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Role of the Mammalian ATG8/LC3 Family in Autophagy: Differential and Compensatory Roles in the Spatiotemporal Regulation of Autophagy

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Abstract

Autophagy, an evolutionarily conserved cellular degradation pathway of the lysosome, is associated with many physiological and pathological processes. The hallmark of autophagy is the formation of the autophagosome that engulfs and degrades cytosolic components via its fusion with the lysosome, in either a selective or a non-selective manner. Autophagy is tightly regulated by proteins encoded by autophagy-related (*atg*) genes. Among these proteins, ATG8/LC3 is essential for autophagosome biogenesis/maturation and it also functions as an adaptor protein for selective autophagy. In mammalian cells, several homologs of yeast Atg8 such as MAP1LC3, GABARAP, and GABARAPL 1/2 have been identified. However, the biological relevance of this gene diversity in higher eukaryotes, and their specific roles, are largely unknown. In this review, we describe the mammalian ATG8/LC3 family and discuss recent advances in understanding their roles in the autophagic process.

Introduction

Autophagy is an evolutionarily conserved, highly regulated lysosomal degradative pathway for the degradation of bulky protein aggregates, which is essential for cellular maintenance and cell viability (1). Autophagy functions to eliminate unnecessary macromolecules, damaged organelles, and intracellular pathogens through the fusion of the lysosome with a double-membrane bound autophagosome, which can also sequester cargo (2). Following degradation, breakdown products are recycled in order to provide a source of metabolites that can be re-used to protect vital cell structure and functions under conditions of stress. This process is also involved in many physiological processes within cells, such as cellular differentiation, development, maintenance, and immunity, and its dysregulation is linked to many different human diseases including cancer, infectious diseases, heart diseases, liver diseases, and several neurological disorders (3). Although autophagy is generally considered as a non-selective degradative pathway, a growing body of evidence suggests that selective autophagy, with cargo specificity, also exists. Examples of the latter include mitophagy, pexophagy, aggrephagy, and xenophagy (4). Besides the degradative role of autophagy, more recently an autophagy-based secretory pathway was reported expanding its functions to non-degradative pathways (5).

Upon autophagy induction by various cellular stresses such as nutrient-deprivation, or accumulation of misfolded proteins, a cup-shaped double membrane structure called the isolation membrane is formed from intracellular membrane sources by the recruitment of multiple Atg proteins to the membrane formation site. After elongation and sealing of the autophagosome, it matures into either an amphisome or an autolysosome through fusion with the endosome or lysosome, respectively. In the autolysosome, the cytosolic cargo is finally

degraded by several lysosomal hydrolases. During the autophagic process, autophagosome formation and maturation is regulated by several autophagy-related (Atg) core proteins in a highly controlled manner (6). Among several Atg core proteins, the Atg8/MAP1LC3 (microtubule associated protein 1 light-chain 3, hereafter referred to as LC3) conjugation system is required for the elongation and maturation of the autophagosome. Atg8/LC3 is cleaved by ATG4(B) protease to expose a C-terminal glycine residue. Cleaved LC3 is then conjugated to phosphatidylethanolamine (PE) by the sequential activation of Atg7 (E1-like enzyme), Atg3 (E2-like enzyme), and the Atg12 complex, to generate LC3-PE (a membrane-bound form of LC3 also referred to as LC-II), the level of which is known to be correlated with the number of autophagosomes (Fig.2). The cellular localization of Atg8/LC3 in the autophagosome has been associated with closure, hemi-fusion, or transport of the autophagosome during its maturation . LC3 can itself be degraded by autophagy indicating that it is an autophagic substrate. Because of these features, LC3 has been widely used to monitor the number of autophagosomes as well as autophagic activity. Moreover, emerging evidence has shown that during selective autophagy, LC3 functions as an adaptor protein to recruit selective cargo to the autophagosome via interaction with cargo receptors (7). LC3 also regulates a process referred to as LAP (LC3A-associated phagocytosis), which does not have the classic double membrane autophagosome. Instead, a single-membrane structure is generated, which is involved in the efficient clearance of dead cells (8). Although there is only one Atg8 protein in yeast, there are several mammalian Atg8 orthologs, which are classified into the LC3, GABARAP (γ-aminobutyric acid receptor-associated protein), and GABARAPL (γ-aminobutyric acid receptor-associated protein like) subgroups (9). Since the discovery of mammalian homologs of Atg8, functional characterization has mostly been focused on LC3. The exact functions of each of the mammalian Atg8 paralogs and their

biological roles in autophagy in various cellular contexts are largely unknown and many key questions remain unanswered. For example; Is there any spatiotemporal specificity in paralog expression? Where are the different paralogs localized? How are the paralogs involved in different types of autophagy? Therefore, an understanding of the role of paralogs in autophagy as well autophagy-associated pathways will provide a more sophisticated understanding of the regulation of autophagic processes. Such knowledge may lead to the identification of new molecular targets that are important in modulating autophagy in several human diseases. In this review, based on recent advances, we will focus on the structures and the physiological roles of the mammalian Atg8/LC3 family in autophagy, and we will finally discuss their potential involvement in the cellular pathogenesis of human diseases.

ATG8/LC3 family: expression & structure

Yeast Atg8, is a ubiquitin-like (Ubl) protein, that, based on amino acid sequence similarity, including LC3 (A, B, C), GABARAP, GABARAPL1(GEC1), GABARAPL2 (also known as GATE-16 (Golgi-associated ATPase enhancer of 16 kDa)), and GABARAPL3 (10). The number of genes has probably arisen owing to gene duplication and loss events during evolution (9).

Among the mammalian homologs of Atg8, LC3 was first identified as the light chain of microtubule-associated protein 1A and 1B in the rat brain (LC3A and LC3B); however, its exact function in cellular transport needed to be clarified. Since then, the role of LC3 in autophagy was first described by Yoshimori's group and it has been well characterized compared to other LC3 paralogs (11). In addition, LC3C has been recently reported to have a role in autophagosome formation and COPII (coat protein complex II)-dependent ER export

via binding to TECPR2 (tectonin β-propeller containing protein 2), which could possibly represent a link between the autophagic and the secretory pathways (12).

GABARAP and GABARAPL were initially characterized as intracellular trafficking factors (9). GABARAP was shown to interact with a myriad of binding partners, including the gamma2 subunit of the GABAA receptor, tubulin and microtubules, the N-ethyl maleimide sensitive factor, gephyrin, and the transferrin receptor (13). GABARAP has been characterized in the context of being a GABA(A) receptor-associated protein and in particular it has been extensively studied in the regulation of neuronal signaling receptors (14). Recently, GABARAP has also been suggested to be a membrane-localized signaling scaffold that regulates TIAM1 (T lymphoma invasion and metastasis 1)-RAC1 signaling via association with the CUL3 (Culin3)-KBTBD6/7 (Kelch repeat and BTB domain-containing protein 6/7) ubiquitin ligase (15). GABARAPL1 (also known as GEC1 (glandular epithelial cell 1)) was first identified as an early estrogen-induced gene in quiescent guinea-pig endometrial GECs (16). Interestingly, GABARAPL1 has been implicated in several cellular processes involved in the specific regulation of estrogen hormones, the forkhead box O (FOXO) family, and circadian rhythm. GABARAPL1 dysregulation is associated with breast cancer, colorectal cancer, and neurodegenerative disease such as Parkinson's diseases (17). GABARAPL2 regulates intra-Golgi transport by linking NSF (N-ethylmaleimide-sensitive factor) and SNAREs (Soluble NSF Attachment Protein Receptors) and plays a role in post-mitotic Golgi reassembly (18).

Although Atg8/LC3 family members are ubiquitously expressed, there is some tissue specificity in their expression. The mRNAs for GABARAPL1 or GABARAPL2 are predominantly expressed in the central nervous system especially the brain. Interestingly,

GABARAPL1 is expressed specifically in the regions important for regulating somatomotor or endocrine function, such as the pons or diencephalon, suggesting a crucial roles in these processes (19). In contrast, the expression of GABARAP is found to be much higher in endocrine glands themselves. Interestingly, among the ATG8/LC3 family members, LC3C expression is found to be relatively low in nearly all tissues examined with the exception of lung where it is highly expressed (18). The expression of each ATG8/LC3 protein has been proposed to be regulated at the transcriptional, post-transcriptional, or post-translational level (20).

All Atg8/LC3 proteins have an ubiquitin-like structure despite differences in their respective amino-acid sequences. The amino-terminus is composed of an α-helix, which differs among the Atg8/LC3 members, whereas the conserved carboxy-terminus consists of an ubiquitin core composed of β-strands with hydrophobic pockets (Fig.1) (7). The C-terminal conserved ubiquitin core region is responsible for the interaction with Atg core machinery proteins such as the conjugation system. Therefore, the divergent N-terminal α-helices might be important for specific functions during autophagy via protein-protein interactions, lipid-protein interactions, or through post-translational modifications (Fig.1). Consistent with this concept, it has been reported that the first α-helix in LC3 is basic in nature, whereas that in GABALAPL2 or GABARAP is neutral or acidic, respectively (21). As is the case for LC3-I and LC3-II, all Atg8/LC3 proteins exist in two forms, namely cytosolic or membrane bound, suggesting that the processing of ATG8/LC3 family members is conserved (Fig. 2). ATG8/LC3 proteins are processed by Atg4 proteins and consistent with the presence of mammalian ATG8/LC3 paralogs, there are multiple homologs of Atg4. Atg4A is specific for GABARAP and GABARAPL2, whereas Atg4B appears to be involved in

processing all ATG8/LC3 family members although with different affinities.

Several ATG8/LC3 protein-interacting molecules have been identified and characterized in selective or nonselective autophagy. Many ATG8/LC3 interacting proteins have a basic hydrophobic AIM/LIR (ATG8-interacting motif/LC3-interacting region) motif with a core sequence of (W/F/Y)XX(L/I/V) (22). The functions of ATG8/LC3 proteins appear to depend on binding to effector proteins in a LIR-dependent manner. In the following section, we will describe the functional role of each ATG8/LC3 protein during autophagy.

Physiological functions of the ATG8/LC3 family in autophagy

Autophagosome biogenesis

1) Initial step

LC3 is localized to both the nucleus and the cytosol implying that there the protein can shuttle between the two cellular compartments. Recent studies have shown that during starvation, deacetylation of LC3 by Sirt1 (Sirtuin 1), and association with the nuclear tumor protein TP53INP2 (p53 inducible nuclear protein 2), promotes LC3 redistribution from the nucleus to the cytosol, where it associates with Atg7 and the autophagy core machinery to form autophagosomes (Fig.2) (23).

LC3 and GABARAP proteins are associated with the serine-threonine kinases ULK1/2 (uncoordinated-51-like kinase 1/2), Atg13, and FIP200 (FAK family kinase-interacting protein of 200 kDa), and they are thought to act as scaffolds for the assembly of the ULK complex (24). Interestingly, GABARAP compared to the LC3 subfamily displays a binding preference for the ULK complex (24). More recently it was reported that, during starvation-

induced autophagy, trafficking of GABARAP, but not LC3, GABARAPL1, or GABARAP2, from the centrosome specifically promotes ULK activation and this has a non-hierarchical function in the formation of the autophagosome via regulation of WAC (WW domain-containing adaptor with coiled coil) and GM130 (Golgi matrix protein of 130 kDa) proteins (25). Moreover, MAPK15/ERK8 stimulates autophagy by interacting with LC3 and GABARAP proteins, indicating that MAPK15/ERK8 is an upstream regulator of autophagosome formation that functions by recruiting LC3 and GABARAP to the autophagosome formation site (Fig.2B) (26).

2) Late step

Based on studies using yeast Atg8, ATG8/LC3 can promote membrane fusion, elongation, and sealing of the autophagosome during autophagosome biogenesis (Fig.2B) (27). Expression of a kinase-dead mutant of ATG4B in mammalian cells resulted in the accumulation of unclosed pre-autophagosomal membrane structures, suggesting that mammalian homologs contribute to the closure of the isolation membrane and to autophagosome formation (28). However, experiments wherein each ATG8/LC3 family member was knocked down using specific siRNAs have indicated that each ATG8/LC3 protein regulates a different step of autophagosome formation. LC3 mediates the elongation step of the isolation membrane, whereas GABARAP and GABARAPL2 are involved in the later steps of autophagosome formation, such as sealing or fusion (29). More recently, it has been reported that GABARAP-mediated targeting of PI4K2A (phosphatidylinositol 4-kinase type 2 alpha, also abbreviated as PI4KIIα) to autophagosomes regulates PtdIns4P-dependent autophagosome-lysosome fusion, supporting the role of GABARAP in the maturation of

autophagosomes (Fig.2B) (30). Furthermore, LC3/GABARAP proteins were reported to be associated with PLEKHM1 (Pleckstrin homology domain containing protein family member 1) and to regulate autophagosome-lysosome fusion through the HOPS (homotypic fusion and vacuole protein sorting) complex (Fig.2B)(31). Interestingly, based on a recent study using a minimally reconstituted system, both enzymatically and chemically lipidated forms of GABARAPL2 and GABARAP proteins were shown to promote extensive membrane tethering and fusion, whereas lipidated forms of LC3 did so to a much lesser extent. This suggests a preferential role for the GABARAP subfamily in membrane fusion compared to that of LC3 subfamily (15). Moreover, LC3 indirectly regulates the intracellular trafficking of the autophagosome for its fusion with the lysosome. Rab7 and its interacting protein FYCO1 (FYVE and coiled-coil domain containing protein 1) are known to regulate the transport of autophagosomes to lysosomes by interacting with LC3 and kinesin motor proteins (Fig.2B) (32).

Intriguingly, several members of TBC (Tre-2/Bub2/Cdc16), a family of GAPs (GTPase-activating proteins), interact with LC3/GABARAP proteins suggesting important roles during autophagy and vesicular trafficking. Indeed, TBC1D5 (TBC1 domain family, member 5), which has two- LIR motifs, could associate with VPS 29 (vacuolar protein sorting protein 29), a retromer complex protein, thereby linking autophagy and intracellular trafficking pathways (Fig.2B) (33).

Cargo recognition/recruitment during selective autophagy of the ATG8/LC3 family

ATG8/LC3 family also has an extensive role in cargo recognition and selective targeting to autophagosomes through specific interactions with autophagy receptors during selective

autophagy (22, 34). Autophagy receptors have a LIR motif, which mediates association with the Ubl domain of ATG8/LC3 proteins. This motif usually recognizes ubiquitinated cargo such as protein aggregates, damaged mitochondria, or peroxisomes, and targets them to autophagosomes. Since the first discovery of p62, a multifunctional protein that is an autophagy receptor for ubiquitinated protein aggregates, many ATG8/LC3 proteins have been identified as selective autophagy receptors. These include NBR1 (neighbor of BRCA1) for protein aggregates, NDP52 (nuclear dot protein of 52 kDa) and optineurin for intracellular pathogens, and Nix, BNIP3 (BCL2/adenovirus E1B 19 kDa interacting protein), BNIP3L, and FUNDC1 (Fun14 domain containing 1) for recognizing outer mitochondrial membrane proteins for the recruitment of the autophagic machinery to damaged mitochondria (9, 35). Moreover, some specificity exists in the association between ATG8/LC3 proteins and these selective autophagy receptors, although the mechanism of this specificity remains to be understood. For example, NDP52 shows preferential binding to LC3C, rather than GABARAP, or GABARAPL, whereas BNIP3L preferentially associates with GABARAPL1, rather than LC3. Binding of GABARAP to ALFY (autophagy-linked FYVE protein) is required for selective binding and the recruitment of ALFY to LC3B-positive structures (36). GABARAP regulates mitophagy via its association with the E3 ubiquitin ligase Mulan, which can interact with multiple E2-conjugating enzymes (37). LC3B/GABARAP/GABARAPL1 associate with Dvl2 (Dishevelled2), which negatively regulates the Wnt pathway (38). How specific interactions between each ATG8/LC3 protein and selective autophagy receptors are regulated, and how these interactions cooperate in a context-dependent manner will be an interesting future area of study.

Regulation of ATG8/LC3 proteins

ATG8/LC3, like other Atg core proteins can be tightly regulated at the transcriptional, post-transcriptional, or post-translational level. Recently, various transcription factors such as ATF4 (activating transcription factor 4), CEBP β (CCAAT-enhancer-binding protein β), CHOP (C/EBP homologous protein), E2F1 (E2F Transcription Factor 1), FOXO 1/3, GATA1 (GATA Binding Protein 1 or Globin Transcription Factor 1), Jun, TFEB (transcription factor EB, SREBF2/SREBP2 (sterol regulatory element-binding protein 2), and ZKSCAN3 (Zinc finger protein with KRAB and SCAN domains 3) were reported as transcriptional regulators of the ATG8/LC3 family as well as other Atg genes (20). Interestingly, among these transcription factors, only GATA1 (which activates genes involved lysosome biogenesis and function) was reported to regulate the ATG8/LC3 family during hematopoiesis (39). In addition, microRNAs such as MiR-204 have been reported to be involved in LC3B-mediated autophagy at the post-transcriptional level in renal clear cell carcinoma (40).

Among post-translational modifications, phosphorylation and acetylation of ATG8/LC3 family members have been reported. The phosphorylation of the N-terminus of LC3B by PKA is known to be negatively regulated upon autophagy induction (41). More recently, it was reported that the hippo kinase STK3 (Serine/threonine kinase 3) phosphorylates LC3 and regulates autophagosome fusion with the lysosome (42). Acetylation and deacetylation of LC3 by EP300 (E1A binding protein 300/p300) can also modulate autophagic activity. Deacetylation of LC3 promotes autophagy, whereas acetylation inhibits the autophagic process (43). Other post-translational modifications such as ubiquitination and SUMOylation of other essential autophagy proteins have also been extensively characterized. Further studies on the spatiotemporal regulation of each ATG8/LC3 at the transcriptional, post-

transcriptional, and post-translational level are required.

Conclusions and perspectives

Each mammalian ATG8/LC3 protein acts as a scaffold for autophagy core machinery and functions in autophagosome biogenesis, as an adaptor of selective cargo for recognition/recruitment to the autophagosome, or as a regulator for the activity of signaling proteins such as kinase/GTPase/GAP, or as a decision maker for cell fate.

However, many key questions remain to be answered. How is the differential interaction between mammalian homologs of Atg8 and other Atg core proteins or other cellular proteins regulated? What determines the specificity of their functions? What is the biological relevance of these interactions in vivo? Do defects in mammalian ATG8/LC3 proteins contribute to pathogenic effects during disease progression in many autophagy-associated human diseases? Recent accumulating evidence showed the alteration of level or distribution of LC3 and GABARAP in neurodegenerative diseases such as Lewy body diseases as well as some cancers (44). Therefore, in the future the elucidation of how specific ATG8/LC3 forms are altered in human diseases could provide insights for the discovery, diagnosis, or targeting of several autophagy-related human diseases.

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Figure legends

Figure 1. Domain structure of mammalian ATG8/LC3 family proteins

ATG8/LC3 family has an amino-terminal helix and a C-terminal ubiquitin core composed of β-strands with hydrophobic pockets. LC3/GABARAP(L)-I cleaved by ATG4 protease is conjugated to phosphatidylethanolamine (PE) to generate LC3II-PE. The arrow indicates the cleavage site of LC3/GABARAP(L) family proteins.

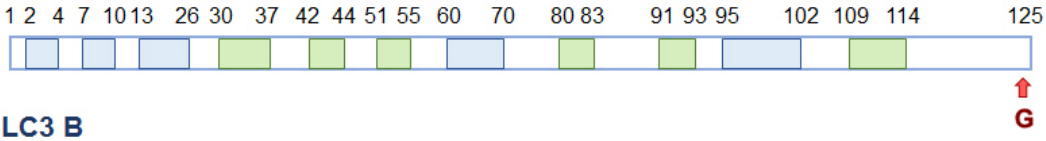
Figure 2. Differential/compensatory roles of the LC3 and GABARAP subfamilies during autophagy.

(A) Autophagy can be induced in response to a variety of cellular stresses. Once autophagy is activated, it is executed in a multi-step processes including induction, formation/elongation/sealing of the isolation membrane, cargo selection/recruitment, transport/fusion of the autophagosome, and cargo degradation in the autolysosome. (B) LC3, GABARAP, or GABARAPL, together with the autophagy core machinery proteins or specific proteins, regulate autophagosome biogenesis, cargo recognition/recruitment, transport, and fusion. Some functions, such as elongation of the membrane or cargo recruitment, are redundant between these proteins; however, other functions such as fusion or recruitment of specific cargo are differentially regulated by each ATG8/LC3 protein together with its interacting partners.

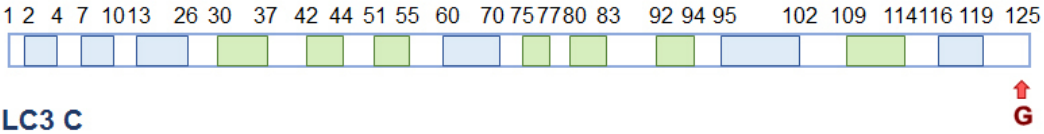
LC3 (A, B, C)

α-helix β-sheet

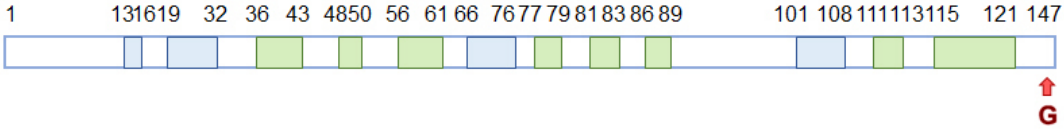
LC3 A



LC3 B

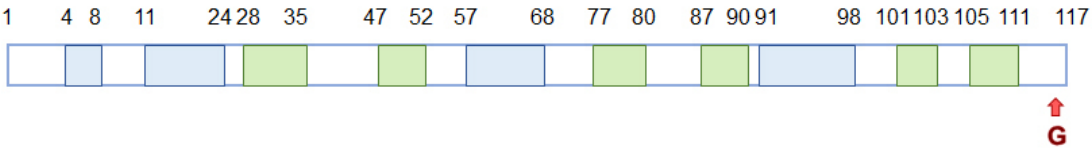


LC3 C



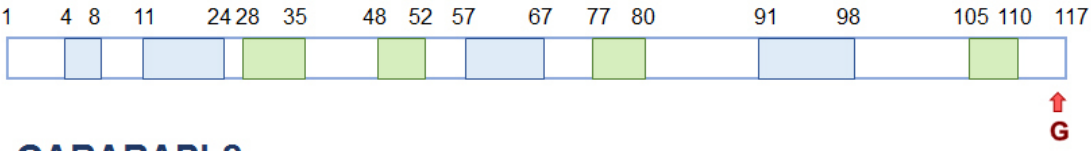
GABARAP

GABARAP



GABARAPL1

GABARAPL1



GABARAPL2

GABARAPL2

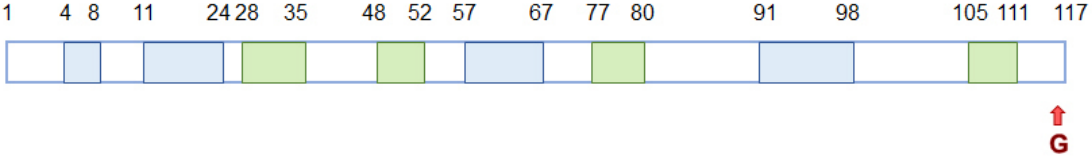
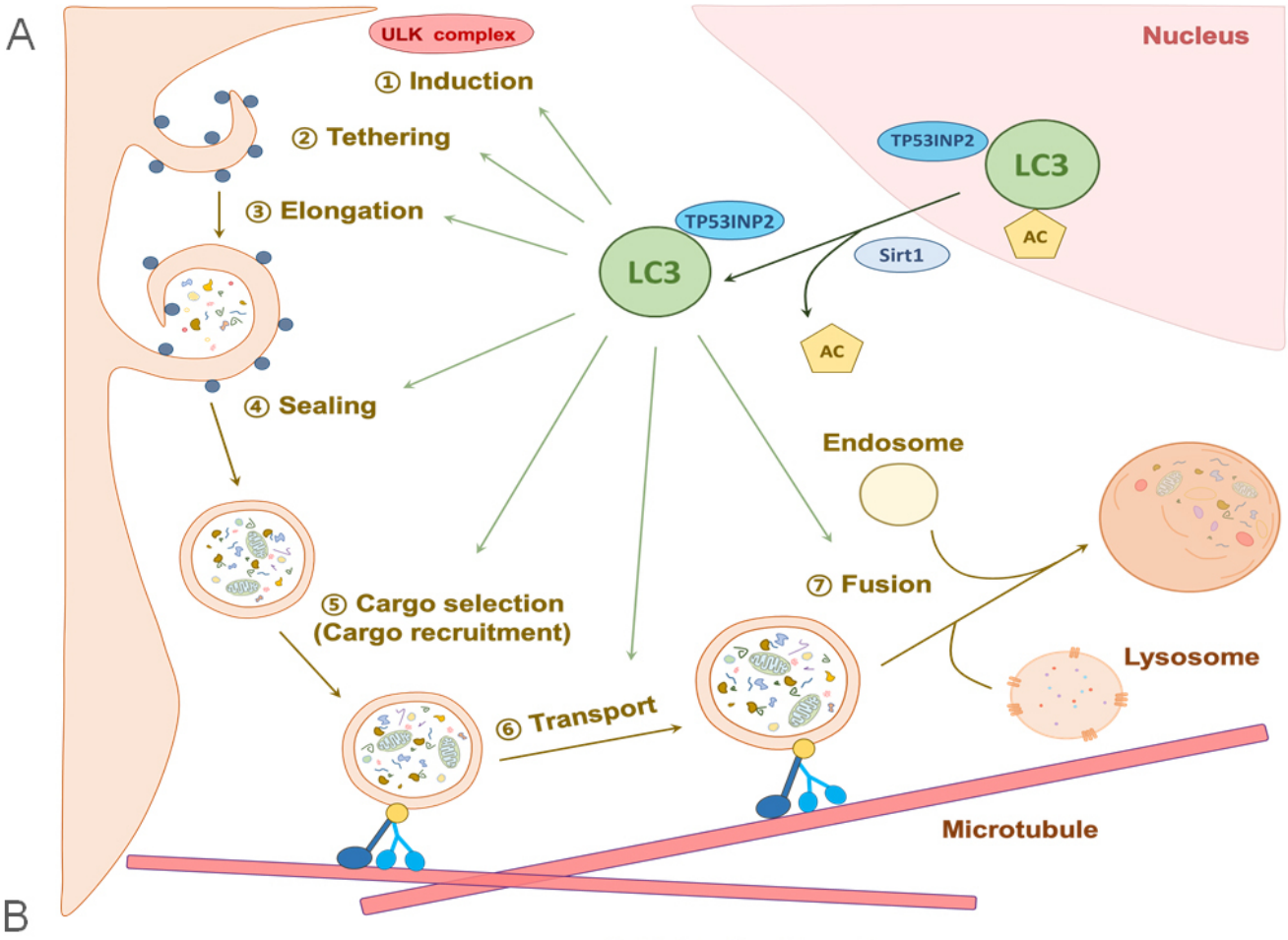


Fig. 1 Figure1

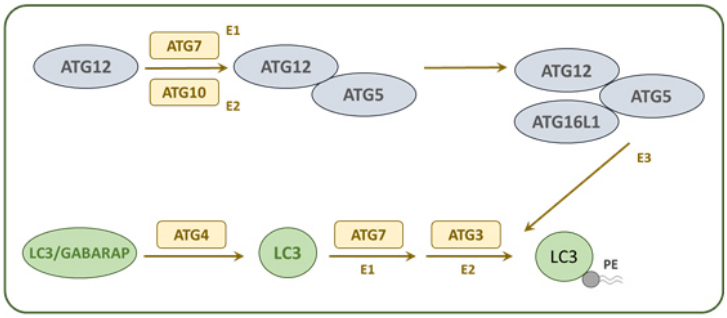


B

① Induction

ATG8/LC3 Family	Interacting Protein	Function
GABARAP/LC3	MAP15 ERK8	Initiation of autophagy
GABARAP/LC3	ULK1/2 ATG13 FIP200	Scaffold and activation for ULK complex
GABARAP	WAC/GM130	Activation of ULK1 Formation of autophagosome

②,③ Tethering / Elongation



⑤ Cargo selection (Cargo recruitment)

ATG8/LC3 Family	Interacting Protein	Function
LC3C	NDP52	Receptor for ubiquitinated bacteria
GABARAP	Multan	Regulation of mitophagy
	NBR1	Cargo receptor for misfolded proteins /aggregates
	ALFY	Recruitment of ALFY into LC3B containing structure
GABARAPL1	BNIP3L	Adaptor for autophagy receptor (mitophagy)
LC3B/ GABARAPL1/2	Dishevelled2	Negative regulation of Wnt

⑦ Fusion

ATG8/LC3 Family	Interacting Protein	Function
GABARAP/LC3	Rab7 FYCO1	Fusion of autophagosome with lysosome
	TBC1D VPS29	Endosome fusion vesicular transport
GABARAP	PLEKHM1	Fusion of autophagosome via HOPS
GABARAP	PI4K2A/PI4KIIα	Targeting of PI4K2A/PI4KIIα into autophagosome

Fig. 2 Figure2