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The multifaceted functions of ATG16L1 in autophagy and related processes

Noor Gammoh*

ABSTRACT

Autophagy requires the formation of membrane vesicles, known as autophagosomes, that engulf cellular cargoes and subsequently recruit lysosomal hydrolases for the degradation of their contents. A number of autophagy-related proteins act to mediate the *de novo* biogenesis of autophagosomes and vesicular trafficking events that are required for autophagy. Of these proteins, ATG16L1 is a key player that has important functions at various stages of autophagy. Numerous recent studies have begun to unravel novel activities of ATG16L1, including interactions with proteins and lipids, and how these mediate its role during autophagy and autophagy-related processes. Various domains have been identified within ATG16L1 that mediate its functions in recognising single and double membranes and activating subsequent autophagy-related enzymatic activities required for the recruitment of lysosomes. These recent findings, as well as the historical discovery of ATG16L1, pathological relevance, unresolved questions and contradictory observations, will be discussed here.

KEY WORDS: Autophagy, Autophagosome, Phagophore, ATG16L1, Atg16, Lipid binding, Membrane recruitment, Single-membrane lipidation

Introduction

Autophagosomes are distinct double-membrane vesicles that form *de novo* to encapsulate and deliver cellular components for lysosomal degradation in a process known as macroautophagy (hereafter referred to as autophagy). The activities of autophagy-related (ATG) proteins are essential for the recognition of degradation substrates and for autophagosome biogenesis (Gatica et al., 2018; Lamb et al., 2013). A subset of ATG proteins is also involved in so-called non-canonical autophagy or autophagy-related processes, which engage pre-formed single-membrane vesicles (Galluzzi and Green, 2019). Regulation of ATG protein activities is essential for ensuring the proper recruitment of lysosomes and to prevent the aberrant degradation of cellular contents.

At the heart of autophagy, two ubiquitin-like conjugation machineries exist (Mizushima, 2019). These act to mediate the conjugation of ubiquitin-like proteins, including ATG12 and members of the ATG8 family of proteins [which comprises the LC3 (LC3A, LC3B and LC3C; also known as MAP1LC3A, MAP1LC3B and MAP1LC3C) and GABARAP (GABARAP,

Cancer Research UK Edinburgh Centre, Institute of Genetics and Molecular Medicine, University of Edinburgh, Crewe Road South, Edinburgh EH4 2XR, UK.

*Author for correspondence (noor.gammoh@igmm.ed.ac.uk)

N.G., 0000-0001-9402-9581

GABARAPL1 and GABARAPL2) subfamilies], to ATG5 and the phospholipid phosphatidylethanolamine (PE), respectively. The two conjugation reactions require the common E1-like ubiquitin ligase ATG7, but distinct E2-like enzymes, ATG10 and ATG3, for the conjugation of ATG12 and ATG8 proteins, respectively. While no E3-like enzyme has been described for ATG12 conjugation, it is now established that the ATG5–ATG12 conjugate, in complex with ATG16L1 (Mizushima et al., 2003), harbours an E3-like activity during ATG8–PE conjugation (Fracchiolla et al., 2020; Lystad et al., 2019). Given that the ATG5–ATG12 conjugate is constitutively formed in cells, it can be predicted that the regulation of ATG8 conjugation, which is indicative of autophagic activity, occurs through the regulation of membrane availability, lipid modification, enzymatic activities and protein localisation (Martens and Fracchiolla, 2020).

ATG16L1 plays a crucial role during various steps leading to the conjugation of ATG8 proteins and accumulating recent data have revealed multifaceted activities of this essential factor for autophagy (Fig. 1). This Review will discuss the function and regulation of ATG16L1 during autophagy and autophagy-related processes in depth. The specific domains of ATG16L1 that mediate its functions, as well as its pathophysiological relevance, will also be examined.

Brief outline of autophagosome formation

The characteristic double-membrane nature of autophagosomes led to their initial identification as distinct organelles (Hollenstein and Kraft, 2020; Kawabata and Yoshimori, 2020; Yang and Klionsky, 2010) (Fig. 2A). Autophagy occurs at basal levels in cells and can be further induced upon various stimuli, such as nutrient and oxygen deprivation, as well as genotoxic and cytotoxic stresses (Antonucci et al., 2015; Kroemer et al., 2010; Mizushima et al., 2004; Russell et al., 2014). A number of protein complexes act to relay autophagy-stimulatory signals and engage in membrane modifications in order to facilitate the recruitment of the core ATG machinery to the pre-autophagosomal membrane (known as the phagophore or isolation membrane) (Box 1). Of these, the ULK complex, comprising the ULK1 or ULK2 kinases and their adaptor proteins FIP200 (also known as RB1CC1), ATG13 and ATG101, can be activated following autophagy-inducing stimuli (Wong et al., 2013), leading to the subsequent stimulation of the VPS34 complex (VPS34 is also known as PIK3C3 in mammals), which catalyses the generation of phosphatidylinositol 3-phosphate [PI(3)P] on the phagophore (Russell et al., 2014). PI(3)P is required for the phagophore recruitment of the WIPI family of proteins (including WIPI1 and WIPI2) and the ATG5 complex (comprising the ATG5–ATG12 conjugate bound to ATG16L1) (Itakura and Mizushima, 2010). The ATG5 complex can stimulate the lipid conjugation of ATG8 proteins (Johansen and Lamark, 2020; Mizushima, 2019). The eventual fusion of lysosomes with autophagosomes (forming autolysosomes) requires components of the endocytic pathway
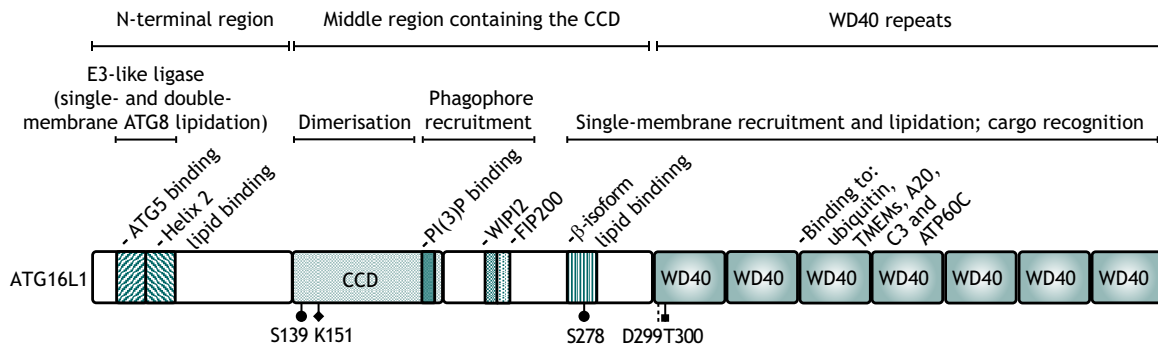
Fig. 1. Domain organisation of ATG16L1. Major domains, interacting partners (proteins and lipids) and activities of ATG16L1 are highlighted. Denoted above the protein are various regions mediating ATG16L1 activities and the locations of the three major regions (the N-terminal region, middle region and WD40 region), including those involved in double-membrane autophagosome and single-membrane vesicle ATG8 lipidations. Interacting partners are also denoted diagonally above the protein. PTMs are shown underneath the protein using oval arrowheads (S139 and S278 phosphorylation), diamond arrowhead (K151 methylation), square arrowhead (T300 Crohn's disease associated variant) and dotted line (D299 caspase cleavage site). Residue numbers correspond to human ATG16L1β sequence.

and is key for the execution of a complete autophagic degradation (known as flux) (du Toit et al., 2018; Loos et al., 2014).

Brief outline of ATG8 conjugation on single membranes
Single-membrane ATG8 conjugation (SMAC) (Durgan et al., 2020 preprint) has been reported and is a topic of emerging interest (Heckmann and Green, 2019; Sanjuan et al., 2007). SMAC involves the lipid conjugation of ATG8 proteins on already formed membranes and does not require membrane nucleation and growth, as is the case for autophagosome biogenesis (Fig. 2B). This non-canonical form of ATG8 lipidation can occur in the absence of the ULK and VPS34 complexes, but invariably requires the core ATG lipidation machinery, including ATG7 and the ATG5 complex (Florey et al., 2015, 2011; Martinez et al., 2011). Our current understanding of the underlying molecular mechanisms that mediate SMAC and the relevance of ATG16L1 will be discussed throughout this Review.

Identification and structural organisation of ATG16L1/Atg16
Identification
The identification of most ATG gene products (Atg1–Atg15) was performed in yeast using a screen for mutants that were unable to grow under nitrogen starvation conditions (Tsukada and Ohsumi, 1993). Atg16 (initially termed Apg16) was identified several years later using a yeast two-hybrid screen for yeast Atg12-interacting partners (Mizushima et al., 1999) and was followed by the identification of mammalian ATG16L1 (initially termed Apg16-like, Apg16L) through co-immunoprecipitation with ATG5 from mouse cells (Mizushima et al., 2003). Mammalian ATG16L1 and yeast Atg16 share high similarity except for the presence of additional sequences within the C-terminal of ATG16L1 that are absent in its yeast counterpart (Fujioka et al., 2010) (Table 1). Three main splice isoforms of mouse ATG16L1 were identified (α, β and γ) that differ in sequences within their middle region as well as exhibit variable tissue expression patterns (Ishibashi et al., 2011; Mizushima et al., 2003).

Domains and structural organisation
ATG16L1 can be divided into three main domains that contribute to its distinct functions: an N-terminal region containing an ATG5-binding domain, a middle region containing a coiled-coil domain (CCD), and seven WD40 domains located within its C-terminal half (Fig. 1). The tertiary structures of these three domains have been individually determined providing a prediction of the overall

structural organisation of the full-length protein (Wilson et al., 2014). Mutations in individual domains of ATG16L1 enable the distinction of various forms of autophagy and autophagy-related processes (Fletcher et al., 2018; Gammoh et al., 2013).

ATG16L1/Atg16 proteins likely exist in dimeric forms (Fujioka et al., 2010; Fujita et al., 2009). Dimerisation of ATG16L1 occurs mainly through CCD sequences (Fujioka et al., 2010; Parkhouse et al., 2013) although dimerisation activities have also been detected in the N-terminal and WD40 domains of ATG16L1 (Bajagic et al., 2017; Kim et al., 2015). Size-exclusion chromatography indicates that ATG16L1 exists in an ~800 kDa complex in cells, which is about three times the predicted size of an individual ATG16L1 dimer bound to two ATG5–ATG12 molecules (~250 kDa in size) (Ishibashi et al., 2011; Lystad et al., 2019; Mizushima et al., 2003). The two factors that are required for formation of this large molecular complex are binding to ATG5 and dimerisation via the CCD (Mizushima et al., 2003; Saitoh et al., 2008). The precise components of this higher molecular mass complex are not fully understood. It is possible that solution-exposed amino acid residues within the CCD can mediate multimerisation of ATG16L1 as suggested by computational modelling (Kaufmann et al., 2014). This, however, remains to be experimentally confirmed as the use of sucrose density gradients as alternative methods to predict complex sizes suggest that ATG16L1 is mainly found as a dimer in cells (Fujita et al., 2009).

N-terminus
The N-terminus of ATG16L1 contains an α-helix (termed helix 1) required for binding to the ubiquitin-folds of ATG5 (Matsushita et al., 2007; Otomo et al., 2013). This interaction requires residues R35 and F46 of yeast Atg16 (corresponding to R24 and I36 of mammalian ATG16L1) and is essential for autophagy (Matsushita et al., 2007). The existence of additional residues in mammalian ATG16L1 (I17 and L21) required for ATG5 binding may suggest a divergence in the interaction between ATG16L1/Atg16 and ATG5/Atg5 between mammalian and yeast proteins (Kim et al., 2015; Mizushima et al., 2003). A second amphipathic helix (designated as helix 2) within the N-terminus of ATG16L1 harbours a membrane-binding activity and is also required for ATG8 lipidation (Lystad et al., 2019) (Fig. 1). The lipidation stimulatory activity of the ATG16L1 N-terminus is further supported by its ectopic plasma-membrane tethering, which was shown to be sufficient to drive LC3 lipidation in cells (Fujita et al., 2008). The E3-like ubiquitin ligase activity of the ATG5 complex is discussed further below.
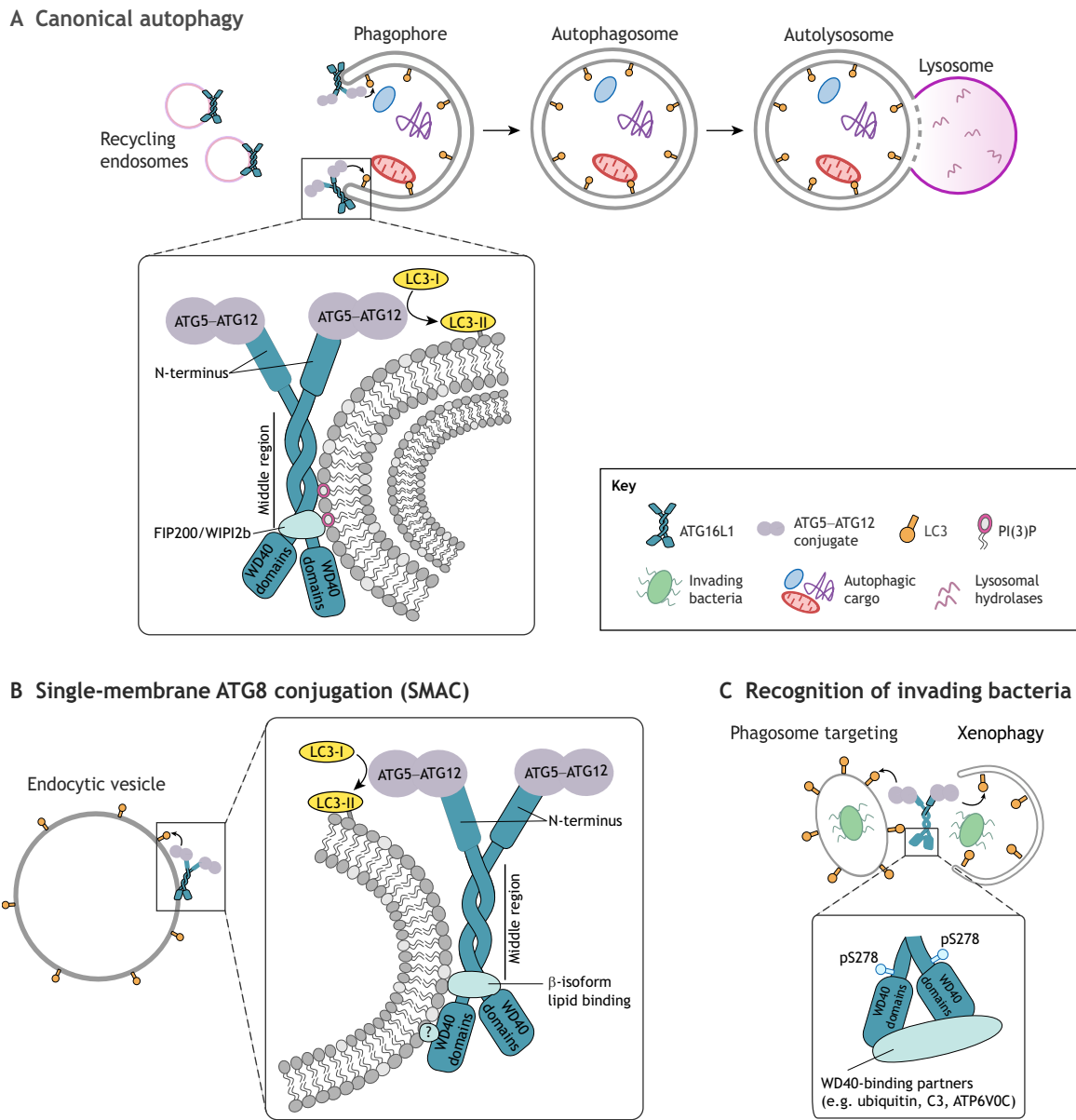
Fig. 2. Relevance of ATG16L1 during autophagosome biogenesis and single membrane ATG8 lipidation. (A) Autophagosome biogenesis requires the formation of a precursor, a double-membrane structure termed the phagophore. ATG16L1 is recruited to the phagophore through its ability to bind FIP200, WIPI2b and PI(3)P. The localisation of ATG16L1 to endocytic vesicles (recycling endosomes) suggests that it functions in contributing to phagophore growth. These events facilitate ATG8 lipidation (LC3-I to LC3-II conversion) on the phagophore and contribute to autophagosome maturation, which subsequently fuse with the lysosome system forming an autolysosome. (B) Single-membrane ATG8 conjugation (SMAC) occurs on pre-formed vesicles such as entotic bodies, phagocytosed bacteria and perturbed endosomes. This requires various domains within ATG16L1, including N-terminal sequences (ATG5 and lipid binding), the β-isoform lipid binding sequence, and sequences within the WD40 domain. (C) ATG16L1 is also involved in initiating autophagosome biogenesis at the site of invading bacteria during xenophagy (double membrane autophagosome, right), as well as initiating SMAC on phagocytosed bacteria (single-membrane vesicles, left). Recognition of invading bacteria by ATG16L1 requires various binding partners of the WD40 domains and is enhanced by S278 phosphorylation (pS278).

CCD and the middle region

Coiled-coil regions are composed of helical structures that are known to contribute to protein–protein interactions. In ATG16L1, hydrophobic sequences within the CCD mediate its homodimerisation (Fujioka et al., 2010), while solution-exposed residues can mediate binding to lipid and protein interactors, including phospholipids (Dudley et al., 2019) and Rab33B (Itoh et al., 2008). Interacting partners of yeast Atg16 CCD were also identified, including the PI(3)P effector Atg21 (Juris et al., 2015). Further downstream of the CCD within the middle region of ATG16L1, sequences that mediate interactions with WIPI2b (an isoform of WIPI2) (Dooley et al., 2014), FIP200 (Gammoh et al.,

2013; Nishimura et al., 2013) and lipids (Lystad et al., 2019) have been described. These sequences are absent from yeast Atg16 and their lengths differ between the ATG16L1 isoforms (highlighted in Table 1).

The CCD is essential for autophagy and its deletion causes neonatal lethality in mice, which mimics what is seen upon knockout of core ATG genes (Saitoh et al., 2008). The importance of the middle region of ATG16L1 has been further highlighted by several observations. First, overexpression of ATG16L1 in some cell lines leads to the inhibition of LC3 lipidation in a manner dependent on its middle region (amino acids 80–265 of mouse ATG16L1) (Fujita et al., 2008; Li et al., 2017a). Second, mutations

Box 1. A brief overview of autophagosome formation
Autophagy occurs at basal levels in cells and can be further induced through various stimuli such as nutrient and oxygen deprivation as well as genotoxic and cytotoxic stress (Antonucci et al., 2015; Kroemer et al., 2010; Mizushima et al., 2004). A number of signalling cascades can regulate autophagy (Russell et al., 2014). In the case of nutrient deprivation, the mammalian target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK) pathways have been most widely studied with respect to their abilities to suppress or activate autophagy, respectively. These sensing complexes act to regulate autophagy by mainly phosphorylating members of the Unc-51-like kinase (ULK) complex, although additional regulatory mechanisms have been described (Wong et al., 2013). The ULK complex is important for the regulation of autophagy during nutrient sensing (Ganley et al., 2009; Hosokawa et al., 2009; Jung et al., 2009; Mercer et al., 2009). Optimal kinase activity of ULK1 requires autophosphorylation and binding to its adaptor proteins, and acts to activate downstream factors including the VPS34 complex (Wong et al., 2013; Zachari and Ganley, 2017). The VPS34 complex, comprising the VPS34 lipid kinase, ATG14, Beclin-1 and VPS15 (also known as PIK3R4) (Itakura et al., 2008), catalyses the production of PI(3)P on the phagophore, which is essential for the recruitment of subsequent ATG players (including WIPI2, the ATG5 complex and ATG2 proteins) as well as for ATG8 lipidation (Dudley et al., 2020). The final stages of autophagy involve autophagosome maturation and closure, followed by lysosome fusion (forming the autolysosome) and require the lipidation of the ATG8 family members for their efficient execution (Johansen and Lamark, 2020; Nakamura and Yoshimori, 2017; Nguyen et al., 2016). Fusion between autophagosomes and lysosomes in the absence of ATG8 lipidation has been reported but occurs at a slower rate (Tsuboyama et al., 2016). The ATG8 family members are also involved in cargo and cargo receptor recognition although upstream ATG proteins (such as FIP200) may also play a role (Kirkin and Rogov, 2019; Turco et al., 2019). Lipidated ATG8 proteins (such as lipidated LC3, LC3-II) that are tethered to the outer autophagosomal membrane can be cleaved by the action of ATG4 proteases to generate delipidated proteins (for example, LC3-I), which can be recycled back to the cytoplasm (Maruyama and Noda, 2017).

in conserved and solvent-exposed residues of yeast Atg16 CCD that do not contribute to the dimer–dimer interface result in autophagy inhibition, suggesting a relevance of the CCD beyond dimer formation (Fujioka et al., 2010). Finally, the structurally related and closely similar ATG16L2 lacks LC3-lipidation-stimulating activity during canonical autophagy and can potentially exhibit inhibitory effects (Ishibashi et al., 2011; Khor et al., 2019; Wible et al., 2019). Elegant domain-swapping experiments suggest that replacing the middle region of ATG16L2 with that of ATG16L1 can reconstitute LC3 lipidation to levels that are comparable to those seen with wild-type ATG16L1 (Ishibashi et al., 2011). The absence of a localisation of ATG16L2 to phagophores is likely to explain its lack of autophagic activity as the ectopic localisation of its N-terminus to the plasma membrane can stimulate LC3 lipidation, as seen with ATG16L1 (Fujita et al., 2008; Ishibashi et al., 2011). ATG16L2 can bind ATG5 and homodimerise, but it does not bind WIPI2b nor FIP200 and has reduced affinity to Rab33B, further highlighting the importance of the middle region of ATG16L1 (Dooley et al., 2014; Gammoh et al., 2013; Ishibashi et al., 2011). As residues that mediate these interactions are absent in yeast Atg16, it remains to be determined whether binding to these factors is sufficient to explain the lack of ATG16L2 autophagic activity.

WD40 domains
Seven WD40 domains span the C-terminal half of ATG16L1 and are absent in Atg16, indicating that they are dispensable for the role of ATG16L1 in canonical autophagy (Fletcher et al., 2018;
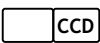
Gammoh et al., 2013) (Fig. 1). Recent studies have begun to unfold a non-canonical SMAC function for this region (Fletcher et al., 2018). The WD40 domains of ATG16L1 form a seven-bladed propeller (Bajagic et al., 2017) and are involved in multiple protein–protein interactions, as discussed further below. Mice harbouring deletions in the WD40 domains of ATG16L1 exhibited no phenotypic abnormalities or neonatal fatality in contrast to what is seen upon the loss of ATG16L1 CCD (Rai et al., 2019; Saitoh et al., 2008). Bone marrow-derived dendritic cells from mice lacking the WD40 domains of ATG16L1 showed defective MHC class II antigen presentation attributed to the non-canonical activity of ATG16L1 in facilitating LC3 lipidation on single membranes (Fletcher et al., 2018). In addition, these animals exhibited an increased sensitivity to influenza A infection and reduced survival when compared to their control counterparts (Wang et al., 2020 preprint). The exact mechanisms through which the WD40 domains mediate SMAC remain to be determined and likely involve as-yet-unidentified interactions that include conserved residues within the top face of the WD40 domains (Fletcher et al., 2018).

Function of ATG16L1 at different stages of autophagy
Phagophore recruitment of the ATG5–ATG12 conjugate
Accumulating recent evidence shows that the phagophore recruitment of the ATG5 complex is mediated through ATG16L1 (Dudley et al., 2019; Juris et al., 2015; Li et al., 2017a) and is therefore a critical regulatory step during autophagosome biogenesis. The phagophore localisation of ATG16L1 requires components of the ULK complex, as well as VPS34-mediated PI(3)P production (Fig. 2A) (Itakura and Mizushima, 2010). Live-imaging studies suggest that ATG16L1 (bound to ATG5) and the ULK complex concurrently localise to the growing phagophore (Koyama-Honda et al., 2013). This is likely mediated by the direct binding of ATG16L1 to the ULK complex component FIP200 (Gammoh et al., 2013; Nishimura et al., 2013). Genetic inhibition of FIP200 or deletion of the FIP200-binding domain of ATG16L1 abrogates puncta formation of the ATG5 complex (Gammoh et al., 2013; Nishimura et al., 2013). The phagophore localisation of ATG16L1, but not the ULK complex, requires PI(3)P (Itakura and Mizushima, 2010), indicating that it may occur in a two-step manner, whereby initial recruitment of ATG16L1 is followed by its stabilisation on membranes.

The requirement of PI(3)P for the phagophore recruitment of ATG16L1 can be mediated by direct interaction of ATG16L1 to phosphoinositides, as well as binding to the PI(3)P effector WIPI2b (Dooley et al., 2014; Dudley et al., 2019). The requirement of multiple interaction partners may be important to prevent the aberrant recruitment of ATG16L1 on other membranes (Dudley et al., 2019; Ravikumar et al., 2010). Yeast Atg21 (a phosphoinositide-binding protein) has been shown to bind the CCD of Atg16 (residues D101 and E102) (Juris et al., 2015). Genetic deletion of Atg21 in yeast does not fully disrupt starvation-induced Atg8 lipidation (Meiling-Wesse et al., 2004; Nair et al., 2010) but reduces autophagy and Atg16 phagophore recruitment under nutrient-rich conditions (Juris et al., 2015). Given that the Atg16^{D101A,E102A} mutant is deficient in autophagy (Fujioka et al., 2010), it is possible that these sites mediate additional autophagic functions of yeast Atg16. Interestingly, similar to the interaction between mammalian ATG5 and ULK complexes that are mediated by binding between ATG16L1 and FIP200 (Gammoh et al., 2013; Nishimura et al., 2013), yeast Atg5 complex can also interact with the Atg1 (ULK1 homologue) complex but requires binding between Atg12 and Atg17 (a functional homologue of FIP200) (Harada et al., 2019). This suggests that diverged but multi-factorial

Table 1. Comparison of ATG16L1-related proteins

Isoform	Dimerisation	ATG5	FIP200	WIPI2	CCD–lipid	Helix 2–lipid	β-isoform–lipid	Notes and distinctive features	Diagram
ATG16L1α	✔	✔	✔	✔	✔	✔	✗	Lacks C-terminal sequences required for single-membrane ATG8 lipidation	CCD — WD40 domains
ATG16L1β	✔	✔	✔	✔	✔	✔	✔	Supports single- and double-membrane ATG8 lipidation	CCD — WD40 domains
ATG16L1γ	✔	✔	✔	✔	✔	✔	✔	Longest ATG16L1 isoform mainly expressed in the brain and heart	CCD — WD40 domains
ATG16L2	✔	✔	✗	✗	?	?	?	Divergence of unspecified middle region sequences results in autophagic inactivity	CCD — WD40 domains
Atg16 (yeast)	✔	✔	n/a	n/a	?	?	n/a	Lacks C-terminal sequences including the WD40 domains	CCD

Comparison of mammalian ATG16L1 (α, β and γ isoforms) and ATG16L2 as well as yeast Atg16 proteins. The relevant binding partners and dimerisation capacity are compared in the table. Activities that have not yet been tested are marked with a question mark (?) and predicted activities based on sequence similarities are marked with a grey tick mark (✔). Diagrams depict major domains and relative length.

mechanisms exist to target the ATG5 and Atg5 complexes in mammalian and yeast systems, respectively.

In mammals, PI(3)P, WIPI2b and FIP200 have been shown to bind adjacent residues in the middle region of ATG16L1 (Fig. 1). How these interacting partners crosstalk to mediate the efficient phagophore recruitment of ATG16L1 remains to be determined. Given that these three factors are dispensable for the role of ATG16L1 in mediating LC3 lipidation during mTORC1-independent autophagy or on single membranes, it is likely that additional means exist to mediate ATG16L1 localisation to membranes (Fletcher et al., 2018; Gammoh et al., 2013; Lystad et al., 2019).

In addition, the displacement of ATG16L1 from membranes also appears to be important for LC3 lipidation with its regulation an open question. Enhancing the lipid-binding activity of ATG16L1 within its CCD, which is required for its phagophore recruitment, leads to the inhibition of LC3 lipidation (Dudley et al., 2019). Similarly, unlike the plasma membrane tethering of the N-terminal region of ATG16L1, tethering of a fragment that also includes the middle region (amino acids 1–249) to the plasma membrane inhibited LC3 lipidation (Park et al., 2016). These findings suggest that persistent membrane localisation of ATG16L1 can impose inhibitory effects on LC3 lipidation and its regulation is therefore crucial for autophagy.

Membrane nucleation
Beyond its role in recruiting the ATG5 complex to the phagophore, ATG16L1 can facilitate phagophore growth by localising to endocytic vesicles, which subsequently undergo fusion events (Fig. 2A) (Puri et al., 2013; Ravikumar et al., 2010). Recycling endosomes that are positive for Rab11 and ATG16L1 are thought to originate from the plasma membrane, thereby potentially acting as a membrane source for autophagosome biogenesis (Puri et al., 2013; Ravikumar et al., 2010). However, the localisation of ATG16L1 to endosomes is not sufficient to drive LC3 lipidation, as transiently expressed ATG16L1 that predominantly localises to Rab11-positive

recycling endosomes results in autophagy inhibition (Li et al., 2017a). Indeed, the recruitment of factors such as WIPI2b to Rab11a-positive endosomes are thought to facilitate the role of ATG16L1 during LC3 lipidation (Puri et al., 2018).

Similar to other membrane fusion events, fusion of autophagy-related membranes appears to rely on the SNARE machinery. The homotypic fusion of ATG16L1-containing structures mainly requires VAMP7 and its accessory proteins, whereas heterotypic fusion events between recycling endosomes containing ATG16L1 or the transmembrane ATG protein ATG9 is mediated by VAMP3 (Moreau et al., 2011; Puri et al., 2013). Knockdown of either VAMP3 or VAMP7 reduces the levels of lipidated LC3 (Moreau et al., 2011; Puri et al., 2013). An involvement of annexin A2 has also been documented (Morozova et al., 2015). It is thought that annexin A2 plays a part by influencing the phospholipid composition of ATG16L1-positive vesicles, as well as by supporting vesicle fusion events. Interestingly, annexin A2 is also present at the plasma membrane and may thereby contribute to the plasma membrane recruitment of ATG16L1 (Morozova et al., 2015).

Autophagosome maturation and ATG8 lipidation
The ATG5 complex components are all essential for its E3-like ubiquitin ligase activity, which is required for the lipidation of ATG8 proteins *in vitro* and in cells. This involves the recruitment of the E2-like ligase ATG3 to the site of autophagosome biogenesis through direct binding between ATG3 and ATG12 (Otomo et al., 2013), as well as the ability of ATG16L1 to bind membranes through its N-terminal amphipathic helix 2 (Lystad et al., 2019). Helix 2 mutants of ATG16L1 form punctate structures similar to wild-type protein, suggesting that its involvement in ATG8 lipidation occurs after its recruitment to phagophores. This N-terminal lipid binding is required for both single- and double-membrane lipidation of ATG8 proteins and may facilitate membrane-remodelling events (Lystad et al., 2019). A distinct membrane-binding activity of ATG16L1 located downstream from its CCD within the middle region termed β-isoform

lipid binding (Fig. 1) has been also described and is required for PI(3)P-independent SMAC, such as on perturbed endosomes (Lystad et al., 2019). SMAC has been shown to involve ATG8 conjugation to phosphatidylserine (PS) as well as to PE (Durgan et al., 2020 preprint), thereby raising the possibility that the β-isoform lipid binding exhibits a capacity to mediate lipidation of ATG8 to additional phospholipids (Lystad et al., 2019). Interestingly, an isoform of ATG16L1 (ATG16L1α) lacks this second membrane-binding activity and, unlike its splice variant ATG16L1β, cannot support the lipidation of ATG8 during endosomal stress (Table 1) (Lystad et al., 2019). The tissue-specific expression relevance of these ATG16L1 isoforms remains largely unknown, and elucidating these may help distinguish the roles of ATG16L1 during ATG8 lipidation on various membranes (Ishibashi et al., 2011).

Interactions with Rab GTPases and relevance in autophagosome–lysosome fusion

Rab GTPases are a family of guanine nucleotide (GTP)-dependent exchange factors involved in various stages of vesicle formation and trafficking (Kiral et al., 2018; Zhen and Stenmark, 2015). They recruit specific downstream effectors when bound to GTP in their active state. Although their function is most extensively studied and linked to the endocytic pathway, accumulating data suggest they are also relevant during autophagosome biogenesis (Ao et al., 2014). Several Rab GTPases that can bind ATG16L1 and influence its subcellular localisation have been identified (Binotti et al., 2015; Itoh et al., 2008). The first indication that ATG16L1 can bind Rab GTPases came from a proteomic search for interacting partners of Rab33B, a Golgi-resident Rab GTPase with poorly defined function (Itoh et al., 2008). Subsequently, an interaction between ATG16L1 and Rab33A has also been described and shown to have autophagy-independent functions (discussed below) (Ishibashi et al., 2012; Itoh et al., 2008).

The relevance of the Rab33B–ATG16L1 interaction during autophagy remains poorly understood. Knockdown of Rab33B does not significantly affect LC3 lipidation but the expression of constitutively active Rab33B appears to elevate the levels of lipidated LC3 and the autophagy receptor p62 (also known as SQSTM1) (Itoh et al., 2008). In addition, expression of OATL1 (also known as TBC1D25), a GTPase-activating protein (GAP) for Rab33B, also enhances LC3 lipidation and ATG16L1 puncta formation (Itoh et al., 2011). More detailed analyses show that OATL1 and Rab33B can act by facilitating autophagosome–lysosome fusion (Itoh et al., 2011). Whether the interaction of Rab33B with ATG16L1 is relevant for its effects during lysosome fusion remains to be determined and may indicate that ATG16L1 can influence downstream events during autophagy. An additional involvement of the ATG5 complex in autophagosome–lysosome fusion was revealed upon the identification of tectonin β-propeller repeat containing 1 (TECPR1) as an ATG5-binding partner (Chen et al., 2012). Mutating ATG5-binding sites in TECPR1 leads to an increase in the number of GFP–LC3 puncta, consistent with a perturbed fusion between autophagosomes and lysosomes (Kim et al., 2015). ATG16L1 is excluded from the ATG5–TECPR1 complex and can be assumed to have an inhibitory effect on lysosome fusion in this setting (Chen et al., 2012).

Regulation of ATG16L1 through post-translational modifications and stability

Our current understanding highlights the importance of protein–protein and protein–lipid interactions in regulating the activities of most ATG proteins, including ATG16L1. Although many of these interactions appear constitutive and not affected by autophagy induction in cells (Dooley et al., 2014; Ganley et al., 2009; Nishimura et al., 2013), it remains plausible that post-translational modifications (PTMs) can play a role in regulating the activities of many ATG proteins. PTMs can provide a quick and reversible regulation of ATG proteins during autophagosome biogenesis. Indeed, a number of PTMs of ATG16L1 have been documented (Fig. 1). For instance, phosphorylation of ATG16L1 (predominantly at S139) is stimulated during hypoxia/reoxygenation (H/R) treatment and requires the activity of casein kinase 2 (CK2) (Song et al., 2015). Mutating the putative CK2 phosphorylation site on ATG16L1 diminished its binding to ATG5 and inhibited autophagy (Song et al., 2015). By contrast, phospho-mimetic mutants of this site or inhibiting its dephosphorylation resulted in enhanced binding to ATG5 and restored autophagy (Song et al., 2015). This phosphorylation event can be regulated by an adjacent methylation of K151, which is decreased during H/R treatment, leading to elevated phosphorylation of S139 and enhanced binding to ATG5 (Song et al., 2018). S278 of human ATG16L1 has also been shown to be phosphorylated by ULK1 (Alsaadi et al., 2019), although its phosphorylation by IκB kinase α (IKKα) has also been described (Diamanti et al., 2017). Antibodies that specifically recognise the S278-phosphorylated ATG16L1 suggest that this site is modified under various conditions and can be used to detect its phagophore localisation (Tian et al., 2020). Phosphorylation of S278 by ULK1 is not essential for starvation-induced autophagy (Tian et al., 2020) but is required for the recruitment of ATG16L1 to the site of invading bacteria during Salmonella infection (Fig. 2C) (Alsaadi et al., 2019). However, these stimulus-dependent modifications of ATG16L1 and the underlying molecular mechanisms that affect its autophagic activities remain to be further uncovered.

Regulation of the protein stability of the core autophagy machinery could provide an alternative means to modulate their activities. ATG16L1, as well as other ATG proteins, have been identified as substrates for protease cleavage (Murthy et al., 2014; Norman et al., 2010). Increased susceptibility of ATG16L1 to caspase-mediated cleavage has been associated with its pathogenic variants in Crohn's disease (discussed below) (Murthy et al., 2014). In addition, caspase cleavage and destabilisation of ATG16L1 is affected by IKKα- or ULK1-mediated phosphorylation of S278 (Alsaadi et al., 2019; Diamanti et al., 2017) and by its protein kinase A (PKA)-mediated phosphorylation (Zhao et al., 2019). Given that ATG16L1 overexpression can inhibit autophagy, it is possible that fine tuning of its levels may enable the regulation of its activity. Indeed, the E3 ubiquitin ligase gigaxonin has been described to bind to the WD40 domains region of ATG16L1 and result in its degradation (Scrivo et al., 2019). In the absence of gigaxonin, ATG16L1 levels are elevated, leading to its aggregation and autophagy inhibition, thereby imposing deleterious effects on neuronal health (Scrivo et al., 2019). Interestingly, gigaxonin overexpression can dramatically reduce ATG16L1 levels and restore autophagic activity in neurons (Scrivo et al., 2019). This is consistent with the notion that low levels of some ATG proteins (such as ATG5) is sufficient to drive autophagy in cells (Hosokawa et al., 2006), and raises the question of whether the regulation of ATG protein stability is a means to modulate autophagy under all conditions.

Relevance of WD40 domains interactions in non-canonical autophagy and cargo selection

Unlike for autophagosome biogenesis, C-terminal sequences of ATG16L1 are vital for ATG8 lipidation on single membranes, such as perturbed endosomes or entotic bodies (Fletcher et al., 2018;

Lystad et al., 2019). The underlying molecular mechanisms that mediate SMAC remain to be further explored, but it has been shown that they require sequences within the WD40 domains and the ATG16L1 β-isoform-specific C-terminal lipid-binding region (Fig. 1) (Fletcher et al., 2018; Lystad et al., 2019). The physiological relevance of this non-canonical ATG8 lipidation also requires further investigation and might be uncovered by utilising transgenic mouse models that express WD40-deficient ATG16L1 (Rai et al., 2019; Wang et al., 2020 preprint) or lack specific ATG16L1 isoforms.

WD40 domains are known to mediate protein–protein interactions. Although several protein binding interactions have been mapped to the WD40-containing C-terminal region of ATG16L1, they have not yet been confirmed to contribute to non-canonical ATG8 lipidation. These WD40-domains-binding partners include a subset of transmembrane-containing proteins (Boada-Romero et al., 2013; Hu et al., 2016; Sun et al., 2017), ubiquitin (Fujita et al., 2013), complement component 3 (C3) (Sorbara et al., 2018) and ATP6V0C (Xu et al., 2019). Many of these ATG16L1-interacting partners are dispensable for starvation-induced autophagy and are likely to have roles in autophagic cargo recognition or potentially autophagy-independent functions.

The interacting partners of the WD40 domains of ATG16L1 suggest it might have a role in the clearance of bacteria by autophagy (known as xenophagy, Fig. 2C), such as the three different transmembrane-containing proteins TMEM59, TMEM74 and TMEM166 (also known as EVA1A) (Boada-Romero et al., 2013; Hu et al., 2016; Sun et al., 2017). Overexpression of any of these proteins was shown to induce LC3 lipidation. In the case of TMEM59, its overexpression together with ATG16L1 leads to the localisation of both proteins to *Staphylococcus aureus*-positive endocytic vesicles (Boada-Romero et al., 2013). Binding of the WD40 domains to ubiquitin has also been described and is required for the recruitment of ATG16L1 and LC3 to invading *Salmonella* in the absence of FIP200 (Fujita et al., 2013). How FIP200 can compensate for the requirement to bind ubiquitin, as well as any unknown factors, remains to be investigated. Additional WD40-binding partners of ATG16L1 that may aid its recruitment to the site of bacterial infection during xenophagy include C3 and ATP6V0C (Sorbara et al., 2018; Xu et al., 2019). The interaction between ATP6V0C and ATG16L1 can be antagonised by the bacterial protein SopF as a mechanism to evade bacterial clearance by autophagy and thus enhance replication (Xu et al., 2019). Furthermore, the binding of C3 to ATG16L1 may have a role in mediating the survival of pancreatic cells in response to diabetogenic stress (King et al., 2019). Taken together, these findings highlight the role of ATG16L1 during xenophagy and suggest that it has a role in cargo recognition and local activation of autophagosome biogenesis. Investigating the effects of smaller deletions or point mutations in the WD40 domains of ATG16L1, instead of deleting its entire C-terminus, might help to enable a better understanding of the contribution of individual interacting partners.

Autophagy-independent roles of ATG16L1

Multiple autophagy-independent roles of ATG16L1 have been reported and are defined here as those that do not involve any form of ATG8 lipidation (see Table 2). These functions of ATG16L1 may or may not require its interaction with the ATG5–ATG12 conjugate. Some of these autophagy-independent roles in mammalian cells are discussed below based on the requirement to bind to ATG5 and additional activities in lower organisms are outlined in Box 2.

ATG5-binding-dependent ATG16L1 functions

Several studies have documented a role for ATG16L1 during exocytosis. ATG16L1, along with ATG5, has been shown to be involved in exosome production in a manner that includes the lipidation-independent recruitment of LC3 to vesicles (Guo et al., 2017). Exosomes can transport material from one cell to another and have been implicated in immune response, tumour cell metastasis and neurodegeneration (Kalluri and LeBleu, 2020). Genetic inhibition of ATG16L1 or ATG5, but not ATG7, in mouse embryonic fibroblasts or human breast cancer cells disrupts the recruitment of LC3 to exosomes and their secretion (Guo et al., 2017). Treating ATG5-null breast cancer cells with exosomes from

Table 2. A summary of the autophagy-dependent and -independent activities of ATG16L1

Activity	Function	Reference(s)
Autophagosome formation		
Binding to FIP200	Relaying upstream signalling from the ULK complex	Dooley et al., 2014; Dudley et al., 2019; Gammoh et al., 2013; Nishimura et al., 2013
Phagophore recruitment of ATG5–ATG12 [binding to WIPI2b, FIP200 and PI(3)P]	Specifying site of ATG8 lipidation	Dooley et al., 2014; Dudley et al., 2019; Gammoh et al., 2013; Nishimura et al., 2013
E3-like ligase activity (ATG5–ATG12 and N-terminal lipid-binding)	ATG8 lipidation and autophagosome maturation	Lystad et al., 2019; Otomo et al., 2013
Localisation to endocytic vesicles	Phagophore growth	Moreau et al., 2011; Puri et al., 2013; Ravikumar et al., 2010
Multimer formation	Scaffolding phagophore growth	Kaufmann et al., 2014
Binding to Rab GTPases	Autophagosome–lysosome fusion?	Itoh et al., 2008; 2011
Non-canonical ATG8 lipidation (SMAC) and cargo recruitment		
Unknown protein/lipid binding partner within the WD40 domains	Recruitment to single membranes and subsequent ATG8 lipidation	Fletcher et al., 2018
C-terminal β isoform lipid-binding activity	ATG8 lipidation on single membranes	Lystad et al., 2019
WD40 domains interactions with: Ubiquitin, TMEMs, C3 and ATP6V0C	Relevance in recognising bacterial infections	Boada-Romero et al., 2013; Fujita et al., 2013; Hu et al., 2016; Sorbara et al., 2018; Sun et al., 2017; Xu et al., 2019
Autophagy-independent		
Regulation of V1V0-ATPase activity via ATG5 binding	Exosome production	Guo et al., 2017
Rab33A binding	Hormone secretion	Ishibashi et al., 2012
Undefined WD40-dependent activity	Lysosome exocytosis and plasma membrane repair	Tan et al., 2018
Nod1 and/or Nod2 binding	Cytokine response	Sorbara et al., 2013

> **Box 2. Relevance of ATG16L1 in autophagy-independent activities in non-mammalian organisms**
> Autophagy-independent roles of ATG16L1 have also been described in invertebrates. *Drosophila* mutants lacking Atg16 or expressing a truncation mutant that does not bind Atg5 exhibit reduced sensitivity to ethanol-induced sedation, whereas flies lacking Atg7 or Atg3 show no altered response to ethanol exposure (Varga et al., 2016). This increased resistance to ethanol is mediated by a defect in Corazonin production and secretion in Atg16-mutant flies (Varga et al., 2016). Individual or combined deletions of Atg16, Atg5 and Atg12 in the social amoeba *Dictyostelium discoideum* lead to similar autophagy-related phenotypes to those observed with other Atg deletions, including developmental abnormalities and reduced survival during nitrogen starvation (Karow et al., 2020). However, *D. discoideum* harbouring a combined triple deletion of Atg16, Atg5 and Atg12 show increased defects in macropinocytosis of fluorescent dextran and phagocytosis of yeast cells when compared to those with Atg5 and Atg12 co-deletion alone, thereby suggesting additional non-autophagic and potentially redundant activities of these proteins (Karow et al., 2020). Taken together, these findings suggest that the autophagy-independent roles of Atg16 are also observed in various species with the underlying molecular mechanisms poorly understood.

wild-type cells before their implantation in mammary pads in mice results in enhanced metastasis to the lungs compared to implantation of untreated wild-type or ATG5-null cells (Guo et al., 2017). The presence of ATG16L1, along with the ATG5–ATG12 conjugate, in secretory vesicles from cultured neuroendocrine cells has also been reported and been shown to depend on the binding of ATG16L1 to Rab33A (Ishibashi et al., 2012). This localisation of ATG16L1 is required for hormonal secretion in a manner that is independent of PI(3)P or components of the ULK complex (Ishibashi et al., 2012). ATG16L1 has also been found to be involved in lysosomal exocytosis, which is required to restrict cell-to-cell spreading of *Listeria monocytogenes* (Tan et al., 2018). Here, ATG16L1, along with the ATG5–ATG12 conjugate, acts to mediate plasma membrane repair in a manner that is dependent on its WD40 domains, but is independent of other autophagy players such as ATG3 (Tan et al., 2018). An additional non-autophagic function of the ATG5 complex has been described in mediating interferon production during viral infection (Hwang et al., 2012). However, the underlying molecular mechanisms and whether this effect is mediated through regulation of the exocytic pathway remain unclear.

ATG5-binding-independent roles of ATG16L1
Fewer non-ATG5-binding dependent roles of ATG16L1 have been described, which might be due to the fact that ATG16L1 is destabilised in ATG5-deficient cells (Dudley et al., 2019; Nishimura et al., 2013). One of these ATG5-independent roles of ATG16L1 is the ability to suppress cytokine production mediated by the intracellular pattern recognition receptors Nod1 and Nod2 (Trindade and Chen, 2020) in response to bacterial infection (Sorbara et al., 2013). This effect of ATG16L1 is suppressed in the Crohn's disease variant ATG16L1T^{T300A} (discussed below), potentially underscoring an autophagy-independent pathological role of this variant in inflammatory bowel disease (IBD) (Sorbara et al., 2013).

Link to pathophysiology
The pathophysiological functions of ATG16L1 are likely mediated through both its autophagy-dependent and -independent activities. The first and most extensively studied disease-relevance of

ATG16L1 was prompted by the discovery of its Crohn's disease-associated variant ATG16L1^{T300A} (corresponding to β-isoform residue number) (Hampe et al., 2007). The link between ATG16L1 and intestinal inflammation was further confirmed in a number of studies in which ATG16L1 was specifically deleted in either the intestine (Aden et al., 2018; Adolph et al., 2013; Matsuzawa-Ishimoto et al., 2017) or hematopoietic cells (Kabat et al., 2016; Saitoh et al., 2008; Samie et al., 2018), and together they demonstrated a protective role of ATG16L1 in IBD.

Despite its discovery as a susceptibility variant over a decade ago (Hampe et al., 2007), research is still ongoing to determine the underlying molecular mechanism resulting in the association of ATG16L1^{T300A} variant with increased susceptibility to Crohn's disease. This threonine to alanine substitution falls within the WD40 domain of ATG16L1 that is in close proximity to its middle region and has been implicated in various functions of ATG16L1; however, the specific protein–protein or protein–lipid interactions that are affected by this variant are still undetermined (Boada-Romero et al., 2016). ATG16L1^{T300A} may also be associated with additional pathological conditions, such as brain metastasis of lung cancer (Li et al., 2017b). Further genetic association studies may help to unravel additional diseases that are affected by this ATG16L1 variant.

The most-studied molecular consequence of the ATG16L1^{T300A} variant is its enhanced susceptibility to cleavage by caspase-3 and thereby destabilisation (Murthy et al., 2014). Activation of caspase-3 by nutrient stress or death-receptor signalling stimulates ATG16L1^{T300A} degradation and reduces the autophagic response as well as clearance of bacterial infection (Murthy et al., 2014). Mice that are hypomorphic for ATG16L1 expression or express an ATG16L1^{T300A} knock-in mutated form exhibit altered intestinal microbiota and abnormalities in Paneth cells, specialised secretory cells that line the intestinal epithelium and play an antimicrobial role in protecting the epithelium (Cadwell et al., 2008; Lassen et al., 2014; Lavoie et al., 2019).

Interestingly, variants of Nod2, an ATG16L1-binding partner, have also been associated with increased susceptibility to Crohn's disease. These Nod2 variants are thought to perturb its ability to recruit ATG16L1 to the site of bacterial infection, thereby affecting pathogen clearance (Travassos et al., 2010). Both ATG16L1 and Nod2 can interact with immunity-related GTPase family M (IRGM) protein, another Crohn's disease-associated gene product (Chauhan et al., 2015). This complex formation is induced by microbial products and is required to supress pathogen-induced inflammation (Chauhan et al., 2015). An additional binding partner of ATG16L1 that may be linked to its role in IBD is the anti-inflammatory protein A20 (also known as TNFAIP3), which binds ATG16L1 through its WD40 domains (Slowicka et al., 2019). Interestingly, mice harbouring combined tissue-specific deletions of both A20 and ATG16L1, but not individual knockouts, exhibit an IBD-like phenotype, suggesting that both proteins can cooperatively regulate inflammation of the bowel (Slowicka et al., 2019). Taken together, these Crohn's disease-associated interactors of ATG16L1 underscore its role in the inflammatory response with the underlying mechanisms likely requiring its activities in autophagy and autophagy-related processes.

Conclusions and final remarks
As research continues to expand our knowledge of how ATG proteins function during autophagy, numerous questions remain unresolved. In terms of ATG16L1, recent studies have highlighted the importance of its various domains that engage in the diverse

steps of autophagosome biogenesis as well as during non-canonical SMAC. This raises the question of whether specific domain mutations of ATG16L1 could be used as tools to determine the physiological relevance of canonical autophagy and non-canonical ATG8 lipidation. Furthermore, it is also unclear what are the tissue-specific functions of the ATG16L1 isoforms. In addition, how ATG16L1 activity is regulated, that is, whether there are any on/off switches, and what mediates its recognition of single membranes are other important aspects that warrant further investigation. Finally, the involvement of ATG16L1 in pathological conditions is also an area of ongoing research, and it remains to be addressed whether the underlying mechanisms involve its canonical or non-canonical autophagic functions, or instead require its largely unexplored non-autophagic activities.

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