Atg101: Not Just an Accessory Subunit in the Autophagy-initiation Complex

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ABSTRACT. The *Saccharomyces cerevisiae* autophagy-initiation complex, Atg1 kinase complex, consists of Atg1, Atg13, Atg17, Atg29, and Atg31, while the corresponding complex in most other eukaryotes, including mammals, is composed of ULK1 (or ULK2), Atg13, FIP200 (also known as RB1CC1), and Atg101. ULKs are homologs of Atg1, and FIP200 is partially homologous to Atg17. However, the sequence of Atg101 is not similar to that of Atg29 or Atg31. Although Atg101 is essential for autophagy and widely conserved in eukaryotes, its precise function and structure have remained largely unknown. Now, structural and cell biological analysis of Atg101 together with its binding partner Atg13 reveal that Atg101 is required for stabilization of “uncapped” Atg13 in most eukaryotes and also for recruitment of downstream Atg proteins through the newly identified WF motif. By contrast, *S. cerevisiae* has stable “capped” Atg13, which does not require Atg101 for its stabilization. Possible roles for other binding partners such as Atg29, Atg31, and Atg28 in different organisms are also discussed.

Key words: Autophagy, Atg101, Atg13, HORMA

Introduction

Macroautophagy (hereafter simply “autophagy”) is an evolutionarily conserved cellular process, by which cytoplasmic constituents are degraded in the lysosome/vacuole (Fig. 1). A part of the cytoplasm is first enclosed by a membrane cisternae termed the isolation membrane (also known as phagophore). Closure of the isolation membrane edge gives rise to the autophagosome, a double-membrane organelle. Finally, the autophagosome fuses with the lysosome to result in the degradation of the engulfed materials. The complicated membrane dynamics of this process is governed by autophagy-related (Atg) proteins (Nakatogawa *et al.*, 2009). To date, 41 Atg proteins have been identified in *S. cerevisiae*, and 18 of them (Atg1-10, 12-14, 16-18, 29 and 31) are essential for starvation-induced bulk autophagy. These Atg proteins are classified into six groups, which function in a hierarchical order: (i) the Atg1 complex (Atg1, Atg13, Atg17, Atg29, and Atg31) (Table 1), which can be activated by starvation through inactivation of TORC1, initiate the autophagy process together with (ii) the Atg9 vesicles, by regulating (iii) the phosphatidylinositol (PtdIns) 3-kinase complex I (Atg14, Vps15, Vps30/Atg6, Vps34, and Atg38). Further downstream, (iv) the Atg2-Atg18 complex, and two ubiquitin-related conjugation systems, (v) the Atg12 conjugation system (Atg5, Atg7, Atg10, Atg12, and Atg16), and (vi) the Atg8 conjugation system (Atg3, Atg4, Atg7, Atg8) function to make autophagosomes.

Although these Atg proteins are mostly conserved in other organisms including mammals and plants, the components of the Atg1 complex differ between budding yeast and most eukaryotes. Atg1 is a protein kinase that phosphorylates multiple substrates such as Atg2 and Atg9 (Papinski *et al.*, 2014). Atg13 is regulated by TORC1, protein kinase A (Alers *et al.*, 2014) and possibly by AMP-activated protein kinase (Wang *et al.*, 2001). Atg17, Atg29, and Atg31 are specifically required for starvation-induced bulk autophagy, but not for selective autophagy such as the cytoplasm-to-vacuole targeting (Cvt) pathway.
Nakatogawa et al. (2009). Mammals have two Atg1 homologs, ULK1 and ULK2, and one Atg13. ULK1/2 can be directly regulated by mTORC1 and AMPK, and phosphorylates several substrates such as Beclin 1, Ambra1, and ZIPK (Russell et al., 2013; Wirth et al., 2013). Atg17 is not conserved in mammals, but a partially related protein FIP200 (also known as RB1CC1) is included in the ULK complex. An analysis of its sequence suggests that FIP200 could be a hybrid molecule of Atg17 and Atg11 (Table I) (Mizushima, 2010). Atg11 is required for selective autophagy in budding yeast, but it is also important for starvation-induced non-selective autophagy in the fission yeast *Schizosaccharomyces pombe* (Sun et al., 2013). By contrast, Atg29 and Atg31 are completely absent in mammals, plants, and even fission yeast.

In 2009, two groups independently identified Atg101, an Atg13-interacting protein required for autophagy in mammals (Hosokawa et al., 2009; Mercer et al., 2009). This was a completely novel autophagy protein, which has no homology to any of the yeast Atg proteins including Atg29 and Atg31. Later, Atg101 homologs were identified in other organisms such as filamentous fungi (Bartoszewska and Kiel, 2011), *Drosophila melanogaster* (Banreti et al., 2012), *Caenorhabditis elegans* (Liang et al., 2012), and *Arabidopsis thaliana* (Li et al., 2014). Interestingly, all these species that have Atg101 lack Atg29 and Atg31, and to date, there is no organism that has both Atg101 and the Atg29-Atg31 complex. Thus, there is clear mutual exclusiveness between Atg101 and Atg29-31 despite a lack of homology (Table I). Even their binding partners are different: Atg29-Atg31 interacts with Atg17, whereas Atg101 interacts with Atg13 (see below and Fig. 4). It was thus of great interest to elucidate the function of these molecules and to understand the molecular evolution of the autophagy initiation complex.

**Structures of the core subunits of the ATG1/ULK complex in yeast and mammals**

Atg1 consists of the N-terminal kinase domain and the C-terminal two tandem MIT (microtubule interacting and transport) domains (MIT1, MIT2). The region that connects the kinase domain and the two MIT domains is predicted to be intrinsically disordered (IDR) (Fig. 2A) (Noda and Fujioka, 2015). Mammalian ULK1/2 has a similar domain organization but with a much longer IDR. Atg13 consists of the N-terminal HORMA (Hop1, Rev7 and Mad2) domain.
and the C-terminal IDR (Fig. 2B). The IDR consists of about 450 residues in budding yeast Atg13, whereas that in mammalian Atg13 is much shorter (about 250 residues). The IDR of yeast Atg13 contains both the MIT-interacting motif (MIM), which interacts with the two MIT domains in Atg1 and the Atg17 binding region (17BR) that interacts with Atg17 (Fujioka et al., 2014). Mammalian Atg13 also seems to have an MIM at the C-terminus of IDR that is used for interaction with the MIT domains in ULK1. In fact, deletion of the last three amino acids of human Atg13 abolishes the interaction with ULK1 (Hieke et al., 2015). Mammalian Atg13 also seems to have an MIM at the C-terminus of IDR that is used for interaction with the MIT domains in ULK1. In fact, deletion of the last three amino acids of human Atg13 abolishes the interaction with ULK1 (Hieke et al., 2015). No motif has been identified that is responsible for the interaction with FIP200. In contrast to the flexible, IDR-abundant architecture of Atg1/ULK and Atg17, Atg11 and FIP200 are predicted to have a more rigid architecture that may enable them to function as a scaffold for the Atg1/ULK complex (Fig. 2C). Atg17 consists of a single helical domain (Ragusa et al., 2012). Atg11 is much longer than Atg17 and has some distinct domains such as a coiled-coil (CC) domain and the C-terminal conserved region (Atg11 domain) in addition to a region that has a weak sequence similarity with Atg17 (Li et al., 2014). Domain organization of FIP200 is more similar to Atg11 than Atg17, consisting of an Atg17-like region, CC and Atg11 domains.

In recent years, structural studies on the core subunits of the Atg1 complex made achieved progress by using homologs from thermotolerant budding yeasts, Lachancea thermotolerans and Kluyveromyces marxianus (Yamamoto et al., 2015). In 2012, the first structural study was performed on full-length Atg17 as a complex with Atg29 and Atg31. It revealed the rigid helical architecture of Atg17 and supported the idea that Atg17 functions as a scaffold for the Atg1 complex (Fig. 2F) (Ragusa et al., 2012). Following it, structural studies on Atg1/ULK1 and Atg13 were performed, whereby the domains/regions were studied individually or as a complex. The crystal structure of the N-terminal domain of Atg13 from yeasts and human revealed that it has a HORMA fold (described below and Fig. 3) (Jao et al., 2013; Michel et al., 2015; Qi et al., 2015; Suzuki et al., 2015). The crystal structure of Atg13(MIM) was determined as a complex with the C-terminal domain of
Atg1, which revealed that the C-terminal domain of Atg1 consists of two MIT domains and that the Atg1-Atg13 interaction is similar to canonical MIT-MIM interaction (Fig. 2E) (Fujioka et al., 2014). The crystal structure of Atg101-Atg13HORMA complex (PDB ID 4YK8). C. Crystal structure of human Atg101-Atg13HORMA complex (PDB ID 5C50). D. Crystal structure of thermotolerant budding yeast Atg13HORMA (PDB ID 4J2G). E. Interaction observed between the WF finger of SpAtg101 and SpAtg13HORMA in the crystal. F. Interaction observed between the WF finger of human Atg101 and Atg13HORMA in the crystal. In E and F, Atg13 is shown with a surface model, where positive and negative electrostatic potentials are colored blue and red, respectively. G. Structural comparison of the WF finger between free and Atg13-bound forms of human Atg101.

**Structural analysis of Atg101**

As mentioned above, the ULK/Atg1 complex from most eukaryotes, except for budding yeast, has Atg101 instead of Atg29 and Atg31 as an essential subunit. In 2014, sequence-based prediction suggested that Atg101 from *D. melanogaster* has a HORMA fold similar to those in Atg13 and Mad2 (Hegedus et al., 2014). Thus far, extensive structural study has been performed on Mad2, a representative of HORMA proteins, and established a unique conformational switch of Mad2 between open (O-Mad2) and closed (C-Mad2) conformations, where the N-terminal and C-terminal segments have characteristic conformations specific to each state (colored orange and purple in Fig. 3A) (Luo and Yu, 2008). O-Mad2 and C-Mad2 form a biologically important asymmetric homodimer (Fig. 3A) that stabilizes the conformation of C-Mad2 (Mapelli et al., 2007). Since coimmunoprecipitation experiments suggested that DmAtg101 self-interacts, it was proposed that DmAtg101
forms a HORMA-HORMA homodimer similar to the O-Mad2-C-Mad2 homodimer (Hegedus et al., 2014). At that point, whether the HORMA fold of DmAtg101 is similar to C-Mad2 or O-Mad2 was yet to be determined. In 2015, crystallographic study on Atg101 was first performed using a homolog from *S. pombe*, as a complex with SpAtg13<sub>HORMA</sub>, which revealed that Atg101 indeed has a HORMA fold (Fig. 3B) (Suzuki et al., 2015). SpAtg101 is topologically similar to O-Mad2 rather than C-Mad2, which is in contrast to C-Mad2-like Atg13<sub>HORMA</sub>. SpAtg101 was shown to behave as a monomer in solution and thus will not form a HORMA-HORMA homodimer. Intriguingly, the Atg101-Atg13 heterodimer is structurally similar to the O-Mad2-C-Mad2 asymmetric homodimer (Fig. 3A, B). It is likely that the closed conformation of Atg13<sub>HORMA</sub> is stabilized by O-Mad2-like Atg101 just as C-Mad2 is stabilized by O-Mad2. Soon after the fission yeast structure was reported, two groups independently reported the crystal structure of human Atg101 alone or as a complex with human Atg13<sub>HORMA</sub> (Michel et al., 2015; Qi et al., 2015). These studies confirmed that human Atg101 also has the O-Mad2-like HORMA fold and stabilizes human Atg13<sub>HORMA</sub> in a similar manner (Fig. 3C). The interface between Atg101 and Atg13<sub>HORMA</sub> is also mostly conserved between fission yeast and human and point mutation of Phe29 and His30 in SpAtg101 and their equivalent Leu30 and His31 in human Atg101 equally impaired the interaction with Atg13<sub>HORMA</sub> (Suzuki et al., 2015). One exception is the salt bridge between Atg13 Arg133 and Atg101 Asp54 in the human complex—it is absent from the fission yeast complex but seems to be highly conserved among animals (Qi et al., 2015).

Atg13 from most eukaryotes requires Atg101 for stabilization; however, Atg13 from budding yeast does not. Crystal structure of Atg13<sub>HORMA</sub> from a budding yeast, *L. thermotholerans* (Jao et al., 2013), clearly shows that stabilization of this protein does not require Atg101. LtAtg13<sub>HORMA</sub> has a unique three-stranded β-sheet, named “cap”, in addition to the C-Mad2-like fold (Fig. 3D). The cap locks the C-terminal segment of LtAtg13 through extensive interactions, which then seems to stabilize the C-Mad2-like conformation of LtAtg13 without Atg101, although the manner of stabilization by the cap is different from that by Atg101: Atg101 interacts with the C-terminal β-strands, whereas the cap interacts with the loop region preceding the C-terminal β-strands. The cap is not observed in the structures of fission yeast and human Atg13<sub>HORMA</sub> (Michel et al., 2015; Qi et al., 2015; Suzuki et al., 2015). Thus, the evidence suggest that Atg13 with a cap insertion is stable on its own whereas lacking a cap is intrinsically unstable and requires Atg101 for stabilization.

Fission yeast Atg13 has a protruding long loop that is bound to a hydrophobic pocket in a neighboring, non-target Atg13 using two aromatic residues, Trp94 and Phe95 in the crystallized complex (Fig. 3E) (Suzuki et al., 2015). Similary, the crystal structure of human Atg101 complexed with Atg13 shows that the equivalent loop region in human Atg101 interacts with a hydrophobic pocket in Atg13 and the hydrophobic compound, benzamidine, using Trp110 and Phe112 (Fig. 3F) (Qi et al., 2015). These interactions are most likely an artifact of crystallization but they indicate the potential of this loop region to interact with hydrophobic pockets in other proteins. Trp and Phe in this loop are strictly conserved among Atg101 homologs, and thus is named the WF finger. The WF finger of free human Atg101 has a distinct conformation compared with that of which found in the Atg13-bound form (Fig. 3G) (Michel et al., 2015; Qi et al., 2015). This is probably caused by the distinct environment in the crystal rather than the presence or absence of Atg13 interaction since the Atg13-binding surface is located far from the WF finger. The variable conformation of the WF finger is advantageous for its interaction with other proteins, and seems to actually mediate biologically important protein-protein interaction *in vivo* (described below).

**Function of Atg101**

ULK1, Atg13, FIP200, and Atg101 form a stable complex that can be collected in ~3-MDa fractions by size-exclusion chromatography, under both starvation and nutrient rich conditions (Hosokawa et al., 2009). The Atg13-Atg101 complex and monomeric Atg101 also exist in larger amounts. In Atg101 knockout cells, the expression levels of Atg13 and ULK1 are reduced and these proteins are dephosphorylated (Hosokawa et al., 2009). Consistently, overexpression of Atg101 prevents proteasomal degradation of overexpressed Atg13 (Mercer et al., 2009). Collectively, these data suggest that Atg101 can stabilize Atg13 through a direct binding.

When residues present on the Atg13 binding surface (e.g., Leu30 and His31) are mutated, Atg101 can no longer interact with Atg13 nor be incorporated into the ~3-MDa complex, suggesting that Atg101 is recruited to theULK complex via Atg13 (Suzuki et al., 2015). Consistently, when expressed in Atg101 knockout cells, these interface mutants of Atg101 cannot restore the stability of Atg13 and autophagic activity, unlike wild-type Atg101 (Suzuki et al., 2015). Thus, interaction between Atg13 and Atg101 is important for the stability of Atg13 and essential for autophagy (Fig. 4A).

However, the complete requirement of Atg101 in autophagy cannot be explained simply by its effect on the Atg13 stabilization because, albeit reduced, a significant amount of Atg13 remains in Atg101-deleted cells. The structural analysis suggests that Atg101 exerts another function on the other surface containing the WF finger (Suzuki et al., 2015). Although the mutations in the WF finger do not affect interaction with Atg13, incorporation
into the 3-MDa complex, and stability of Atg13, the Atg101 WF finger mutant cannot restore the autophagic activity in Atg101-deleted cells (Suzuki et al., 2015). In these cells, the Atg101 mutant is normally recruited to the ULK-FIP200 punctate structures, but the recruitment of downstream Atg proteins such as LC3 (a mammalian homolog of Atg8), WIPI1 (a mammalian Atg18 homolog), and DFCP1 is inhibited (Fig. 4A). Therefore, in addition to Atg13 stabilization, Atg101 plays a critical role in recruitment of downstream Atg factors to the autophagosome formation site through the WF finger.

Unshared subunits in other organisms

Although it remains largely unknown why Atg101 and Atg29-31 are mutually exclusive, both may have similar functions in recruitment of downstream factors. Atg29 is required for the PAS formation and recruitment of Atg2 and Atg5 in atg11-deficient yeast cells (Kawamata et al., 2008). Similarly, Atg101 is required for the recruitment of WIPI1 and LC3 in mammalian cells (Suzuki et al., 2015). Recruitment of these factors in yeast and mammalian cells is dependent on the autophagy-specific PtdIns 3-kinase activity, suggesting that Atg29-31 and Atg101 may regulate recruitment or activity of the enzyme.

Why are similar functions assigned to these totally different proteins? A possible scenario is as follows. In most eukaryotes including fission yeast, Atg13 itself is an unstable protein. As described above, Atg13 requires the additional factor Atg101 to be stable. Atg101 also enables this complex to recruit downstream Atg factors through the WF finger (Fig. 4A). On the other hand, Atg101 is lost through evolution in budding yeasts but these have compensated for this loss by acquiring the cap structure in the Atg13 HORMA domain to achieve a stable Atg13 (Fig. 4B). However, as the simple cap structure lacks the ability to recruit downstream Atg factors, Atg29 and Atg31 developed in budding yeasts, serving to recruit downstream Atg factors in the place of Atg101. Alternatively, it is also possible that capped Atg13 is the prototype, and fission yeast and higher eukaryotes acquired Atg101 and lost the Atg13 cap during evolution.

However, these models may be too simplified and cannot be applied to some organisms such as Pichia pastoris (also known as Komagataella pastoris). The primary sequence of PpAtg13 suggests of a stabilizing cap structure, similar to S. cerevisiae Atg13. Accordingly, it does not have Atg101 (Table I). However, P. pastoris does not have Atg29 and Atg31 either. Instead, P. pastoris has another Atg17-interacting protein, Atg28, which is not found in fission yeasts and animals (Fig. 4C) (Nazarko et al., 2009). Furthermore, Candida albicans, Debaryomyces hansenii, and Hansenula polymorpha have Atg28, but not Atg29 or Atg31, whereas S. cerevisiae, Candida glabrata, Ashbya gossypii, and Kluyveromyces lactis have Atg29 and Atg31, but not Atg28 (Nazarko et al., 2009). None of them have Atg101.

Atg28 is a CC protein that is required for both micropexophagy and macropexophagy (Stasyk et al., 2006) and for the recruitment of the microautophagy-specific protein Atg35 (Nazarko et al., 2011). Atg28 is also important for the Cvt pathway in P. pastoris (Farre et al., 2007; Nazarko et al., 2009). This is in contradiction to an earlier report that suggested that Atg28 can be dispensable for starvation-induced bulk autophagy in P. pastoris (Stasyk et al., 2006). However, in this report, the requirement of Atg28 in bulk autophagy was determined based on cell viability during

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**Fig. 4.** Diversity in the autophagy initiation complex. (A) The ULK1/2–Atg13–FIP200–Atg101 complex. This type is found in most eukaryotes including mammals, plants, fly, worms, and fission yeast. Atg13 in this complex is unstable and, consequently requires Atg101 to be stabilized. The WF finger motif in Atg101 is also important for recruitment of downstream Atg proteins. (B) The Atg1–Atg13–Atg17–Atg29–Atg31 complex. This type is found in S. cerevisiae, C. glabrata, A. gossypii, and K. lactis. Atg13 in this complex is stable because the “cap” (red) can stabilize the C-Mad2-like Atg13 without Atg101. (C) The Atg1–Atg13–Atg17–Atg28 complex. This type is found in P. pastoris, C. albicans, D. hansenii, and H. polymorpha. As in the complex (B), Atg13 has the “cap”. Atg28 seems to exert a function similar to that of Atg29 and Atg31.
starvation. An \textit{atg28} mutant, in fact, shows a decrease in viability during nitrogen starvation but was considered to be nearly normal as the defect was much milder than that of a \textit{pep4/prb1} double mutant. Considering that the vacuolar enzymes Pep4 and Prb1 are important not only for autophagy but also for other pathways such as endocytosis, it would be possible that the phenotype of \textit{atg28} mutant can be milder even if it has a complete block in bulk autophagy. Consistent with this assumption, Nazarko et al. showed that Atg28 is at least partially required for starvation-induced autophagy as well (Nazarko \textit{et al.}, 2009). Hence, Atg28 should not be specific to selective autophagy. Moreover, Nazarko et al. found a homology between ScAtg29 and the N-terminal half of PpAtg28 and between ScAtg31 and the C-terminal half of Atg28 and proposed that Atg28 is a hybrid molecule functionally equivalent to the Atg29 and Atg31 complex (Nazarko \textit{et al.}, 2009). All these data suggest that Atg101, Atg29-Atg31, and Atg28 alone is sufficient to act as a subunit/complex that associates with the core subunits of the autophagy-initiation complex, i.e., Atg1 (ULK1/2), Atg13, and Atg17-Atg11 (FIP200).

\section*{Conclusion}

Recent advances in the structural and functional studies of Atg101 shed light on the direct, important role of Atg101, an autophagy initiation protein whose function has been relatively unknown. The next step will be to clarify how Atg101 exerts its function using the newly identified WF finger. Collaborative structural and cell biological studies would be a key to unveil the molecular mechanisms of Atg101-mediated autophagy initiation.

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