ATG16L1 functions in cell homeostasis beyond autophagy

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Running title: ATG16L1 functions in cell homeostasis

Abbreviations:

Atg, autophagy-related; cGAS, Cyclic GMP-AMP synthase; DC, dendritic cell; ER,endoplasmic reticulum; IFN, interferon; IKKa, Inhibitor of nuclear factor kappa-B kinase subunit alpha; IL, interleukin; LAP, LC3-associated phagocytosis; LIR, LC3-interacting region; LPS, lipopolysaccharide; MEF, mouse embryonic fibroblasts; mTOR, mechanistic target of rapamycin; NLR, NOD-like receptor; NOD, Nucleotide-binding oligomerization domain-containing protein; PAS, phagophore assembly site; PKA, cAMP-dependent protein kinase A; PE, phosphatidylethanolamine; PI3K, phosphoinositide 3-kinase;; PI3P, phosphatidylinositol 3-phosphate; Rab, Ras-related protein; STING, Stimulator of interferon genes protein; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter molecule 1; ULK, Unc-51 like autophagy activating kinase; UPEC, uropathogenic *Escherichia coli* ; UPR, unfolded protein response; V-ATPase, vacuolar-type HC-ATPase; WDD, ATG16L1's WD40 domain

Keywords: ATG5-ATG12/ATG16L1 complex, LC3-lipidation, autophagy, LC3-associated phagocytosis, WD40 domain, Crohn's disease, T300A variant

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Abstract

Atg16-like (ATG16L) proteins were identified in higher eukaryotes for their resemblance to Atg16, a yeast protein previously characterized as a subunit of the Atg12-Atg5/Atg16 complex. In yeast, this complex catalyzes the lipidation of Atg8 on pre-autophagosomal structures, and is thereby required for the formation of autophagosomes. In higher eukaryotes, ATG16L1 is also almost exclusively present as part of an ATG12-ATG5/ATG16L1 complex, and has the same essential function in autophagy. However, ATG16L1 is three times bigger than Atg16. It displays in particular a carboxy-terminal extension, including a WD40 domain, which provides a platform for interaction with a variety of proteins, and allows for the recruitment of the ATG12-ATG5/ATG16L1 complex to membranes under different contexts. Furthermore, detailed analyses at the cellular level have revealed that some of the ATG16L1-driven activities are independent of the lipidation reaction catalyzed by the ATG12-ATG5/ATG16L1 complex. At the organ level, the use of mice that are hypomorphic for *Atg16l1*, or with cell-specific ablation of its expression, revealed a large panel of consequences of ATG16L1 dysfunctions. In this

review, we recapitulate the current knowledge on ATG16L1 expression and functions. We emphasize in particular how it broadly acts as a brake on inflammation, thereby contributing to maintaining cell homeostasis. We also report on independent studies that converge to show that ATG16L1 is an important player in the regulation of intracellular traffic. Overall, autophagy-independent functions of ATG16L1 probably accounts for more of the phenotypes associated with ATG16L1 deficiencies than currently appreciated.

Introduction

The term autophagy refers to a degradation pathway involving dynamic membrane rearrangements that sequester cargo for delivery to the lysosome; where the sequestered material is degraded and recycled. Most of the molecular players were discovered through genetic screens in yeast in the last years of the twentieth century [1]. Atg16 , formerly Apg16p, was initially identified through its association with the first identified autophagy protein, Atg5 [2]. Atg5 is covalently conjugated to Atg12, and interacts with Atg16 to form a Atg12-Atg5/Atg16 complex. Atg16 is required for the location of the complex to the pre-autophagosomal structure (PAS), where the complex catalyzes the lipidation of Atg8 [3]. The mammalian homolog of *ATG16* was first cloned in mice in 2003 [4], but raised little interest until two studies identified a susceptibility variant to Crohn's disease in the human homolog, *ATG16L1*, in 2007 [5,6]. Since then, many groups have tried to understand the roles of ATG16L1 in cellular homeostasis, in particular using mice hypomorphic for *Atg16l1*, or afflicted with cell-type specific *Atg16l1* deficiency. The emerging view is that ATG16L1 exerts multiple functions. While its most studied and core activity relates to its contribution to the lipidation of Atg8 homologs, it appears that it also exerts unrelated functions, which are less well understood. In this review, we will recapitulate the current knowledge on ATG16L1 structure and expression. The steps of the autophagy process in which ATG16L1 is required, and the molecules involved will be recapitulated only briefly, since this best understood aspect of ATG16L1 activity has recently been reviewed [7]. We will emphasize how, in mammalian cells, ATG16L1 has evolved to a more versatile molecule than its yeast ortholog, capable of directing the ATG12-ATG5 complex, and thus its lipidation capacity, to a variety of intracellular membranes. We will also see how ATG16L1 has gained a prominent role in counteracting inflammation in a variety of cell types. Finally, we will see that ATG16L1 contributes to cellular homeostasis by regulating a number of membrane trafficking events in mammalian cells, in some cases independently of the autophagy cascade.

I –ATG16L1: a well-established role in the LC3 conjugation machinery.

I-A. ATG16L1 domain composition

The 17 kDa Atg16 protein of yeast is composed of an Atg5-binding domain, followed with a coiled-coil domain [8]. Genomes of higher eukaryotes, including *Caenorhabditis elegans*, *Dictyostelium discoideum* or *Oryza sativa*, code for larger proteins of 55-68 kDa which display only weak homology

with yeast Atg16 in their N-terminus, and were therefore designated as Atg16-like proteins (ATG16L) (Figure 1 A and B). Most notably, ATG16L proteins has a carboxy-terminal extension, including a domain made of seven WD40 repeats, that forms a β-propeller, which we will refer to as the WDD [4,9]. Genomes of mammals code for two ATG16L proteins, called ATG16L1 and ATG16L2 [10]. ATG16L2 also forms complexes with ATG12-ATG5, but displays only limited sequence similarity with ATG16L1, and lacks LC3-lipidation-stimulating activity during canonical autophagy [10]. Recently, generation of *Atg16l2* knock-out mice revealed that that ATG16L1 and ATG16L2 contribute very distinctly to autophagy and cellular ontogeny in myeloid, lymphoid, and epithelial lineages, and ATG16L2 functions will not be discussed in this review [11]. The α and β isoforms are two isoforms of *ATG16L1* which are largely expressed, with some tissue specificity, with the latter (also called isoform 1) carrying an 18 amino acid insertion. These isoforms are also expressed in mice, with more than 90% sequence identity. Mice express an additional isoform, γ, which carries a 16 amino acid insertion that has no equivalent in humans [4,10]. Other isoforms might be expressed, lacking for instance the coiled-coil domain or the WDD domain, but their expression in specific tissues remains to be demonstrated [12].

Two independent genome wide association studies identified a susceptibility variant for Crohn's disease in *ATG16L1*, corresponding to a single nucleotide substitution (A to G, rs2241880) [5,6]. This substitution leads to the presence of an alanine residue at position 300, instead of threonine (T300A, equivalent to T316A for the γ isoform in mice). It was later shown that amino acids 296–299 of ATG16L1 constitute a caspase cleavage motif, and that the T300A variant significantly increased ATG16L1 sensitization to caspase-3-mediated processing [13]. The sensitivity to caspase-3 cleavage suggests that possession of the 300A allele might, under certain circumstances, result in reduced expression of full-length ATG16L1, and increased expression of cleaved fragments such as the WDD, possibly with dominant negative effects. This intriguing possibility that the T300A variant may display dominant negative activity on the wild type variant is supported by the observation that mice bearing a wild-type *Atg16l1* allele and a null allele exhibit a phenotype equivalent to that of wild type mice, while mice expressing the T300A variant, on either one or two alleles, do not [14]. However, it is important to keep in mind that the T300A variant is very common in the human population [5], and that heterozygosity at this locus (GA genotype) was not associated to a higher risk of developing ileal Crohn's disease [15]. Nevertheless, contradictory results on this question were reported when studying pediatric cohorts [16-18]. Furthermore, individuals heterozygotes and homozygotes with the risk allele (GA or GG genotypes) display increased endoplasmic reticulum (ER) stress in their Paneth cells [19]. A positive correlation between the cumulative number of ATG16L1 risk alleles and the proportion of abnormal Paneth cells has been reported in two North American cohorts of Crohn's disease patients, but not in a Japanese cohort [20,21]. Finally, urothelial vesicular abnormalities were associated with the GA and GG genotypes both in mouse and human bladder urothelium samples [22]. These data support the hypothesis that the expression of the T300A variant may induce phenotypic changes, even in an heterozygous context, and this question deserves further investigation.

I-B. ATG16L1 is mainly expressed as part of the ATG12-ATG5/ATG16L1 complex

ATG16L1 forms a homo-dimer through its coiled-coil region, and potentially through interactions with the WDD (Figure 1 A) [4,9,23,24]. Its amino-terminal end binds to ATG5, which is itself covalently bound to ATG12 (through a conjugation reaction mediated by ATG7 and ATG10). Differential centrifugation analysis showed that ATG16L1 mostly migrates as part of an 800 kDa complex, likely containing eight sets of ATG12-ATG5 and ATG16L1 [4]. This behavior is similar to that observed in yeast, where Atg16 forms a ~350 kDa multimeric complex with Atg12-Atg5 [25]. However, the existence of this multimeric form remains to be confirmed. Most importantly, monomeric ATG16L1 or ATG12-ATG5 conjugates are not detected, meaning that ATG16L1 is mainly, possibly exclusively, expressed as part of the ATG12-ATG5/ATG16L1 complex. The interdependence of the three proteins is also revealed by the effect of knocking-out the expression of just one of the partners on the expression of the others. In *Atg5*$^{-/-}$ cells, ATG16L1 is almost undetectable [26]. Mice KO for *Atg7* in epithelial cells, which is necessary for ATG12 conjugation to ATG5, exhibit a total loss of ATG12 and ATG16L1, and a strong reduction in ATG5 expression [27]. One possible explanation is that these proteins have a short half-life, unless they stabilize each other once assembled in the ATG12-ATG5/ATG16L1 complex [26]. Also, it is possible that the loss of autophagy in these experimental set-ups leads to an aberrant over-activation of anabolic pathways such as mechanistic target of rapamycin (mTOR), which controls the expression of multiple autophagy genes [28,29]. In any case, it is important to keep in mind when interpreting loss of function data that the absence of single components of the ATG12-ATG5/ATG16L1 complex, or of the proteins implicated in the formation of the complex, affects the expression of all subunits. Thus, the phenotypes observed are not necessarily due to a loss in autophagy, but could be due to the loss of autophagy-independent activities of each component of the ATG12-ATG5/ATG16L1 complex.

I-C. Transcriptional and post-translational regulation of ATG16L1 expression and activity

ATG16L1 is present in all tissues, though specific transcription factors regulating *ATG16L1* expression have not yet been described. In *C. elegans* the transcription factor HLH-30 (TFEB in mammals) regulates the transcription of a number of autophagy genes, among which is *atg16.2* in response to *Staphylococcus aureus* infection [30]. Whether this transcriptional control is conserved in mammalian cells is unknown. BRG1, an ATPase subunit of the SWI/SNF chromatin remodeling complex, was shown to regulate the transcription of several autophagy genes, including *Atg16l1* in intestinal epithelial cells [31].

Several microRNAs, including miR-142-3p, miR223, mir-106b, miR93, miR-96, miR-410, miR-20a, and miR-874, were reported to target the 3'-untranslated region of the *ATG16L1* gene and regulate its transcription, in different tissues [32-38]. The post-transcriptional regulator Human antigen R (HuR) was proposed to regulate hypoxia-induced autophagy by enhancing the expression of *ATG7* and *ATG16L1* [39]. Post-transcriptional regulation of *Atg16l1* translation was also recently reported in intestinal epithelial cells [40].

Post-translational modifications of ATG16L1 have also been shown to regulate its activity. Phosphorylation at Ser139 and methylation at Lys151 are antagonistic marks that affect ATG16L1

binding to the ATG12–ATG5 conjugate [41,42]. Methylation occurred upon hypoxia/reoxygenation in cardiomyocytes, indicating that these post-translational modifications allow to adjust the activity of the ATG12-ATG5/ATG16L1 complex to oxygen availability. In endothelial cells, the cAMP-dependent protein kinase A (PKA) reduces ATG16L1 half-life by phosphorylation of Ser268, thereby tuning down endothelial autophagy and contributing to the regulation of angiogenesis [43]. In intestinal epithelial cells IKKα-driven phosphorylation at a different residue, Ser278, has the opposite, stabilizing, effect, by protecting ATG16L1 from caspase-dependent degradation [44]. However, this result was later disputed by the observation that, in mouse embryonic fibroblasts, phosphorylation of this Ser278 residue still occurred in the absence of IKKα, but required another kinase, ULK1, in conditions of starvation or in the context of an infection [45]. Phosphorylation by ULK1 at Ser278 enhances ATG16L1 engagement in xenophagy (discussed further below), and this modification was recently proposed as a readout for autophagy level [46]. Finally, Gigaxonin is an E3 ligase that binds ATG16L1 through its WDD, poly-ubiquitinates ATG16L1 and controls its degradation through the proteasome and lysosomal pathways [47]. Gigaxonin depletion results in the accumulation of ATG16L1 and impairs LC3 lipidation. This phenocopies the inhibition of starvation-induced LC3 lipidation upon overexpression of ATG16L1 or ATG12 [48].

I-D. Role of the ATG12-ATG5/ATG16L1 complex in LC3 lipidation reaction

The ATG12-ATG5/ATG16L1 complex forms the core machinery which catalyzes the addition of phosphatidylethanolamine (PE) to the homologs of yeast Atg8, globally referred to as LC3 (Figure 2). Mammalian cells express six paralogs of LC3, (LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2), that require processing by ATG4 cysteine proteases (ATG4A, B, C, and D) prior to activation by ATG7 (E1 ligase). Cleaved LC3 is then transferred to ATG3 (E2 ligase). The ATG12-ATG5/ATG16L1 complex subsequently serves as a noncanonical E3 ligase by attaching LC3 to PE, thereby anchoring LC3 to the target membrane. All these proteins implicated in LC3-PE conjugation are globally designated as the LC3 conjugation machinery [49]. LC3-PE possesses fusogenic properties and mediates the growth of the phagophore and its closure to form the autophagosome. Autophagosomes engulf cytosolic components that eventually fuse with lysosomes for degradation of the cargo. The cargo itself can be bulk cytoplasmic components, or specifically selected components via autophagy receptors such as the ubiquitin-binding protein p62 (SQSTM1), optineurin (OPTN) and the calcium-binding and coiled-coil domain-containing protein 2 (NDP52). Autophagy thereby has been linked to a number of essential functions that conserve cell homeostasis such as: the recycling of organelles and protein aggregates which protects from neurodegeneration, the control of cell signaling, cell metabolism and defense against invading pathogens [49]. *In vitro,* ATG16L1 might be dispensable to the enzymatic reaction of lipidation *per se*, by analogy to the observation that the Atg5-Atg12 conjugate is able to bind to Atg3 and to catalyze the transfer of PE to activated LC3 [8,50]. Yeast Atg16 core function is to localize multimers of Atg5-12 to the PAS and enhance Atg8 lipidation [3,51]. Furthermore, the association of Atg8–PE with Atg12–Atg5/Atg16 forms a membrane scaffold, that was proposed to be critical for phagophore biogenesis [52]. *In vitro* studies were also performed with purified

mammalian proteins and showed that ATG16L1 is required for efficient LC3 conjugation [53]. In mammalian cells, the lipidated form of LC3 is completely lost in the absence of ATG16L1, a phenotype also observed in yeast [26,54]. We will see below that, in higher eukaryotes, the role of ATG16L1 in targeting the LC3 conjugation machinery to target membranes became more versatile than in yeast. Indeed, additional domains, and in particular the acquisition of the WDD, one of the most abundant protein interaction domains in the human proteome [55], increased the levels of regulation for the location of the ATG12-ATG5/ATG16L1 complex to autophagosomes, and opened the possibility to target the LC3 conjugation machinery to other kinds of membranes (Table 1).

II- ATG16L1 drives LC3 lipidation to target membranes through its lipid and protein binding modules.

II-A. ATG16L1's contributions to directing the LC3 conjugation machinery to the PAS

Several features in ATG16L1 contribute to the recruitment of the LC3 conjugation machinery to the PAS. Autophagy is turned off under basal conditions by mTOR. Starvation, oxidative stress, and other environmental perturbations inhibit mTOR and activate a complex made of ULK1, FIP200, ATG13, and ATG101. The ULK1 complex activates the phosphatidylinositol 3–kinase catalytic subunit type 3 (PI3KC3) complex (including Beclin-1, ATG14, VPS15 (PI3KR4), and VPS34 (PI3KC3)), which is recruited to the PAS. The PI3KC3 complex produces phosphatidylinositol 3-phosphate to mark the location for autophagosome biogenesis and create a platform for recruiting lipid binding proteins such as WIPI2b. WIPI2b and FIP200 recruit the ATG12-ATG5/ATG16L1 complex to the PAS through their affinity for adjacent sites located between the coiled-coil domain and the WDD of ATG16L1 [56-58] (Table 1). Other membrane proteins described to interact directly with ATG16L1 and thereby contribute to its recruitment to the PAS include the membrane interacting protein SNX18 [59] and the transmembrane protein TMEM166/EVA1[60]. In addition, association of the ATG12-ATG5/ATG16L1 complex to membranes is favored by ATG16L1's intrinsic membrane-binding features [53,61]. One of those is a conserved N-terminal amphipathic helix adjacent to the ATG5 binding site, required for membrane binding but dispensable for PAS targeting [53]. A second membrane-binding region present only in the β isoform is dispensable for binding to highly curved membranes, but becomes important when membrane curvature is low [53]. Interestingly, this C-terminal membrane-binding region of ATG16L1 is dispensable for canonical autophagy but essential for LC3B lipidation at perturbed endosomes (see below). Finally, the coiled-coil domain also seems to contribute to lipid binding and recruitment to the PAS [61]. Once located at the PAS, ATG16L1's propensity to oligomerize favors the local amplification of LC3 conjugation to PE.

II-B. ATG16L1's contributions to cargo selection

As the phagophore grows through the coordinated action of autophagy proteins, cargos are incorporated into it. Many cargos such as invasive microbes or damaged organelles are marked with ubiquitin. Autophagy receptors, that contain both ubiquitin binding domains and a LC3-intreracting region (LIR), make the link between ubiquitinated cargo and the phagophore [49]. ATG16L1 can also be implicated in the recognition of ubiquitinated cargo, as its WDD binds directly to ubiquitin, decorating damaged endosomes containing *Salmonella* [62]. A recent study revealed that phosphorylation of ATG16L1 at Ser278, catalyzed by ULK1, was necessary to target ATG16L1 to intracellular bacteria in fibroblasts [45].

Complement component C3 was also shown to also interact with ATG16L1, thereby contributing to the clearance of C3-coated invasive bacteria [63]. The interaction between C3 and ATG16L1 was confirmed in a different physiological context, with the discovery of its contribution to cytoprotective autophagy in pancreatic β cells [64].

Another axis between invasive bacteria and ATG16L1 mobilization was discovered a decade ago with several concomitant studies reporting on the contribution of the intracellular sensors NOD1 and NOD2 to the autophagic response to invasive bacteria [65-67]. NOD-like receptors (NLRs) are part of the cytosolic surveillance system for the detection of intracellular pathogens. The founding members, NOD1 and NOD2, detect intracellular bacteria through their ability to sense bacterial peptidoglycan. Activation of NOD1 and NOD2 initiates a proinflammatory response dependent mainly on activation of the transcription factor NF-κB and on recruitment of the adaptor protein RIP2. Both sensors were shown to recruit ATG16L1 at the bacterial entry site, and to co-immunoprecipitate with ATG16L1 [65,68]. We will see below that the interaction between ATG16L1 and NOD proteins is complex, as it was later shown that ATG16L1 suppresses NOD driven inflammation [69].

Recently, it was also reported that ATG16L1 was recruited to damaged vacuoles bearing internalized bacteria via a direct interaction between its WDD and one subunit of the vacuolar ATPase [70]. The WDD alone did not interact with this protein, and the data indicate that ATG16L1 oligomerization, mediated by its coiled-coil domain, was needed.

II-C. ATG16L1's WDD recruits the LC3-conjugation machinery to lipidate PE at specific single membranes.

Multi-layered phagophores and autophagosomes are not the only sites of LC3 conjugation to PE. "Non-canonical autophagy" is often used to designate processes that lead to LC3 lipidation on single, non-autophagosomal membranes. Examples of this non-canonical autophagy pathway include LC3-associated phagocytosis (LAP), where LC3 is lipidated at single-membrane phagosomes following the engulfment of bacterial and fungal pathogens or apoptotic and necrotic cells (review in [71]), and LC3 lipidation at damaged endosomal membranes (experimentally provoked by drugs possessing lysosomotropic or ionophore properties) . Unlike in canonical autophagy, the ULK1 complex is dispensable for LAP, which depends on PI3P generation, and on Rubicon, a Beclin-1-binding protein

[72,73]. The molecular requirements for LC3 lipidation at damaged endosomes have been less investigated than in LAP, but one thing that is common to the two situations is that ATG16L1 WDD is required for lipidation to occur [53,74]. Point mutations in amino acids F467 and K490 impaired ATG16L1 recruitment to latex bead-containing single-membrane phagosomes upon monensin treatment, indicating that these residues, located on the top face of the WDD, are implicated. Importantly, the WDD alone was not recruited to phagosome membranes, indicating that the domain is necessary but not sufficient for the recruitment of the ATG12-ATG5/ATG16L1 complex [74].The membrane-binding properties of ATG16L1 (and in particular its central membrane-binding region, which is dispensable for canonical autophagy but essential for LC3B lipidation at perturbed endosomes [53]), together with the possibility that the WDD behaves as a protein binding platform, amplified by ATG16L1 oligomerization, might be sufficient to target the LC3 lipidation machinery to cognate membranes. A number of potential WDD interacting proteins have been identified (Table 1) and one can speculate that one or several WDD binding partners, possibly transmembrane proteins, may contribute to LAP activity by recruiting ATG16L1 to single membrane compartments. One transmembrane protein containing a WDD binding domain is TMEM59, that is associated with several compartments, including the Golgi apparatus and late endocytic compartments [68,75]. Overexpression of TMEM59 is sufficient to induce LC3 lipidation of the compartment in which the protein resides, through its ability to attract ATG16L1 [68]. Furthermore, TMEM59 depletion prevented LAP induction upon *Staphylococcus aureus* infection [68]. The Pimentel-Muiños' laboratory identified in TMEM59's short cytoplasmic tail a WDD binding motif defined as [YW]-X3-[ED]-X4-[YWF]-X2-L [68]. This motif was found in several other proteins that bind to ATG16L1, such as TLR2 and the CARD domain of NOD2, both possibly involved in LAP. However, a role for TMEM59 in LAP remains questionable since the T300A variant of ATG16L1 displays impaired TMEM59 trafficking and binding to ATG16L1 [76] but has no impact on LAP [74].

A different pathway for LC3B lipidation on single membrane was described very recently [77]. The detection of cytosolic DNA by Stimulator of Interferon Genes (STING) resulted in LC3B lipidation onto single membrane perinuclear vesicles. LC3 lipidation required the recruitment of ATG16L1, via its WD40 domain, and bypassed canonical autophagy players. Instead, it implicated V-ATPase complexes, i.e. proton pumps that are localized throughout the secretory pathway and regulate pH in various vesicular and organellar compartments. This process was distinct from LAP as it did not require PI3K activity and formation of a phagocytic vacuole, and was called VAIL for V-ATPase–ATG16L1—induced LC3B lipidation [77]. The dissection of the VAIL pathway benefited from the previous identification of a bacterial effector required for *Salmonella typhimurium* virulence, SopF, that inhibited LC3B lipidation of the *Salmonella*-containing vacuole by ADP-ribosylation of the C subunit in the V0 complex of the V-ATPase, and could then be used as a tool to impair V-ATPase–mediated LC3B lipidation without disrupting lysosomal acidification [70]. This earlier study showed that the V-ATPase co-immunoprecipitated with ATG5 and ATG16L1, and that the interaction was mediated by the WDD of ATG16L1, illustrating the role of the WDD in directing the LC3 lipidation machinery to a variety of membranes in different contexts [70]. The recent discovery of the VAIL pathway led its authors to propose that the emergence of the ATG16L1's WDD in metazoa allowed to mediate the recruitment of

the LC3B conjugation machinery independently of autophagy, with the VAIL pathway representing an evolutionarily ancient mechanism of cell-autonomous innate immunity [77].

Proteomic studies suggest that the WDD interactome has considerable cell type and developmental state specificity [78,79]. It is likely that other WDD binding proteins remain to be discovered, whose roles are to recruit the ATG12-ATG5/ATG16L1 complex to drive LC3 lipidation of specific membranes. Importantly, most of the interactions listed in Table 1 were detected in systems using protein over-expression and remain to be confirmed in more physiological systems.

II-D. Variation on this theme: the ATG12-ATG5/ATG16L1 complex is required to direct INFγ-driven anti-microbial responses to the microbial niche

Among the most prominently IFN-induced proteins are dynamin-like GTPases, that are classified in four families [80]. Among those, immunity-related GTPases (IRGs, in mice) and guanylate-binding proteins (GBPs, in mice and humans) are targeted to the membrane of cytoplasmic vacuoles containing bacteria, protists, or fungi, to orchestrate their destruction. One of the best studied examples of this is the parasitophorous vacuole of the protist *Toxoplasma gondii*. The ATG12-ATG5/ATG16L1 complex is required for the localization of LC3, and of several members of the IRG and GBP families, to the parasitophorous vacuole. What recruits the ATG12-ATG5/ATG16L1 complex to this compartment is not known, apart from the fact that neither the autophagy cascade nor IFN are necessary. Only a small proportion of parasitophorous vacuoles are positive for LC3 or IFN-induced GTPases at any given time, indicating that the parasite may partially counteract this defense pathway [81]. Several parallels can be drawn between these observations and those made in a very different context, i.e IFNγ-mediated defense against murine norovirus (MNV) infection. The MNV replication complex, which consists in an arrangements of convoluted and sometimes closely apposed membranes without a concentric double membraned structure [82]. The ATG5-ATG12/ATG16L1 complex localizes to this site, allowing for the LC3 lipidation on these membranes, even in the absence of IFN. IFNγ-exposure leads to the expression INF-inducible GTPases, which are recruited to the MNV replication complex in an ATG5-ATG12/ATG16L1 dependent manner, and eventually lead to the disappearance of the site of viral replication [82,83]. Here again what brings the ATG5-ATG12/ATG16L1 complex to the viral replication membranes remains to be elucidated.

III – ATG16L1 acts as a break on inflammation.

III-A. ATG16L1 suppresses type I interferon signaling

The first insight of the potential for ATG16L1 to suppress inflammation came from virology studies. Following viral infection, autophagy is often initiated to curtail infection by delivering viral particles for lysosomal degradation and further integrating with innate pattern recognition receptor signaling to induce type I interferon (IFN-I)-mediated viral clearance. In parallel, some viruses have evolved anti-

autophagy strategies to escape host immunity and to promote viral replication [84]. Mechanistic studies have revealed a variety of mechanisms by which proteins engaged in the autophagy cascade interfere with the IFN-I response of the infected cells (reviewed in [85]). Here we will focus on investigations of the consequences of ATG16L1 deficiencies on IFN-I production. We will see that the suppressive effect exerted by ATG16L1, initially observed in viral infections, also applies to other physiological contexts.

Upon infection with a RNA virus, virus-related RNA species are recognized by retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), including RIG-I and MDA5 (melanoma differentiation associated gene 5) The ATG5-ATG12/ATG16L1 complex regulates the RLR signaling pathway and negatively impacts IFN-I activation during viral infection. This was first demonstrated through the resistance of ATG5-deficient mouse embryonic fibroblasts (MEFs) to vesicular stomatitis virus replication, which was largely due to hyperproduction of IFN-I in response to immunostimulatory RNA. Molecular interaction studies indicated that the ATG5–ATG12/ATG16L1 complex negatively regulated the IFN-I production pathway by direct association of ATG5 with the retinoic acid-inducible gene I (RIG-I) and the mitochondrial antiviral signaling protein (MAVS, also called IPS-1), a signaling intermediate that aggregates at the mitochondria to activate TBK-1 (tank-binding kinase 1) and IRF3 (interferon regulatory factor 3) downstream of the cytosolic RNA sensors [86]. An alternative, non-exclusive explanation for the amplification of RLR signaling in ATG5 deficient cells builds on the accumulation of mitochondria in the absence of functional autophagy, resulting in increased levels of reactive oxygen species that are sufficient to potentiate RLR signaling [87].

Because of the potential detrimental effects of IFN-I, several molecular machineries keep the RLR pathway in check. A unique member of the nucleotide-binding domain (NBD) and leucine-rich-repeats (LRRs)-containing proteins (NLRs), NLRX1, resides in the mitochondria and attenuates the activation of MAVS by binding to its CARD domain, possibly precluding its engagement with RIG-I [88]. One link between cytosolic RNA sensors and the autophagic machinery is provided by the mitochondrial Tu translation elongation factor (TUFM), through its interactions with the ATG5-ATG12/ATG16L1 complex (the precise partner was not solved) and with NLRX1 [89]. Like NLRX1, TUFM negatively regulates RLR-induced IFN-I and promotes autophagy during viral infection.

These, and many other studies on the interplay between autophagy and the immune response to viral infection, converge to a suppressive role exerted by autophagy on excess IFN-I production in response to viral nucleic acids. Two recent works investigated how this relates to the outcome of host-microbiote interaction at the intestinal barrier [90,91]. Using several mouse models, the first study showed that mice deficient in autophagy proteins were protected from the intestinal bacterial pathogen *Citrobacter rodentium* in a manner dependent on IFN-I signaling and nucleic acid sensing pathways. Importantly, increased IFN-I signaling in *Atg16l1* hypomorphic (*Atg16l1*^{HM}) mice was observed even in the absence of *C. rodentium* infection and was lost in germ free animals, indicating that the IFN-I signaling is a spontaneous response of epithelial cells to the microbiota, which is normally suppressed by the autophagy machinery. Strikingly, the same machinery that senses viral nucleic acids is required to confer the resistance to *C. rodentium* infection, as *Atg16l1*^{HM}*Mavs*$^{-/-}$ mice, or *Atg16l1*^{HM}*Sting*$^{-/-}$ mice lose the protection conferred by ATG16L1 deficiency, indicating that both DNA and RNA moieties from the microbiota (possibly from its viral component) are sensed. The second study, using mice lacking

ATG16L1 in myeloid cells, also showed increased clearance of intestinal *Salmonella typhimurium* in an IFNR-dependent manner [91]. Mechanistically, the authors showed that loss of ATG16L1 promoted accumulation of the adaptor TRIF, and identified the proteins TaxBP1 and SQSTM1 (p62) as intermediates between TRIF and the autophagy machinery. Knockdown of *Tax1bp1* phenocopied the effect of ATG16L1 depletion, i.e. increased production of the cytokines IFN-β and IL-1β upon engagement of TLR3 or TLR4, indicating that the phenotype of ATG16L1 deficient cells is due to the impairment of the core autophagy machinery, resulting in sustained signaling downstream of TRIF. Mice lacking ATG16L1 in myeloid cells succumbed to lipopolysaccharide (LPS)-mediated sepsis, emphasizing the importance of ATG16L1 for an adequate, balanced immune response.

The dampening action of ATG16L1 on INF-I signaling was also recently illustrated by the role of this protein in tuning IL-22 signaling in the intestinal epithelium [92]. IL-22 is a protective cytokine of the IL-10 cytokine family that contributes to intestinal immune response towards pathogen infection and epithelial wound healing. However, IL-22 stimulation leads to transient ER stress [92]. One model to simulate ER stress, and trigger the consequent unfolded protein response, is to deplete cells of the transcription factor X-box binding protein-1 (Xbp1). This model was used to demonstrate the compensatory roles played by the unfolded protein response (UPR) and autophagy to resolve ER stress [27]. As expected, mice deficient for both ATG16L1 and Xbp1 in their intestinal epithelial cells presented at baseline with significant ileal inflammation and cell death. This phenotype was aggravated upon IL-22 stimulation, with a strong increase in INF-β and, subsequently, TNF expression. These findings may explain why, under certain circumstances, IL-22 may aggravate intestinal inflammation rather than resolve it [92]. Importantly, IL-22 induced INF-I signaling was completely abrogated in the absence of STING or of cGAS, two molecules implicated in the detection of cytosolic DNA sensing, demonstrating that, like in the viral context, IFN-I signaling upon IL-22 stimulation proceeds in a STING-dependent manner, although the nature and origin of the signal(s) that triggers this signaling pathway remains unclear. Also, the part played by the ATG5/ATG12/ATG16L1 complex is not fully understood. Pharmacological inhibition of autophagy with bafilomycin A phenocopied the enhanced IL-22–induced ISG induction in wild type organoids, indicating that autophagy plays a role, but additional autophagy-independent modes of action of ATG16L1 cannot be excluded.

III-B. ATG16L1 deficiency stimulates interleukin-1β (IL-1 β) production in macrophages

The second insight on an anti-inflammatory role of ATG16L1 came from the observation that macrophages expressing a truncated form of ATG16L1 lacking the coiled coil domain produced high amounts of the inflammatory cytokines IL-1β and IL-18, two proteins that are processed into a mature form by cleavage by caspase 1 [93]. The regulation was likely post translational since the level of immature pro-IL-1β was almost unaffected. Indeed, LPS stimulation led to an increase in caspase-1 activation and IL-1β production in ATG16L1-deficient cells compared to control cells. ATG7-deficient macrophages, and overexpression of ATG4B, which inhibits LC3 lipidation, also enhanced LPS-induced IL-1β production, indicating that the phenotype observed in the ATG16L1-deficient macrophages is linked to the loss of LC3 lipidation capacity [93].

The dampening of IL-1β mediated inflammation exerted by ATG16L1 was further illustrated in an infectious context, with the demonstration that *Atg16l1*^{HM} mice cleared urinary tract infection by uropathogenic *Escherichia coli* (UPEC) more rapidly and thoroughly than the control animals [94]. Mechanistic studies confirmed that loss of ATG16L1 enhanced the release of IL-1β in response to UPEC in a caspase-1 and NLRP3 inflammasome-dependent manner, and that augmented IL-1β signaling is the primary mechanism responsible for enhanced clearance of UPEC from the urinary tract in ATG16L1-deficient mice [95]. These data argue for a suppressive role of ATG16L1 on the IL-1β response in macrophages, that would otherwise protect the host from UPEC infections, in line with the more recent report showing that absence of ATG16L1 in the myeloid lineage leads to increased production of IL-1β and INF-β downstream of TRIF [91].

III-C. ATG16L1 is required for a balanced dendritic cell response to infection

Similar to what was reported on macrophages, ATG16L1 deficiency is associated to dendritic cell (DC) hyperactivity. This was first described in a mouse model of allogeneic hematopoietic stem cell transplantation model, in which ATG16L1-deficiency led to increased DC numbers and alloreactive T cell proliferation [96]. Antibody-mediated blockade of IL-1 β or its receptor did not affect the increased capacity of *Atg16l1*^{HM} DCs to stimulate alloreactive T cell proliferation, indicating that a mechanism distinct from the above-mentioned overstimulation of IL-1β production in *Atg16l1*^{HM} macrophages was at work. One phenotype of the *Atg16l1*^{HM} DCs was an increase in lysosomal structures, indicative of impaired lysosomal functions. The authors also reported an increase in Laptm5, a lysosomal transmembrane protein that promotes the degradation of several targets involved in immune signaling including the anti-inflammatory, ubiquitin-editing enzyme A20 in *Atg16l1*^{HM} DCs. Interestingly, it was recently shown that A20 is an ATG16L1 WD40-binding protein, and that, in epithelial cells, absence of A20 promoted ATG16L1 accumulation, while elimination of ATG16L1 stabilized A20 [79]. The reverse observation was made in *Atg16l1*^{HM} DCs, in which A20 abundance also decreased [96], indicating that the outcome of the interplay between these two proteins is cell-type dependent.

A second illustration of the importance of ATG16L1 in regulating DC-mediated stimulation of the immune system came from the study of the response of DCs to immunostimulatory molecules present in *Bacteroides fragilis* outer membrane vesicles (OMVs) [97]. ATG16L1-deficient dendritic cells failed to induce regulatory T cells (T_{regs}) upon OMV stimulation. The same was observed in *Rubicon*$^{-/-}$ DCs, while other proteins involved in canonical autophagy upstream of the ATG5-ATG12-ATG16L1 complex were dispensable, indicating that defective LAP is implicated in the lack of response to OMVs in ATG16L1-deficient DCs. Importantly, stimulation with OMVs resulted in an increased transcription of multiple proinflammatory cytokines in ATG16L1-deficient DCs compared to WT cells, consistent with an hyperinflammatory response. Altogether, these data suggested that ATG16L1 deficiency in DCs altered the quality of the T cell response to OMVs, with a failure to induce T_{regs} and to suppress mucosal inflammation.

III-D. ATG16L1 is a negative regulator of NOD signaling

Beyond its contribution to tagging invading bacteria with LC3, the interaction of ATG16L1 with NOD proteins has a strong anti-inflammatory role, independent of its engagement in autophagy. Indeed, knock down of ATG16L1 expression, but not that of ATG5 or ATG9a, specifically enhanced NOD-driven cytokine production, indicating that ATG16L1 had a suppressive role on NOD signaling. Mechanistically, ATG16L1 interfered with the recruitment of the adaptor protein RIP2 into large signaling complexes, thus dampening the downstream signaling [69]. Interestingly, while both NOD1 and NOD2 interact with ATG16L1, different domains are implicated as only NOD2 CARD domain is able to co-immunoprecipitate with the WDD [68].

III-E A perilous equilibrium

All these studies show that the level of ATG16L1 conditions the amplitude of the innate immune response to an injury or an infection. Globally, the emerging view is that the innate response is exacerbated when ATG16L1 is low or absent, implicating that, overall, ATG16L1 suppresses inflammation. In many of the experimental systems used, the phenotypes associated with loss of ATG16L1 result from impaired autophagy, but it has not always been investigated in detail, and has sometimes been demonstrated that a non-autophagy related function of ATG16L1 was implicated. Notwithstanding the mechanism(s) at work, the outcome is that the presence of ATG16L1 appears to have an adverse effect in some situations, since its absence helps clear certain infections or injuries. First of all, this is not always the case; we emphasized earlier the beneficial role of ATG16L1 in bacterial clearance through xenophagy for instance. Secondly, the adverse effects of the suppression of inflammation by ATG16L1 likely comes in exchange of the benefit of preventing deleterious over-activation of this pathway. This was demonstrated upon several circumstances, such as the susceptibility of mice lacking ATG16L1 in myeloid cells to LPS-mediated sepsis [91] or to *Chlamydia pneumoniae* infection [98]. In that last example, the bacterial burden was the same in ATG16L1 deficient and control mice, indicating that mortality occurred as a consequence of the loss of control normally exerted by ATG16L1 over inflammation rather than failure to clear the infection. Still, there are circumstances under which lower expression of ATG16L1 is advantageous. This protective effect of ATG16L1 deficiency might have contributed to the selection of the T300A ATG16L1 allele in a large proportion of the population (Table 2).

IV –ATG16L1, an actor in the regulation of intracellular trafficking

IV-A. ATG16L1 participates to secretory autophagy

Secretory autophagy facilitates extracellular release of cytosolic cargo such as leaderless cytosolic proteins, aggregate-forming proteins, or organelle material (reviewed in [99]). Several molecules identified for their contribution to autophagy contribute to these non-degradative trafficking pathways.

This includes ATG16L1, whose hypomorphic expression in intestinal cells provided the first cue of this unexpected role for "autophagy proteins" in intracellular traffic (Figure 3). Indeed, Paneth cells of *Atg16l1*^{HM} mice exhibit notable abnormalities in the granule exocytosis pathway and a notable lack of lysozyme staining in the mucus, whereas other intestinal epithelial cells appeared normal [100]. A later study confirmed the role of secretory autophagy for the secretion of lysozyme by Paneth cells during bacterial infection, and identified some of the extrinsic signals involved in this process [101]. Ultrastructure analysis of Paneth cells of infected mice contained lysozyme filled large LC3-positive granules surrounded by a double membrane, while granules from uninfected mice were small and surrounded by a single membrane. Secretory autophagy was triggered in Paneth cells by bacteria-induced ER stress and required extrinsic signals from innate lymphoid cells. Another illustration of the role of the ATG12-ATG5/ATG16L1 complex in specialized lysosome secretion was provided by the directional release of lysosome-residing resorptive molecules by osteoclasts [102]. However, in that case secretory lysosomes remained single membraned and did not appear to be LC3 coated, meaning the qualification of "secretory autophagy" might not be appropriate here, and the phenomenon might be more related to the pathways described below.

IV-B. ATG16L1 participates to secretory pathways that are not related to autophagy

The best documented demonstration that ATG16L1 can participate to secretion independently of autophagy was made by the study of hormone secretion by neuroendocrine PC12 cells [103]. ATG16L1 is localized on hormone-containing dense-core vesicles in the neurites of these cells. LC3 is not enriched on these granules, and ATG16L1 recruitment depends on its interaction with the small GTPase Rab33A. Knockdown of ATG16L1 causes a dramatic reduction in the level of hormone secretion, while a ULK1 kinase-dead mutant or an ATG4B mutant have no effect, demonstrating that ATG16L1 does not act downstream of the canonical autophagy cascade. Rab33A is abundantly expressed in the brain, lymphocytic system, and hematopoietic system, indicating that ATG16L1 could contribute to cell-specific secretion events in these tissues.

More recently it was shown that the ATG12-ATG5/ATG16L1 complex was necessary for plasma membrane repair upon bacterial toxin-mediated damage [104]. Here again, ATG16L1 promoted lysosomal exocytosis at the plasma membrane, but loss of the autophagy components Rubicon, ATG3, ATG9, and ATG14 did not affect toxin resistance, demonstrating that canonical autophagy was not implicated. The precise role of the ATG12-ATG5/ATG16L1 complex in plasma membrane repair remains to be determined. The WDD was required to suppress toxin damage, indicating that WDD binding protein(s) are implicated. Absence of ATG16L1 caused intracellular cholesterol accumulation, which in itself might be sufficient to impair repair upon toxin injury.

IV-C. ATG16L1 is associated to Rab6 dependent intracellular traffic

A recent study showed that, in normal conditions, TMEM59 positive compartments capture vesicles positive for the small GTPase Rab6 in a ATG16L1 dependent manner [105]. Interestingly, the intracellular pathogen, *Chlamydia trachomatis,* secretes a protein, TaiP, that binds the ATG16L1's

WDD, thereby competing with TMEM59. Secretion of TaiP allows the bacteria to hijack the Rab6 positive pool of vesicles to support the growth of the compartment in which the bacteria develop, the inclusion. In its absence, ATG16L1 restricts inclusion growth, and the effect is independent of the role of ATG16L1 in autophagy since a construct encompassing the WDD (aa 266-607) is sufficient for this effect, while a truncated form lacking the WDD is not.

IV-D. Loss of ATG16L1 or expression of the T300A variant affects endocytic events

This role of ATG16L1 in trafficking indicates that ATG16L1 deficiency (or expression of the T300A variant) might affect trafficking events. This has not been thoroughly investigated, but some observations suggest that it is indeed the case (Table 2). Morphological studies showed that the architecture of urothelial cells of T300A mice, or of urothelial cell devoid of ATG16L1, was different from that in wild type mice, for instance they were observed to have a higher density of multivesicular bodies [22]. This phenotype was not observed in cells lacking ATG14, a component of the PI3KC3 complex (see II.A), or EPG5, that regulates lysosome degradative capacity [106]. These observations indicate that defects in autophagy might not account for the morphological changes observed in ATG16L1 deficient urothelial cells [22]. The T300A variant is associated with increased expression of several small GTPase of the Rab family including Rab33B, an ATG16L1 interactant. The T300A variant restricts the ability for the UPEC to establish persistent intracellular reservoirs, thereby protecting the mice against urinary tract infections. An earlier study had shown that macrophages expressing low levels of ATG16L1 engulfed more UPEC within one hour than the wild type cells, indicating that ATG16L1 level might also affect phagocytosis efficiency and subsequent bacterial clearance [95]. On the other hand, the entry rate of the bacterium *Salmonella* in epithelial cells, measured 10 minutes after infection, showed a two-fold reduction in the absence of ATG16L1, or when the T300A variant was expressed, indicating that the overall effect of ATG16L1 on endocytosis/phagocytosis depends on the cell type and cargo [107].

Another illustration of the consequences of the absence of ATG16L1 on intracellular traffic comes from the observation that exosome production was strongly reduced in cells lacking *Atg5* or *Atg16l1*, but not in cells lacking *Atg7*, implicating that this phenotype was independent of canonical autophagy. The scenario proposed in that study involved the ATG5/ATG16L1 dependent dissociation of a subunit of the V-ATPase, and its degradation in exosomes, thereby controlling the acidification of exosomes and their release [108]. The interaction between the WDD and the V-ATPase observed in the context of an infection and discussed in II.C might be implicated in the mechanism by which ATG5 and ATG16L1 regulate exosome maturation and release [70,77]

Finally, recent work in the amoeba *Dictyostelium discoideum* confirmed the essential function of the ATG12-ATG5/ATG16L1 complex in canonical autophagy, but also supported an autophagy-independent functions of the complex and its individual components in the regulation of phagocytosis and pinocytosis. The data indicate that engagement of the complex in endocytic events might have evolved early [109].

CONCLUSIONS AND PERSPECTIVES

Most, if not all, components of the molecular machinery for autophagy also mediate autophagy-independent functions [110]. ATG16L1 gives a strong illustration of this diversification of activities, mostly mediated by its carboxy-terminal extension, in particular the WDD. However, mice lacking the linker domain and the WDD of ATG16L1 survive neonatal starvation, are fertile, and show no obvious phenotype in several examined tissues [111]. These observations revealed that, unlike the core "autophagy"-module, the carboxy-terminal extension in ATG16L1 was dispensable for development. *In vitro* experiments reported in this review indicate that the carboxy-terminal extension might function redundantly with other pathways, for instance in the regulation of intracellular trafficking, and becomes important when cells need to respond to a disruption of host cell homeostasis such as an infection or an injury. This novel animal model will allow for further testing of this hypothesis. Regarding the roles of ATG16L1 in intracellular trafficking, which rely, at least in part, on WDD interactions with small GTPases or transmembrane proteins, more detailed investigation of the consequence of the loss of the linker and WDD in specific cell types are needed, while keeping in mind that compensatory pathways might explain a lack of phenotypes.

The allele variant associated to Crohn's disease, T300A, affects an amino acid that is beyond the coiled-coil domain, and is thus not expected to influence canonical autophagy. This is indeed what most experimental set-ups observed, although some cell type specificity might arise (Table 2). In contrast, most, but not all, studies report on a defect in bacterial clearance associated with the T300A allele. Discrepancies might be due to the different models used, as residual ATG16L1 expression might have confounded interpretation of some of the early results, before knock-out or knock-in models became the norm. Also, because of the ability for ATG16L1 to dimerize, it is important to work in an *ATG16L1* KO background when re-expressing different forms of the molecule. At this stage, a variety of phenotypes, in different cell types, have been associated to the T300A allele, and it remains unclear as to which are relevant to the susceptibility to Crohn's disease (Table 2). In many instances, the phenotype observed in homozygous T300A cells or individuals had the same trend as what was seen with low or no ATG16L1 expression. This supports the possibility that decreased ATG16L1 expression in individuals expressing the T300A allele (due to increased cleavage by caspase 3) explains the T300A phenotype. However, this is not systematically the case, and when heterozygous populations have been investigated, which is not very often, they are sometimes grouped with the T300A homozygous genotype, and sometimes with the "wild-type" homozygous phenotype. Also, as mentioned earlier, comparison of the phenotypes of *Atg16l1*$^{KO/+}$ and *Atg16l1*$^{T300A/+}$ bone marrow derived macrophages argue for a dominant negative activity of the T300A variant over the wild type variant [14]. This possibility should be more systematically investigated, for instance by looking at the complementation of *ATG16L1* KO cells with the two fragments resulting from the caspase 3 cleavage, individually or together. Along that line, the carboxy-terminal domain of ATG16L1 was recently found to be necessary and sufficient to recapitulate the activity of the full-length protein, thus supporting the idea that this domain might have physiological activity [105]. The same study showed that a bacterial protein evolved to counteract ATG16L1's activity on intracellular trafficking, not on autophagy as one might have expected, emphasizing that these

"secondary" activities of ATG16L1 may be more important than initially thought. Given the quality of the tools available (various knock-in mice, or cell-specific KO, complementation with various forms, including the WDD only) our understanding of the contributions of ATG16L1 to the maintenance of homeostasis should continue to progress rapidly.

Author contributions

DH and AS wrote the MS, DH drew the figures.

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900 Table 1: **ATG16L1 binding proteins**
901

Name	Interaction domain	Proposed function	Ref.
A20	WDD (320-607)	Control of intestinal homeostasis	[79]
ATG16L1	126-207 (Coiled-coil)	Dimerization	[4,9,23,24]
ATG5	11-28	Formation of the ATG12-ATG5/ATG16L1 complex	[4,112]
C3	not determined	Targeting to autophagy	[63,64]
DEDD2	not tested, presumably WDD	unknown	[68]
FIP200	230-246 (and 247-607)	Recruitment to PAS	[56 ,57 ,62]
IRGM	85-341 and WDD (286-607)	Coupling to innate immunity receptors	[113]
Nod1	not determined	Negative regulation on cytokine response	[68]
Nod2	WDD (320-607)	Negative regulation on cytokine response	[68,69]
Rab26	1-265 (murine)	Promotion of selective autophagy of synaptic vesicles	[114]
Rab33B & Rab33A	141-265	Modulation of autophagosome formation/Contribution to hormone secretion	[103,115]
T3JAM	not tested, presumably WDD	unknown	[68]
TaiP	WDD (266-607)	Competition with TMEM59 binding	[105]
TLR2	WDD (320-607)	unknown	[68]
TMEM166/EVA1	WDD (301-588)	Promotion of autophagy and cell death	[60]
TMEM59	WDD (320-607)	Recruitment to TMEM59 positive compartment	[68]
TMEM74	WDD (320-607)	Promotion of autophagy in tumor cells	[116]
TRIM20	85-341 and WDD (266-607)	Target inflammasome components to autophagic degradation	[117]
Ubiquitin	WDD (245-607)	Increased xenophagy	[62]
V-ATPase	139-588 (WDD necessary but not sufficient)	Recruitment to pathogen-damaged vacuoles	[70]
WIPI2b	207-230	Recruitment to PI3P rich membrane	[58]
Gigaxonin	WDD (266-623)	Ubiquitination of ATG16L1 for targeting to degradation	[47]
SNX18	not determined	Production of ATG9A and ATG16L1 vesicles for delivery to PAS	[59,118]

902

903 Table 2: **Phenotypic manifestations of the expression of ATG16L1 T300A variant**. The studies
904 are presented in chronological order. Approaches using overexpression were progressively replaced in
905 many laboratories by more accurate models involving knock-in cells or patient-derived primary cells.
906

Subject, tissue or cell type	Model	Read-out	Phenotype observed in 300A variant compared to the 300T allele	Ref.
Human monocytes and epithelial cell line (HT29)	• Monocytesderived from homozygous or heterozygous subjects • Re-expression of either WT or T300A ATG16L1 in siRNA treated HT29 cells	• TNF-α secretion upon MDP-stimulation • *Salmonella* clearance upon MDP stimulation (gentamycin protection assay)	No difference in primary macrophages, impaired MDP stimulated *Salmonella* clearance in epithelial cells (no comparison of expression levels)	[66]
Human MDAMC epithelial cells	Re-expression of either WT or T300A ATG16L1 in *ATG16L1* KO cells	Rescue of the exaggerated IL-8 expression observed in the absence of ATG16L1	Partial rescue with WT form, not with the T300A form	[69]
Human epithelial cell lines (HeLa, Caco-2)	Silencing of endogenous ATG16L1 expression and complementation with WT or T300A ATG16L1	• *Salmonella* in LC3 positive compartment • LC3 lipidation	• 2-fold decrease in *Salmonella* contained in LC3 positive compartment • No effect on basal LC3 lipidation flux	[119]
Mouse fibroblasts	MEFs expressing ATG16L1-ΔCCD complemented with WT or T300A ATG16L1	LC3 positive compartments and *Salmonella* survival	• No effect on canonical autophagy • No effect on the elimination of *Salmonella*	[26]
Human monocytes	Monocytes derived from homozygous subjects	• *In vitro* infection with *Mycobacterium avium* • Cytokine production	• No difference in bacterial growth • Elevated production of IL-6 and IL-10, no difference for IL-8 or TNFα	[120]
Human monocytes	Monocytes derived from subjects homozygous or heterozygous subjects	Cytokine production upon contact with *Mycobacterium tuberculosis*	No difference in TNFα or IL-1b production (slightly less INFγ production)	[121]
Human epithelial cell line (HCT116)	Knock-in cells	• *S.typhimurium* invasion (10 min) • Bacterial clearance (8h)	• Reduced invasion capacity • Defective bacterial clearance	[107]
Human DCs	Monocytes derived DC from pediatric homozygous or heterozygous patients	• *E. coli* bioparticles uptake • DC maturation (HLA-DR and CD86 expression upon exposure to bacteria particles) • Formation of protrusions through CaCo2 epithelial layers	• Reduced bacterial particle uptake and DC maturation in heterozygous or T300A homozygous DC • Defective protrusion formation for the T300A homozygous DCs, not for the heterozygous DC	[122]
Human PBMC and mouse macrophages	• PBMC derived macrophages from human homozygous donors • Macrophages isolated from knock-in mice	• Sensitivity to caspase-mediated cleavage at the D299-T300 scissile site • Clearance of *Yersinia enterocolitica* infection by macrophages *in vitro* • Cytokine production	• Increased sensitivity to caspase cleavage (induced by TNF-α) • No difference for LC3-lipidation upon rapamycin treatment • Impaired *Yersinia* clearance by macrophages (but no defect in phagocytosis per se), resulting in elevated inflammatory cytokine response	[13]
Mice, MEFs, primary CD11b+ cells	Knock-in mice and derived cells for *in vitro* assays	• Morphological analysis • Autophagy flux in MEFs • IL-1β levels in culture supernatants of CD11+ cells stimulated with LPS and MDP or with *Shigella flexneri* • Infection of mice with *S. typhimurium*	• Abnormalities in Paneth cell lysozyme distribution and in goblet cell morphology in the colonic epithelium (not in the small intestinal epithelium) • Modest effects on basal autophagy • Decreased bacterial clearance and increased IL-1β production in primary cells and *in vivo*	[78]
Human PBMC Humans	• PBMC derived from healthy controls and CD patients • CD patient cohort	• TNF-α and IL-1β production upon stimulation with 5 different pathogenic bacterial species • Clinical data	• Increase in IL-1β production for all tested pathogens and in TNF-α production for 3 out of 5 bacterial species tested (only in homozygous T300A) • No significant association between disease-specific infectious complications and genotypes, or non-disease-specific infections and genotype	[123]
HCT116, MEFs	• Knock-in cells or complemention with WT or T300A variant • Purified proteins	• Number of TMEM59-GFP positive vesicles/cell • Pull-down of different ATG16L1 WDD partners (full length or peptides) LC3-positive phagosomes containing S. aureus, bacterial cfu, ATG16L1/TMEM59 co-immunoprecipitation (2 hpi)	• Altered TMEM59-GFP trafficking • Some WDD partners (i.e. TMEM59, DEDD2, T3JAM) pulled down less efficiently with the A300 variant while others (TLR2, NOD2) did equally well or better. • Reduced ability to respond to bacterial infection	[76]
Bone marrow derived DCs Mice and Humans	• Bone marrow derived DCs from knock-in mice	• T_{reg} reponse to outer membrane vesicles	• Impaired T_{reg} response • No protection against colitis	[97]

	• Human DCs from homozygous subjects	• Protection from chemically induced colitis		
Bone marrow derived macrophages	• Homozygous and heterozygous knock-in mice • Comparison of Atg16L1$^{T300A/+}$ with Atg16L1$^{KO/+}$	• LC3 lipidation • IL-1β production upon various stimulations • Clearance of ingested bacteria in vitro and in vivo • Susceptibility to Caspase 3 cleavage	• T300A homozygous and heterozygous have defect in canonical autophagy in macrophages, increased IL-1β production, increased susceptibility to cleavage • Atg16L1$^{KO/+}$ behaves as WT, not like Atg16L1$^{T300A/+}$ or Atg16L1$^{T300A/T300A}$	[14]
Mice	Knock-in mice	• Susceptibility to *C. rodentium* infection upon Caspase-3 activation • Susceptibility to murine norovirus induced pathology in the intestinal epithelium	• Decreased susceptibility to *C. rodentium* infection • Increased susceptibility to murine norovirus induced pathology in the intestinal epithelium	[90,124]
	Healthy and CD patients, homozygous and heterozygous	IRE1α expression in noninflamed areas of the ileum	Increase in the percentage of IRE1α positive crypts on biopsies (not in heterozygous individuals)	[125]
Resections specimens	Japanese and North American CD patients	Paneth cell morphology	No correlation between the number of T300A allele and the percentage of abnormal Paneth cells in the Japanese cohort, in contrast with North American cohort	[21]
Macrophages	Monocytes derived macrophages from healthy homozygous donors	IFN-β and IL-1β production upon LPS or Poly(I:C) stimulation	Increased IFN-β and IL-1β production	[91]
Fibroblasts	MEFs expressing ATG16L1-ΔCCD complemented with WT or T300A ATG16L1	• Resistance to toxin mediated plasma membrane damage • Cell to cell spread by *Listeria monocytogenes*	• Decreased resistance to damage • Increased cell to cell spread	[104]
Mice, bone-marrow derived macrophages, human biopsies	• Knock-in mice with wild-type or T300A variant • Human bladder tissue biopsies (homozygous or heterozygous for *ATG16L1)*	• Quantification of quiescent intracellular reservoirs (QIRs) of UPEC Architecture of superficial urothelial cells • IL-6, TNF and IL-1β production • Expression of Rab GTPases	• No difference in infection or early colonization but decrease in QIRs • Change in urothelial cell morphology in mice, and in human biopsies (T300A homozygous and heterozygous group together) • No change in the cytokine response to infection • Increase expression of some Rab proteins	[22]
Mice	• Knock-in mice with wild-type or T300A variant • Oral human stool inoculation	• Gut microbial composition • Composition of the population of immune cells	• Different microbiota at steady state and upon gut-induced inflammation • Human microbiota from CD patients show susceptibility to the presence of T300A (not the case for microbiota from ulcerative colitis patients) • Enhanced presence of Th17 and Th1 cells when *Bacteroides ovatus* added to the flora	[126]
Hek293, HCT116	Overexpression of ATG16L1 WT or T300Ai n *ATG16L1* KO cells	ATG16L1 cleavage upon ULK1 overexpression or upon Salmonella infection	Increased cleavage upon infection or metabolic stress, dependent on ATG16L1 phosphorylation at S278 by ULK1	[45]
T cells, DCs, mast cells	ATG16L1 T300A knock-in mice and mice KO for ATG16L1 in specific lineages (DCs, T cells, mast cells)	Transcriptional profile of different cell types at basal level or upon different stimulation	Stimulation-dependent and cell-type dependent programs regulated by the *Atg16l1* genotype.	[127]

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Fig. 1:ATG16L1 and its partners

A- Schematic representation of ATG16L1. Binding sites for some of its interactors and the caspase cleavage site are indicated. Ser designates serine residues 139, 268 and 278 that undergo phosphorylation and Lys indicates lysine 151 that can be methylated. LB stands for lipid binding. The WD40 domain forms a 7-blade beta-propeller. Residue numbers correspond to the β isoform of human ATG16L1, the second lipid binding domain (266-284) is absent from the α isoform. This figure was prepared using the IBS illustrator [128]

B- Comparison of ATG16L1 orthologs in different species. Domains were defined using PFAM. The peptide sequences (Uniprot) used were Q676U5 (*H. sapiens*), Q8C0J2 (*M. musculus*), A0A0R4IBY0 (*D. rerio*), B7Z0R7 (*D. melanogaster*) and Q03818 (*S. cerevisiae*). The percentage of similarity compared to the equivalent domain in *H. sapiens* ATG16L1 is indicated.

Fig. 2: The LC3 conjugation machinery.

1- ATG12 is activated by the E1-ligase protein ATG7 and conjugated to ATG5 through the E2-like activity of ATG10. ATG16L1 binds to ATG5, leading to the formation of the ATG12-ATG5/ATG16L1 complex

2- LC3 is activated by the cysteine protease ATG4 (exposing a glycine residue on its C-terminal domain).

3- ATG7 transfers activated LC3 to the E2-like protein ATG3, followed with the catalysis, by the ATG12-ATG5/ATG16L1 complex, of the transfer of phosphatidylethanolamine (PE) to LC3, resulting in the synthesis of lipidated LC3. ATG16L1 orchestrates the spatiotemporal targeting of the LC3 conjugation machinery to specific membranes.

4- The LC3 conjugation machinery operates on double membranes in canonical autophagy, and on a variety of single membranes. The two processes display several differences, in particular the WDD is dispensable for canonical autophagy but not for LC3-lipidation on single membranes.

Fig. 3: ATG16L1 regulates intracellular trafficking events independently of autophagy.

A- ATG16L1 regulates the exocytosis of lysozyme positive stress granules in Paneth Cells. Hypomorphic mice with highly reduced expression of ATG16L1 exhibit defects in lysozyme secretion leading to chronic infection by enteric pathogens in the gut.

B- ATG16L1 regulates the secretion of cholesterol rich compartments to the plasma membrane in case of damage. This activity limits L. *monocytogenes* cell to cell dissemination.

C- ATG16L1 controls the flux of Rab6 positive vesicles to TMEM59 positive compartments. The pathogen *C. trachomatis* hijacks Rab6 trafficking by secreting a bacterial proteins that blocks the interaction between ATG16L1 and TMEM59.

D- The secretion of hormone-containing dense core vesicles in neuroendocrine PC12 cells is dependent on ATG16L1, presumably through its interaction with Rab33a. The loss of ATG16L1 drastically reduces hormone secretion.

Figure 1

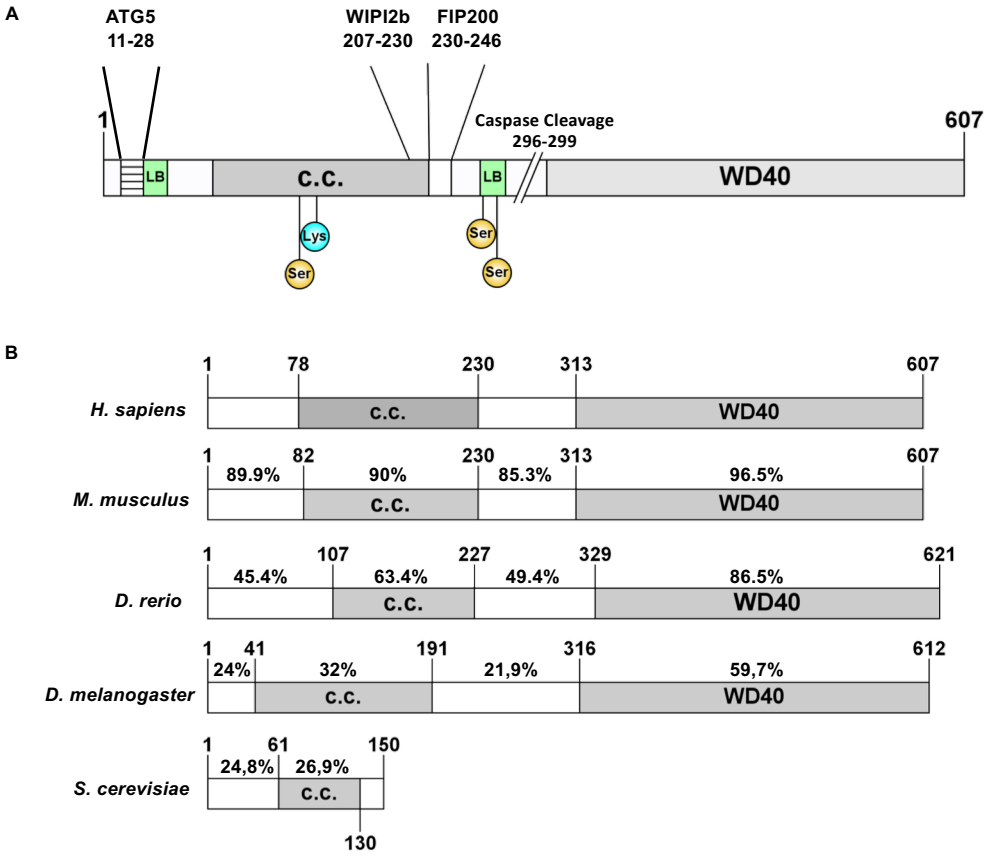
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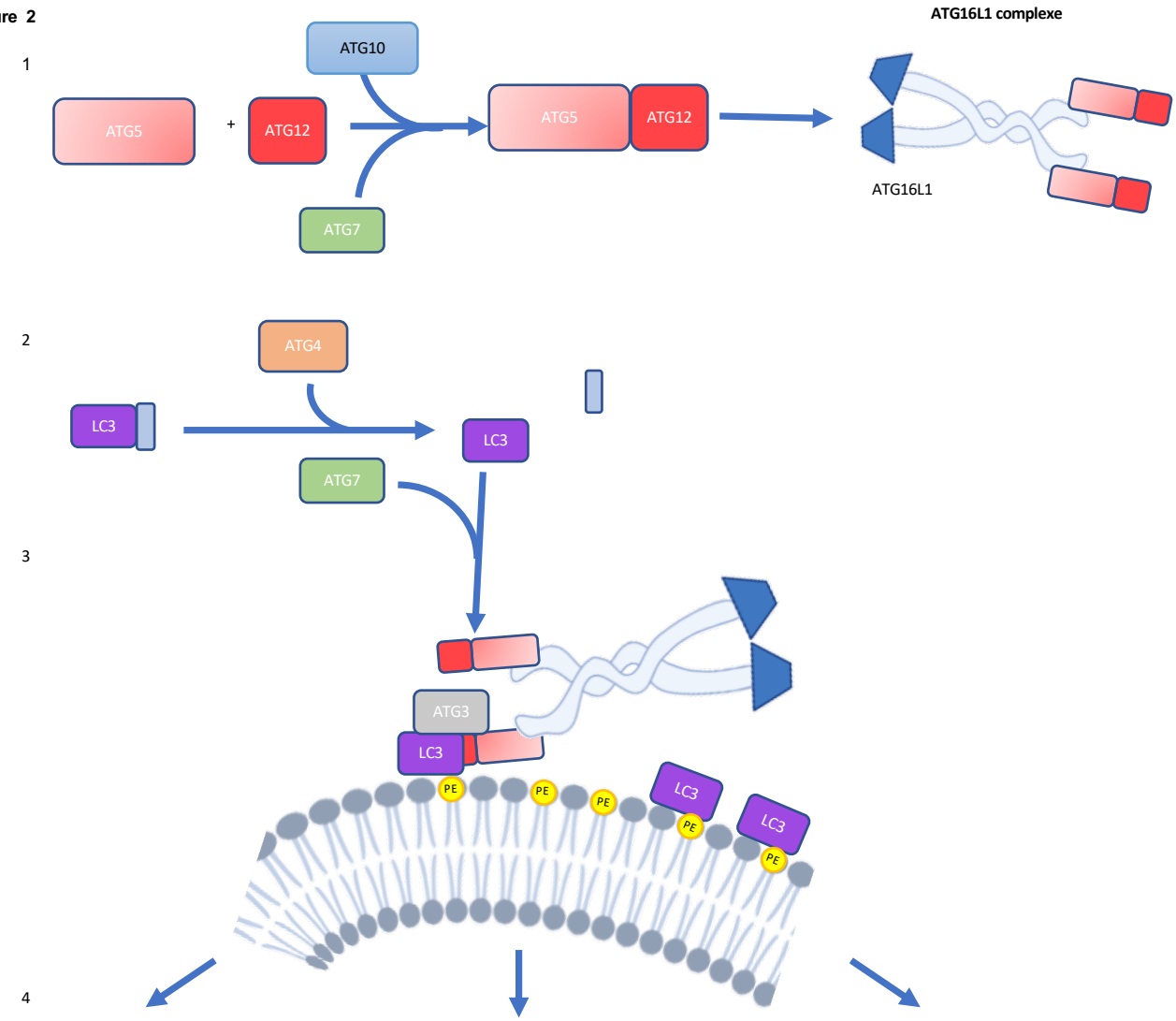
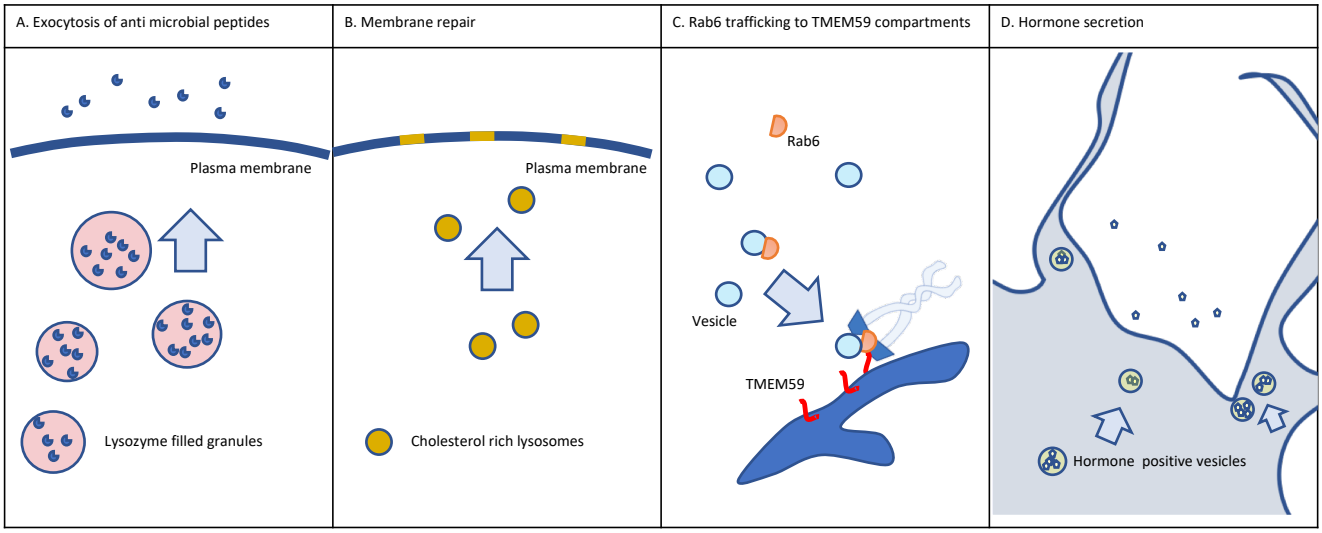
Figure 2



On double membranes (canonical autophagy)	On phaogosomes (LC3 associated phagocytosis)	Other single membranes
- VPS34 dependent - Beclin dependent - WIPI2B dependent - WDD independent	- Single membrane phagosomes - VPS34 independent - Beclin dependent - WDD dependent	- Damaged endosomes - Vesicular remnants after phagosomal escape - LC3 associated endocytosis.

Figure 3


A. Exocytosis of anti microbial peptides
B. Membrane repair
C. Rab6 trafficking to TMEM59 compartments
D. Hormone secretion
Plasma membrane
Plasma membrane
Rab6
Vesicle
TMEM59
Lysozyme filled granules
Cholesterol rich lysosomes
Hormone positive vesicles