autophagy but apparently act at different steps of autophagosome biogenesis. It has been suggested that LC3s are involved in the elongation of the phagophore membrane, whereas GABARAPs act at a later stage possibly in the sealing of autophagosomes [163]. Next to the regulation of membrane dynamics during autophagosome biogenesis, LC3 plays an important role in target recognition. In recent years, a new class of cargo-recognition receptors, termed autophagy receptors or adaptors, have been identified and characterized (reviewed in [174-178]). These autophagy receptors are especially important for selective forms of autophagy such as mitophagy and xenophagy, i.e. the autophagic control of intracellular pathogens. The prototype of autophagy receptors is p62 (also termed SQSTM1 or sequestome 1), and it could be shown that p62/SQSTM1 directly binds to Atg8/LC3 to facilitate the degradation of ubiquitinated protein aggregates by autophagy [179, 180]. Three features have been reported to be important for autophagy receptors: 1) direct interaction with LC3 via a so-called LC3-interacting region (LIR), 2) the inherent ability to polymerize or aggregate, and 3) the specific recognition of cargo [175]. The list of autophagy receptors is steadily growing, including p62/SQSTM1 [179, 180], NBR1 [181], NDP52 [182], and optineurin [183]. These four proteins

have in common the simultaneous binding of ubiquitin-decorated cargo and LC3/GABARAP [175, 183]. However, there exist additional autophagy receptors which interact with either ubiquitin or LC3/GABARAP or which indirectly associate with ubiquitin and LC3/GABARAP [176]. Examples are the mitochondrial protein NIX, which is a selective autophagy receptor for mitochondrial clearance, or Tecpr1, which binds Atg5 and WIPI-2 [184-186].

5.1. Atg12-Atg5/LC3(Atg8)-PE conjugation systems and protein phosphorylation

Two research groups independently reported the phosphorylation of LC3 [187, 188]. In 2010, Cherra III et al. reported that LC3B is phosphorylated at S12 by PKA. During autophagy induction, this site is dephosphorylated. Apparently, phosphorylation of S12 regulates the incorporation of LC3 into the autophagic vesicle. Notably, this PKA site is not present in Atg8 orthologs of yeast and *Drosophila*, and is also absent in the GABARAP/GATE16 subfamily [187].

Jiang et al. could show that LC3 is phosphorylated at T6 and T29 by PKC. Mutations of these residues significantly reduced LC3 *in vitro* phosphorylation by purified PKC. Notably, HEK293 cells stably expressing LC3 with these sites mutated to A, D or E did not reveal any altered autophagy. The authors suggested that PKC regulates autophagy through a mechanism independent of LC3 phosphorylation [188].

Recently, the regulation of autophagy receptors by phosphorylation emerged as an important theme for the control of selective autophagy. S403 phosphorylation of p62/SQSTM1 leads to increased affinity of the ubiquitin-associated domain of p62 for polyubiquitin chains. This residue is directly phosphorylated by casein kinase 2 (CK2) [189]. Finally, the work of two groups confirmed the importance of the TANK-binding kinase 1 (TBK1) for the xenophagy of *Salmonella*. Ubiquitin-coated *Salmonella* bacteria recruit the autophagy receptor NDP52, which in turn recruits TBK1 to the bacterial loci [182]. The autophagic adaptor optineurin constitutively associates with TBK1, indicating that also optineurin is translocated to the bacteria. The autophagic receptors NDP52 and optineurin bind to LC3 and thereby target the bacterium for autophagosomal degradation. TBK1-mediated phosphorylation of optineurin increases LC3-binding affinity and autophagic clearance of *Salmonella* [183, 190].

Ulk1 itself has been shown to interact with several members of the LC3 and GABARAP/GATE16 subfamily [66, 191]. It has been speculated that these interactions possibly play a role of vesicular transport during axonal elongation [191]. Interestingly, the recruitment of LC3 to the autophagosome formation site is dependent on FIP200 in starvation-induced canonical autophagy [131]. In contrast, recruitment of LC3 to *Salmonella*-containing vacuoles or to depolarized mitochondria is independent of FIP200 [156, 157]. Accordingly, the dependency of LC3 recruitment on the Ulk1 kinase complex appears to be different between starvation-induced autophagy and mitophagy/xenophagy.

The phosphorylations of LC3 and associated autophagy receptors are summarized in figure 3.

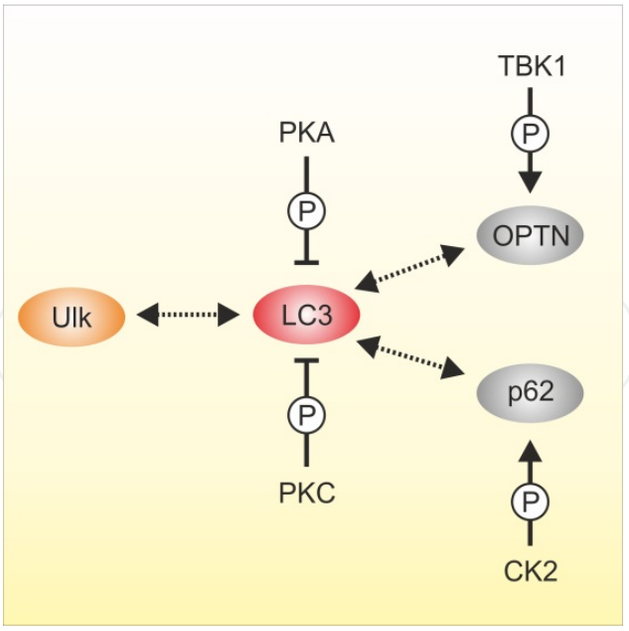

Figure 3. Phosphorylations of LC3 and autophagy receptors. CK2, casein kinase 2; LC3, microtubule-associated proteins 1A/1B light chain 3; OPTN, optineurin; PKA, protein kinase A; PKC, protein kinase C; TBK1, TANK-binding kinase 1; Ulk, Unc-51-like kinase.

6. Conclusion

In recent years, the signal transduction of autophagy has been centered in the focus of many different research areas. Autophagic processes occur at basal levels in any cell. However, under stress conditions, autophagy is dynamically induced and contributes to cellular homeostasis. This dynamic is supported by the network organization of autophagy-relevant proteins [66] and by their rapid and reversible post-translational modifications such as phosphorylation. It is likely that in the future additional phosphorylations (and alternative post-translational modifications) of autophagy proteins will be discovered, which will contribute to our deeper understanding of the autophagic machinery.

Autophagy has been implicated in different human pathologies, including cancer, infectious diseases and neurodegeneration. Accordingly, the modulation of autophagy signaling pathways represents an attractive means of therapeutic intervention. Historically, kinase inhibitors have frequently been used as therapeutic agents, and current clinical trials also target kinases involved in autophagy signaling, e.g. Akt, AMPK, or mTOR. We assume that the development of highly specific and potent Ulk1/2 kinase inhibitors will significantly contribute to the therapeutic success in settings where the blockage of autophagy is desired.

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