

REVIEW ARTICLE

Autophagy-regulating protease Atg4: structure, function, regulation and inhibition

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Autophagy is an intracellular degradation system that contributes to cellular homeostasis through degradation of various targets such as proteins, organelles and microbes. Since autophagy is related to various diseases such as infection, neurodegenerative diseases and cancer, it is attracting attention as a new therapeutic target. Autophagy is mediated by dozens of autophagy-related (Atg) proteins, among which Atg4 is the sole protease that regulates autophagy through the processing and deconjugating of Atg8. As the Atg4 activity is essential and highly specific to autophagy, Atg4 is a prospective target for developing autophagy-specific inhibitors. In this review article, we summarize our current knowledge of the structure, function and regulation of Atg4 including efforts to develop Atg4-specific inhibitors.

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INTRODUCTION

Autophagy is an intracellular bulk degradation system conserved in eukaryotes from yeast to mammals and contributes to maintaining cellular homeostasis.¹ Its degradation targets are not only cytosolic proteins, but also organelles such as endoplasmic reticulum, nucleus and mitochondria, and even pathogenic microbes,^{1–4} in contrast to another major degradation system, the ubiquitin-proteasome system, whose targeting is restricted to proteins. Autophagy is induced strongly in response to environmental changes such as starvation and pathogen infection, however, it is believed that autophagy is constitutively active at a low level and contributes to intracellular homeostasis.¹ Due to these critical physiological roles, dysfunction in autophagy can play a role in or cause various diseases such as infections, neurodegenerative diseases and cancers, and thus autophagy is attracting attention as a new therapeutic target.⁵ In the autophagy process, a cup-shaped membrane structure known as an isolation membrane or phagophore suddenly appears, which elongates and seals into a double-membrane structure called an autophagosome (Figure 1a).⁶ In this process, cytosolic components are selectively or non-selectively encapsulated in the autophagosome. The autophagosome then fuses with a lysosome (vacuole in the case of yeast and plants), enabling its contents to be degraded by lysosomal hydrolases. These events are strictly regulated by autophagy-related (Atg) proteins which were identified by genetic analyses in budding yeast.^{6,7} Atg proteins are classified into 6 functional units: (1) Atg1-kinase complex, (2) phosphatidylinositol (PI) 3-kinase complex, (3) membrane protein Atg9, (4) Atg2-Atg18 complex, (5) Atg12 conjugation system and (6) Atg8 conjugation system.^{6,7} It has been supposed that the initial autophagic step is the formation of the pre-autophagosomal structure (PAS) where most Atg proteins are gathered, and that

autophagosomes are generated from the PAS by collaborative functions of the Atg proteins.^{8,9}

Atg4 is the sole protease among dozens of Atg proteins and functions as an essential factor in the Atg8 conjugation system, one of the unique mechanisms in autophagy (Figure 1b).^{10,11} In the Atg8 conjugation system, nascent Atg8 is first processed by Atg4 to expose a glycine residue at the C-terminus. The C-terminus of processed Atg8 is adenylated by the E1 enzyme, Atg7, in an ATP-dependent manner, forming an Atg8~Atg7 thioester intermediate. This intermediate enables Atg8 to form a thioester intermediate with the E2 enzyme, Atg3. Finally, Atg8 is specifically transferred to the amino-group of phosphatidylethanolamine (PE), resulting in the Atg8-PE conjugate where the C-terminal carboxyl moiety of Atg8 is covalently attached to the amine group of PE via an amide-bond. The final conjugation reaction requires the E3-like Atg12-Atg5-Atg16 complex that is also formed through ubiquitin-like conjugation reactions (Figure 1b).^{12,13} LC3-PE, a mammalian counterpart of Atg8-PE, is widely used as a marker of autophagic membranes because of its specific localization on isolation membranes and autophagosomes.¹⁴ It is believed that Atg8-PE mediates at least two functions: one is elongating and/or sealing isolation membranes and another is recognizing selective cargoes through cargo receptors/adapters.¹⁵ Besides processing Atg8 precursors, Atg4 plays another important role, that is, cleaving Atg8-PE, which is referred to as delipidation or deconjugation, between the C-terminal carboxyl moiety and the amine group of PE. Deconjugation of Atg8-PE by Atg4 has at least two roles: one is to recycle Atg8 for the next round of the conjugation reaction,¹⁶ and another is promoting the elongation step of the isolation membrane directly.¹⁷ Since both processing and delipidation reactions of Atg8 by Atg4 are important for autophagosome formation, inhibition of Atg4 leads to inhibition of autophagy at the step of autophagosome

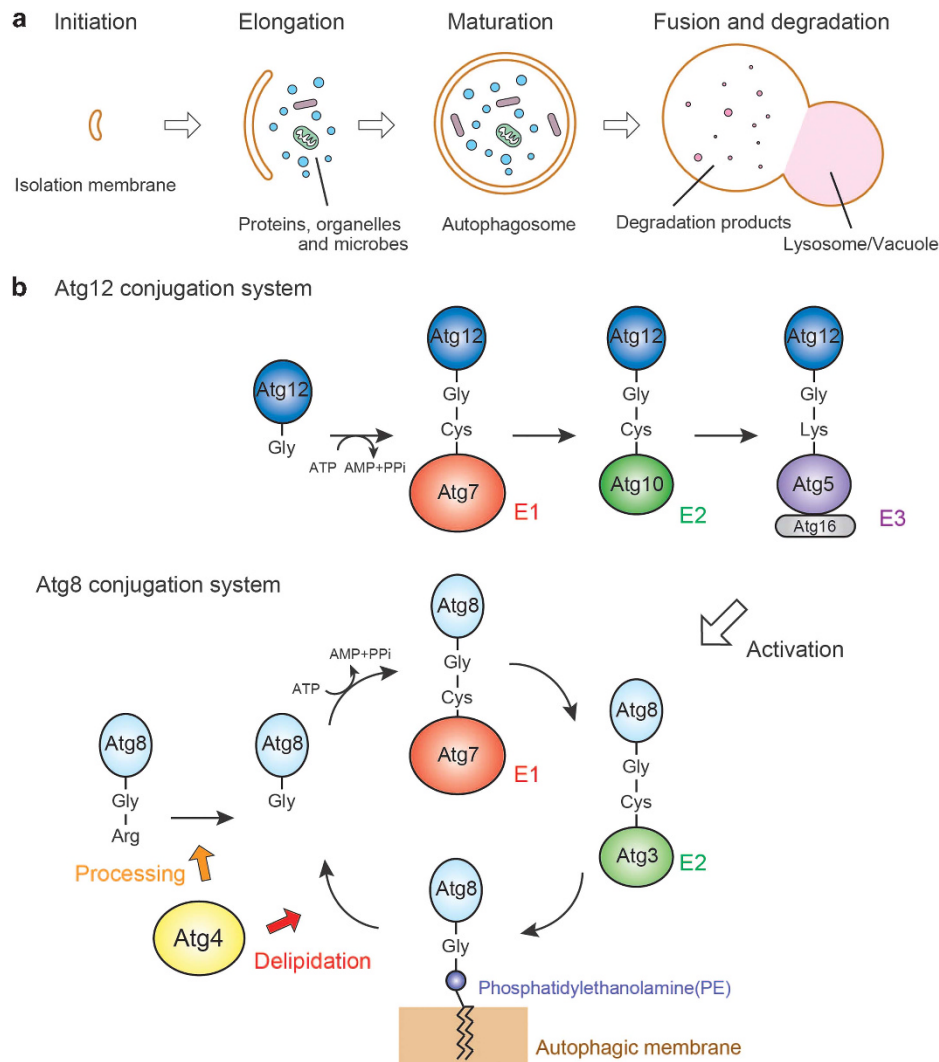


Figure 1 Basic mechanism of autophagy. (a) Membrane dynamics of autophagy. (b) Atg8 and Atg12 conjugation systems essential for autophagy.

formation, and thus Atg4 is an attractive target for developing autophagy inhibitors.¹⁸

In this review article, we summarize our current knowledge of the structure and molecular function of Atg4-family proteases. Furthermore, we will also introduce another Atg8 deconjugase, RavZ, that is not evolutionarily related to Atg4 but has a unique deconjugase activity toward Atg8-PE. Finally, we will summarize the regulation of Atg4 activity in cells and the efforts to develop Atg4-specific inhibitors. As for more physiological or medical aspects of Atg4, please refer to other excellent reviews.^{18–20}

ATG4-FAMILY CYSTEINE PROTEASES

Atg4 was originally identified in budding yeast as a sole cysteine protease specific to Atg8¹⁰ and classified as family C54 in clan CA in the protease database MEROPS.²¹ Together with Atg8, Atg4 is evolutionarily conserved from yeast to mammals. Although yeast has only one Atg4 and one Atg8 gene, higher eukaryotes conserve multiple homologs for both proteins: worms conserve two Atg4 (ATG-4.1 and ATG-4.2) and two Atg8 homologs (LGG-1 and LGG-2),²² whereas mammals conserve at least four Atg4 (Atg4A/autophagin-2, Atg4B/autophagin-1, Atg4C/autophagin-3 and Atg4D/autophagin-4)²¹

and six Atg8 homologs that are grouped into LC3-family and GABARAP-family with slightly different, open and closed conformations.^{23,24} It was reported that the protease activity is markedly different among Atg4 homologs and the susceptibility to each Atg4 homolog is also different among Atg8 homologs.^{25–29} For example, ATG-4.1 processes LGG-1 about 100-fold more efficiently than ATG-4.2,³⁰ and Atg4B processes LC3B 1500-fold or more efficiently than the other Atg4 homologs.²⁵ Atg4A prefers GABARAP-L2 to LC3B, whereas Atg4B processes all Atg8 homologs efficiently.^{25,31} Atg4C and Atg4D show little protease activity toward any types of mammalian Atg8 homologs, whereas Atg4B and Atg4C, but not Atg4A and Atg4D, complemented yeast Atg4 in autophagy.²¹ Interestingly, these four homologs show similar binding affinity to Atg8 homologs in spite of their extremely different catalytic efficiency.²⁵ Knockout mice experiments revealed both redundant and specific roles of these homologs.^{32–34} Although it has not been established, higher eukaryotes may use different homologs depending on the cell types and situations.

Although not evolutionarily related to Atg4, RavZ was identified as another Atg8-specific deconjugase from an intracellular pathogen *Legionella pneumophila*.³⁵ The activity of RavZ has two significant

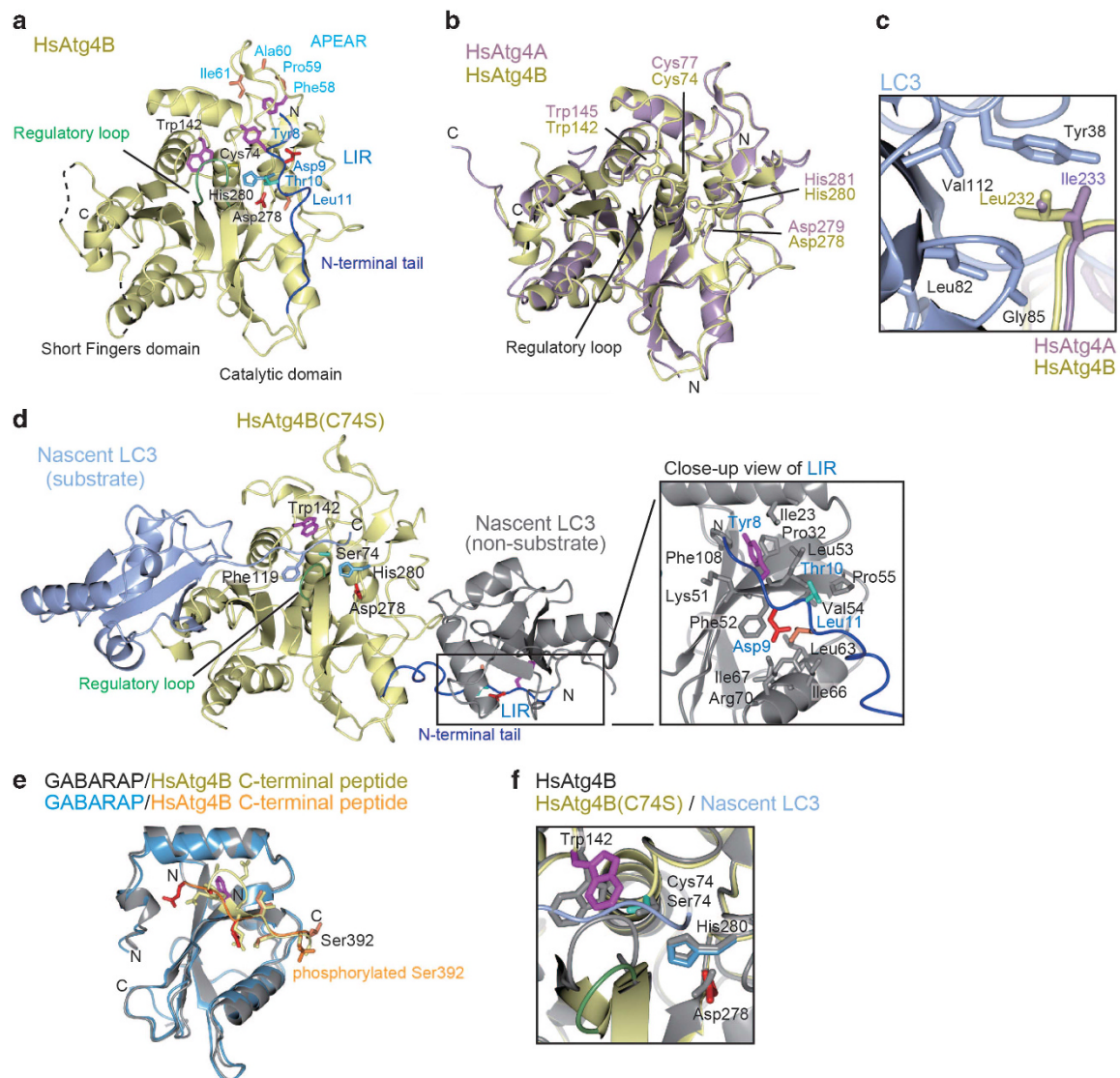


Figure 2 Structural basis of Atg4 and its interaction with Atg8. (a) Crystal structure of HsAtg4B (PDB 2CY7). The side-chains of the catalytic triad, APEAR, LIR and the regulatory Trp142 are shown with stick models, where aromatic, acidic and basic residues are colored purple, red and cyan, respectively. (b) Structural comparison between HsAtg4A and HsAtg4B. Crystal structure of HsAtg4A (PDB 2P82) was superimposed on that of the HsAtg4B-LC3 complex (PDB 2ZZP). (c) Close-up view of the interaction of Leu232 of Atg4B with LC3, where Atg4A structure is superimposed on the Atg4B-LC3 complex structure. (d) Crystal structure of HsAtg4B-LC3 complex (PDB 2ZZP). In the crystal, HsAtg4B interacts with both a substrate LC3 and a non-substrate LC3. (e) Structural comparison between Ser392-phosphorylated (PDB 5LXI) and unphosphorylated (PDB 5LXH) C-terminal tail of HsAtg4B bound to GABARAP. (f) Structural comparison of the catalytic site between LC3-bound and unbound HsAtg4B. Throughout the manuscript, protein structural models were prepared using CCP4mg.⁶⁹

differences with Atg4: one is the specific activity toward PE-conjugated forms with little activity toward precursor forms, and the other is that RavZ cleaves the peptide bond at the N-terminal side of the C-terminal glycine.³⁵ The latter activity irreversibly inactivates Atg8 and Atg8 cannot be recycled anymore, leading to the inhibition of autophagy.³⁵

ARCHITECTURE OF ATG4

Among Atg4-family proteases, crystallographic studies have been limited to *Homo sapiens* (Hs) Atg4A and HsAtg4B. The first reported structure was HsAtg4B, whose structure is composed of the two domains: the catalytic domain conserved among papain-family cysteine proteases and the short fingers domain that is inserted into the catalytic domain and is unique to Atg4 (Figure 2a).^{36,37} The active

site of HsAtg4B is composed of Cys74, Asp278 and His280, whose geometry corresponds to the canonical catalytic triad of cysteine proteases. Structure of Atg4B in a free form has an autoinhibited conformation: the regulatory loop and Trp142 interact with each other to cover the catalytic Cys74, thereby Cys74 is completely shielded from the solvent (Figure 2a).^{36,37} In addition, the N-terminal tail is bound to the exit of the catalytic site of HsAtg4B. The former autoinhibition, but not the latter, was also observed for the structure of Atg4A, which shows high structural similarity with Atg4B (PDB 2P82; Figure 2b). Among the Atg4B residues that are directly involved in LC3 binding, Leu232 is the sole residue that is not conserved in Atg4A (Leu232 in Atg4B corresponds to Ile233 in Atg4A; Figure 2c). Ile substitution of Leu232 in the HsAtg4B-LC3 complex would cause steric clash, which seems to be one reason why LC3 is more efficiently processed by

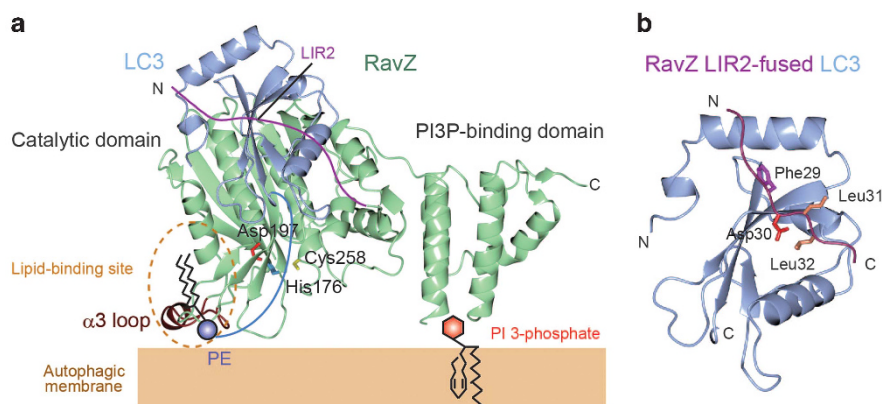


Figure 3 Structural basis of RavZ-mediated deconjugation. (a) Model of LC3-PE recognition by RavZ. Crystal structure of RavZ (PDB 5CQC) was used for model preparation. (b) Crystal structure of the LIR2 of RavZ bound to LC3 (PDB 5MS5).

Atg4B than Atg4A. Indeed, Leu mutation at Ile233 enhanced the activity of Atg4A toward LC3.³⁸ Crystal structures of the HsAtg4B-LC3 complex revealed that Atg4 recognizes Atg8 using at least two mechanisms: one is using Atg8-interacting motif (AIM)^{39,40} or LC3-interacting region (LIR)⁴¹ located at N- and C-terminal flexible tails^{38,42,43} and the other is using the enzyme body (Figures 2d and e).³⁸ Upon LC3 binding, the autoinhibitory regions mentioned above undergo a large conformational change.³⁸ The regulatory loop is lifted up by Phe119 in LC3, so that a groove is formed along which the LC3 tail enters the active site (Figure 2f). The N-terminal tail is detached from the enzyme core and is bound to another LC3 in the crystal using the Tyr-Asp-Thr-Leu sequence (Figure 2d), whose sequence and interaction manner with LC3 is a canonical type of AIM.³⁸ These observations suggest that the substrate LC3 itself possesses an ability to release the autoinhibition of Atg4 to be processed/deconjugated by this enzyme. Such mechanism would endow the enzyme with high specificity toward Atg8-family proteins. Atg4B shows much higher processing activity (10^6 times or more) toward LC3 precursors than the peptides derived from the C-terminal tail of LC3,⁴⁴ suggesting that in order to release the autoinhibited conformation of Atg4B, the ubiquitin core region of LC3 is required.

Budding yeast Atg4 also possesses multiple AIMs and uses them for efficient catalysis.⁴³ Four putative AIMs in Atg4 were analyzed, which showed that one N-terminal and one C-terminal AIM mediate interaction with unlipidated Atg8, while one AIM in the enzyme body, Phe102-Val103-Pro104-Ile105, is not responsible for unlipidated Atg8 recognition but mediates an essential role in the deconjugation of Atg8-PE, thus was named Atg8-PE association region (APEAR) motif.⁴³ Phe and Ile side-chains in the APEAR motif are not exposed and involved in hydrophobic interactions with the enzyme body (Figure 2a). Moreover, there is a proline residue (Pro104) in the APEAR motif. These features are not observed in other canonical AIMs and thus the APEAR motif would not bind to Atg8 in a way similar to canonical AIMs. They might be important for targeting Atg4 to the membrane surface where Atg8-PE exists.

ARCHITECTURE OF RAVZ, AN IRREVERSIBLE DECONJUGASE OF ATG8

As mentioned above, RavZ from *L. pneumophila* is another Atg8 deconjugase that inhibits autophagy through irreversibly deconjugating Atg8-PE.³⁵ Structural studies of RavZ revealed that RavZ is structurally related to Ulp family proteases that belong to clan CE and structurally different from Atg4-family that belongs to a different

clan, CA.⁴⁵ Structure of RavZ is composed of the N-terminal catalytic domain and the C-terminal domain, both of which are important for binding to the autophagosomal membranes (Figure 3a).⁴⁵ An extended loop with a short α -helix is inserted into the N-terminal domain, which plays an important role in both lipid-binding and catalysis. The C-terminal domain possesses the binding site for phosphatidylinositol 3-phosphate (PI3P).⁴⁵ PI3P is known to be abundant in the autophagic membranes and plays essential roles in autophagy.⁶ RavZ localizes to autophagic membranes through the interaction with PI3P.⁴⁵ In addition to the two domains, the N- and C-terminal flexible tails of RavZ contain two and one canonical LIRs, respectively, among which the N-terminal second AIM (LIR2) is the most important for binding and deconjugating LC3-PE (Figure 3b).^{46,47} It was proposed that RavZ extracts the PE moiety of LC3-PE from the membrane and deconjugates LC3-PE while binding the acyl chain of PE (Figure 3a),⁴⁶ which is in contrast to Atg4-family which is thought to deconjugate Atg8-PE without extraction. It is interesting that both RavZ and Atg4 possess multiple AIMs at the terminal tails although they are structurally and sequentially different to each other. Such information would be useful for understanding the molecular mechanism of the deconjugation of Atg8-PE by these enzymes.

ATG4 AS A TOOL TO STUDY AUTOPHAGY

Overexpression of Atg4B in mammalian cells impairs formation of LC3-PE, which indicates that excess Atg4B cleaves LC3-PE faster than the conjugation reaction of LC3-PE.⁴⁸ Interestingly, overexpression of inactive mutant (C74A or C74S) of Atg4B also impairs LC3-PE formation and inhibits autophagy through forming a stable complex with LC3, that is, sequestering LC3 from the cytosol.⁴⁸ Thus overexpression of Atg4B mutant can be used as a tool to specifically inhibit autophagy in cells, and actually many researchers have been using this method for autophagy studies.⁴⁹

Processing of Atg8 precursors by Atg4 is highly specific and efficient, and it is considered that almost all Atg8 molecules are processed by Atg4 soon after translation. Recently developed autophagic flux probe GFP-LC3-RFP-LC3 Δ G utilizes endogenous Atg4 activity in cells.⁵⁰ When this probe is expressed in mammalian cells, it is cleaved by Atg4, resulting in equimolar amounts of GFP-LC3 and RFP-LC3 Δ G. GFP-LC3 is conjugated with PE and is degraded in lysosomes by autophagy, whereas RFP-LC3 Δ G remains in the cytosol due to its inability to be conjugated with PE. Therefore, the GFP/RFP

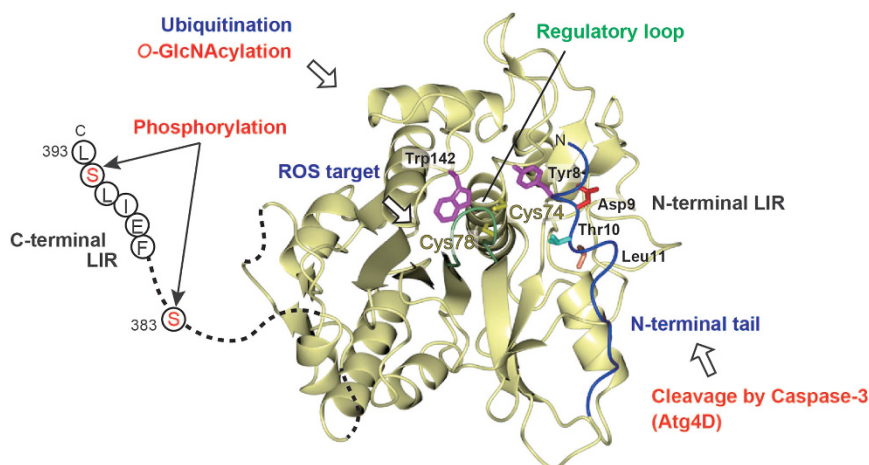


Figure 4 Regulation of Atg4B activity. Red and blue letters indicate positive and negative regulation of Atg4 activity, respectively.

fluorescence ratio reversely correlates with the autophagic activity, enabling this probe to monitor autophagic flux quantitatively.⁵⁰

REGULATION OF ATG4 ACTIVITY IN CELLS

As mentioned above, Atg4 is essential for both conjugation and deconjugation between Atg8 and PE. If the deconjugation activity of Atg4 were always high, Atg8-PE would be deconjugated before it could function in autophagy. Therefore, there must be some mechanisms that regulate deconjugation activity of Atg4. In yeast, when the processed form of Atg8, which exposes Gly116 at the C-terminus, was expressed in *atg4Δ* cells, Atg8-PE accumulated on various organelle membranes whereas co-expression of Atg4 restricted the accumulation of Atg8-PE to the PAS, suggesting that Atg4 plays a role in recycling inappropriately generated Atg8-PE.^{16,51,52} This observation suggests that deconjugation of Atg8-PE by Atg4 is suppressed specifically at the PAS. PI3P binding proteins Atg18 and Atg21, as well as the Atg12-Atg5-Atg16 complex, were shown to be required for the accumulation of Atg8-PE at the PAS, suggesting that these factors may protect Atg8-PE from Atg4 directly or indirectly.⁵³ However, it remains to be elucidated how these molecules regulate Atg4 activity at the PAS. The N-terminal tail of Atg4B negatively regulates the activity,^{25,38} and in the case of Atg4D, processing of the N-terminal portion of Atg4D by caspase-3 increases the activity against GABARAP-L1.⁵⁴ Conformational regulation of the N-terminal tail of Atg4-family through interaction with other proteins might be one mechanism that regulates PAS-specific Atg8-PE accumulation.

It was reported that reactive oxygen species, especially H₂O₂, accumulate in mammalian cells upon starvation.⁵⁵ Under the existence of antioxidants such as *N*-acetyl-L-cysteine or catalase, H₂O₂ production and localization of Atg8-family proteins to autophagic membranes were impaired, indicating that H₂O₂ is required for autophagy.⁵⁵ One of the targets for H₂O₂ is Atg4, whose catalytic Cys (Cys74 in Atg4B) or its adjacent Cys (Cys78 in Atg4B) forms a reversible sulfenic acid and loses catalytic activity, which is canceled by addition of DTT (Figure 4).⁵⁵ Later, the REDD1-TXNIP pro-oxidant complex was shown to regulate Atg4B activity through regulation of reactive oxygen species.⁵⁶ In the case of yeast, it was indicated that a disulfide bond formed between Cys338 and Cys394, both of which are not a catalytic Cys, suppresses Atg4 activity and its reduction by thioredoxin impairs autophagy through activating Atg4.⁵⁷ These observations suggest that autophagy is regulated by redox through modifying the Atg4 activity both in mammals and in yeast.

Post-translational modifications were also reported to regulate Atg4 activity (Figure 4). In mammals, Atg4B is phosphorylated at Ser383 and Ser392, which increases the activity of Atg4B and promotes deconjugation of LC3-PE, leading to autophagy progression possibly through promoting the maturation step.⁵⁸ However, it was not clear how phosphorylation at Ser392 affects the activity of Atg4B from a structural study.⁴² Atg4B is also modified with O-linked β-*N*-acetylglucosamine under metabolic stress condition, which was reported to increase Atg4B activity and activates autophagy although modification sites were not determined.⁵⁹ Ubiquitination also regulates Atg4 activity. Atg4B is recognized by a membrane-associated E3 ligase RNF5 and ubiquitinated, and finally degraded by the proteasome.⁶⁰ It was proposed that degradation of membrane-bound Atg4B through the ubiquitin-proteasome system negatively regulates autophagy through impairing the processing of LC3 that is requisite for LC3-PE formation.⁶⁰ These reports are contradictory to the reactive oxygen species-mediated regulation of Atg4B where loss of Atg4B activity leads to autophagy induction. Because Atg4 is required for both formation and deconjugation of Atg8-PE, it is not easy to expect what effect the reduction in Atg4 activity has on the level of Atg8-PE. It will be important to know when, where, and how much the modifications affect Atg4 activity in cells. More quantitative studies about the relationship among the levels of Atg4 activity, Atg8-PE accumulation, and autophagy progression are also required to understand the entire picture of autophagy regulation through Atg4.

DEVELOPING INHIBITORS FOR ATG4

Loss of function of Atg4 leads to autophagy impairment. Therefore, Atg4 can be a target for developing autophagy inhibitors. In general, it is considered that growing tumor cells activate autophagy to produce nutrients in cells. Therefore, Atg4 has been regarded as a potential therapeutic target for cancers.¹⁸ Among four mammalian Atg4 homologs, Atg4B exhibits much higher activity than the other homologs²⁵ and thus is widely used as a target for developing Atg4 inhibitors. Some studies actually showed the relationship between Atg4 and cancer: a positive role of Atg4B in the growth of osteosarcoma tumor cells⁶¹ and high-level expression of Atg4B in chronic myeloid leukemia stem/progenitor cells.⁶² Each human tumor has distinct patterns of genetic alterations in the four Atg4 genes.¹⁸ Various methods have been developed for measuring Atg4 activity *in vitro* for screening Atg4 inhibitors,¹⁹ which include fluorogenic peptides whose sequence is derived from the C-terminal tail of Atg8,⁴⁴ LC3B-PLA₂

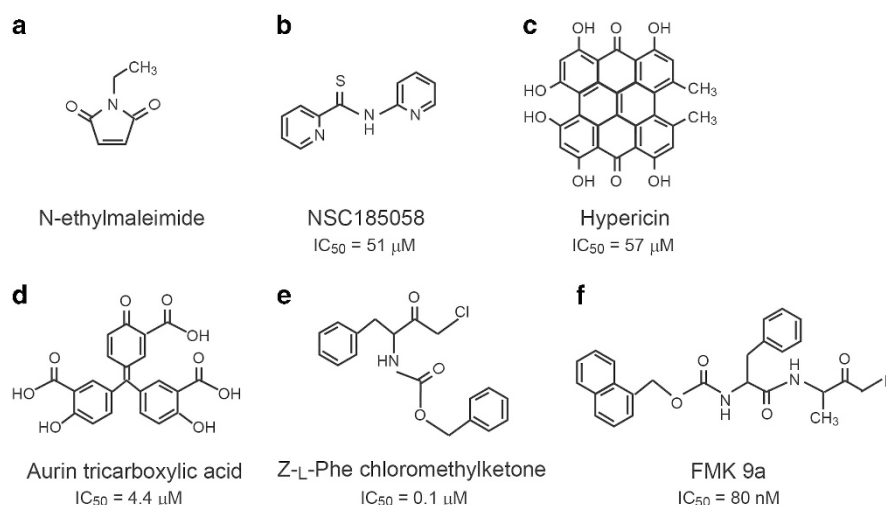


Figure 5 Summary of Atg4 inhibitors. (a) Structure of N-ethylmaleimide. (b) Structure of NSC185058. (c) Structure of Hypericin. (d) Structure of aurin tricarboxylic acid. (e) Structure of Z-L-Phe-chloromethylketone. (f) Structure of fluoromethylketone (FMK) 9a.

fusion protein,^{44,63} as well as FRET-based assays.^{64,65} Methods to measure Atg4 activity in living cells have also been developed.^{66,67} Using these methods, various inhibitors for Atg4B have been developed (Figure 5). N-ethylmaleimide is known to inhibit various cysteine proteases by irreversible alkylation of the active site cysteine (Figure 5a); however, N-ethylmaleimide was shown to be an inefficient inhibitor for Atg4B and complete inhibition of Atg4B required 20,000 molar equivalents of N-ethylmaleimide.⁶⁵ By *in silico* docking simulation of a small chemical compound library to the Atg4B catalytic site, NSC185058 was identified as an Atg4B inhibitor (Figure 5b).⁶¹ However, the inhibitory activity of NSC185058 is weak, with an IC₅₀ value of ~50 μM estimated from LC3-GST cleavage assay. High-throughput screening of Atg4B inhibitors have been performed using FRET-based assays in which CFP-LC3B-YFP or YFP-LC3B-GFP fusion protein is used.^{64,65} FRET occurs between the two fluorescent tags of these fusion proteins because the N- and C-termini of Atg8-family proteins are proximal to each other. However, the FRET signal is lost when the C-terminal fluorescent tag is removed from the fusion proteins by Atg4B, which specifically cleaves the peptide bond at the C-terminal Gly of LC3B. Thus measuring the FRET signals, Atg4B activity can be easily and quantitatively analyzed.^{64,65} Using FRET-based assays, hypericin and aurin tricarboxylic acid were identified as an Atg4B inhibitor with an IC₅₀ of 57 and 4.4 μM, respectively (Figures 5c and d).⁶⁵ By using time-resolved FRET, Z-L-Phe-chloromethylketone was identified as a more potent Atg4B inhibitor, with an IC₅₀ of 0.1 μM (Figure 5e).⁶⁸ However, the chloromethylketone moiety of the compound has high chemical reactivity, which shows cytotoxic activity. Compounds that possess both a fluoromethylketone moiety and a moiety that mimics the C-terminal sequence of LC3 were developed, among which a naphthalene-1-carboxamide analog fluoromethylketone 9a inhibited Atg4B with an IC₅₀ of the order of 100 nM (Figure 5f).⁶⁹ These types of compounds were shown to bind covalently to the catalytic Cys of Atg4B by LC-MS analysis. They do not inhibit asparagine protease, serine proteases, metalloprotease and 20S proteasome, but inhibit cysteine proteases such as cathepsin B and calpain. Thus, the improvement of specificity toward Atg4-family is an important future subject.

CONCLUSIONS

Recent structural and functional studies greatly increased our knowledge on Atg4-family proteases. However, the regulation of Atg4 in cells seems to be too complicated to be understood, and further extensive studies through combinational usage of structural biological, biochemical and cell biological techniques are required to comprehend this unique protease. Such basic knowledge will contribute to developing more specific, valuable compounds that regulate autophagy through the regulation of Atg4 activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DEDICATION

This article is dedicated to Professor Hamao Umezawa.

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