

# Non-autophagic roles of autophagy-related proteins

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**Autophagy and autophagy-related processes are fundamentally important in human health and disease. These processes are viewed primarily as cellular degradative pathways that recycle macromolecules and dysfunctional or redundant organelles into amino acids, sugars and lipids, especially during starvation. However, the ubiquitin-like autophagy proteins and other components of the autophagic machinery additionally participate in cellular reprogramming. We highlight these non-autophagic roles of autophagy proteins with the aim of drawing attention to this growing, but unexplored, research topic. We focus on the non-autophagic functions of autophagy proteins in cell survival and apoptosis, modulation of cellular traffic, protein secretion, cell signalling, transcription, translation and membrane reorganization.**

Keywords: autophagy, non-autophagic roles, unconventional protein secretion, signalling, Atg protein function

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See the Glossary for abbreviations used in this article.

## Introduction

Thirty-six known ATG genes that encode autophagy (Atg) proteins coordinate autophagy-related processes. During autophagy, non-selective or specific cargos are captured in a double membrane vesicle (DMV) known as the autophagosome, which forms by the expansion of an isolation membrane from a phagophore assembly site. The autophagosomes fuse with the lysosome (vacuole in yeast), in which hydrolases degrade the cargo for reuse by the cell. The canonical formation of autophagosomes involves five steps [1]: initiation, nucleation, elongation and closure, recycling, and autophagosome fusion with the lysosome to form an autolysosome (Fig 1).

Ubiquitin and ubiquitin-like-molecules constitute a broad class of protein and lipid modifiers the diverse cellular roles of which continue to be discovered [2]. Atg8 and Atg12 are two ubiquitin-like proteins that are integral to general and selective autophagy. Atg12 is coupled with Atg5 to form an Atg12–Atg5 conjugate,

whereas Atg8—or its mammalian orthologues MAP1LC3, GATE16 and GABARAP, often referred to as LC3—is conjugated to a lipid, phosphatidylethanolamine. Conjugation of Atg12 and Atg8—or its orthologues—to Atg5 or phosphatidylethanolamine, respectively, requires an E1 enzyme, E2 enzymes, and an E3-like ligase activity supplied by the Atg12–Atg5/Atg16L complex [3,4], similar to the conjugation of ubiquitin and its relatives.

This review focuses on the ever-expanding array of non-autophagic biological functions for Atg proteins (Fig 2). We define these processes as those generally distinct from, or occasionally in addition to, an involvement in autophagosome formation or lysosomal cargo degradation. Our focus is distinct from ‘non-canonical’ autophagy, in which the biogenesis of conventional autophagosomes involved in macroautophagy proceeds either in the absence of key Atg proteins that originally defined the canonical process, or originates from membrane sources that differ from the traditional phagophore assembly site [1]. The growing appreciation for other functions of Atg proteins has paralleled the explosion of research activity in the ubiquitin field, which began with a narrow focus on the role of ubiquitination in protein turnover, but has now broadened to study its tremendous impact on many aspects of cellular physiology, including protein sorting, DNA repair, gene regulation, protein retrotranslocation, apoptosis and the immune response [5].

We begin by bringing together information on the non-autophagic roles of the ubiquitin-like Atg proteins and then highlight the roles of other Atg proteins.

## Non-autophagic roles of ubiquitin-like Atg proteins

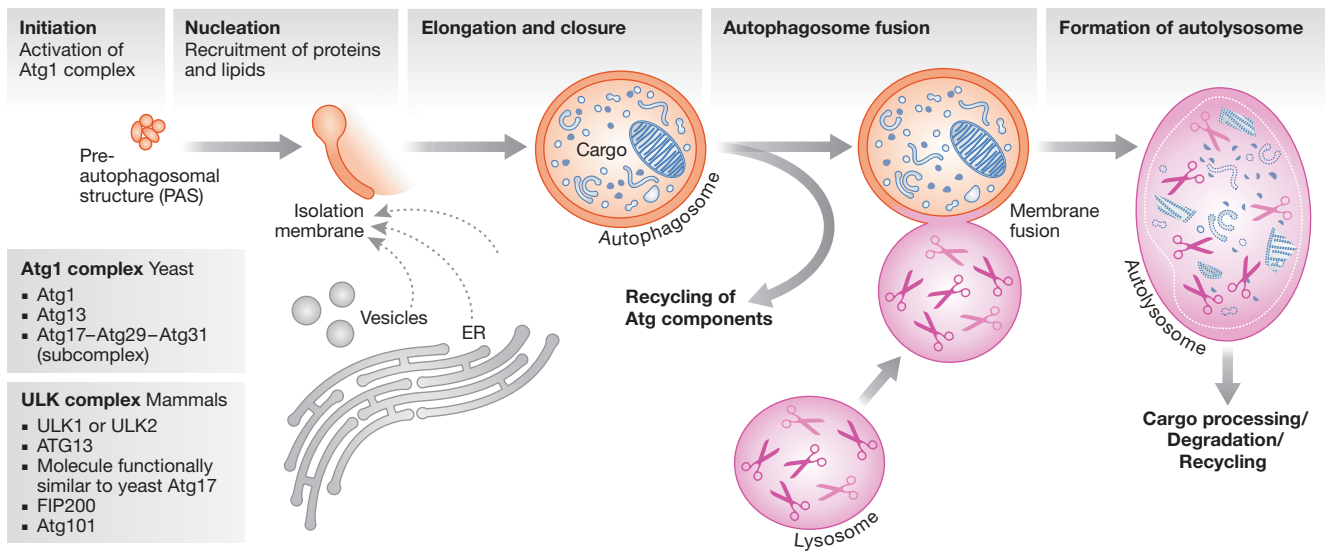
Various analyses of the partners of Atg proteins, particularly of Atg8 and LC3, have revealed many new interacting components, including those that affect other cellular pathways. This has raised the question of whether the ubiquitin-like Atg proteins and their conjugates have a broader role that goes beyond autophagy.

As with the interaction of ubiquitin with many protein domains [6–8], Atg8 and LC3 bind to many proteins either through Atg8-interaction motifs (AIMs) or LC3-interacting regions (LIRs), respectively [9–11]. The six ATG8 orthologues in humans—encoded by the *MAP1LC3A*, *MAP1LC3B* and *MAP1LC3C*, *GABARAP*, *GABARAPL1* and *GATE16* genes—interact with at least 67 other proteins. Furthermore, several cryptic AIMs and LIRs are activated by the phosphorylation of serine and threonine residues in the vicinity of the AIM and LIR [12], thereby potentially expanding the number of putative Atg8/LC3-interacting proteins. Among the non-autophagy-related

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**Fig 1** | Steps in autophagy—initiation occurs by activation of the Atg1 complex (ULK complex I in mammals). In yeast, this complex includes Atg1, Atg13 and the Atg17–Atg29–Atg31 subcomplex. The mammalian Atg1 complex has the yeast Atg1 homologue, Unc-51-like kinases 1 or 2 (ULK1 or ULK2, respectively), the autophagy-related 13 homologue (ATG13), a molecule functionally similar to yeast Atg17, FIP200 (focal adhesion kinase (FAK)-family-interacting protein of 200 kDa) and Atg101 [72]. Nucleation is the step that recruits proteins and lipids for autophagosome biogenesis. It begins with the recruitment of Atg proteins, such as ATG14L and WD-repeat proteins interacting with phosphoinositides (WIPIs), to the PAS—a step requiring the activity of the class 3, PI3K complex, as well as phosphatidylinositol 3-phosphate. Membrane expansion and closure—during autophagosome formation, an isolation membrane expands from the PAS, either by direct flow of membranes from a source such as the endoplasmic reticulum (ER) or by vesicle fusion. The isolation membrane surrounds the cargo, and fusion of the growing edge of the isolation membrane completes autophagosome formation. The two UBL-conjugation systems (Atg8/LC3 and Atg12) are involved in vesicle expansion and closure, and SNAREs are also probably involved. After autophagosome formation, several Atg proteins are recycled in a process involving Atg9. Autophagosome and lysosome fusion—the autophagosome fuses with the lysosome (the vacuole in yeast) to form an autolysosome, in which the cytosolic cargos are degraded and recycled. This process also involves SNAREs. PAS, phagophore assembly site; SNARE, soluble NSF-attachment protein receptor; UBL, ubiquitin-like.

proteins that interact with Atg8 and LC3 are GTPases [13], GTPase-activating proteins (GAPs; [14,15]) and guanine-nucleotide exchange factors (GEFs; [16]). Although these are not direct targets of the autophagic process, they might serve to regulate events involved in the cellular response to autophagy, as illustrated in Table 1.

**Atg8 and LC3 interactions with GTPases.** The Rho family of small GTPases regulate diverse signalling pathways that affect cytoskeletal dynamics, cell cycle progression, gene expression, cell polarity, migration and cell transformation [5]. These and other GTPases behave as two-state molecular switches, comprising an active GTP-bound and an inactive GDP-bound form, and controlled by many proteins including GEFs, GAPs and guanine-nucleotide dissociation inhibitors. The ubiquitin–proteasome pathway regulates several Rho GTPase family members and modulates regulators of the cycling of Rho between its GTP- and GDP-bound states. Although many Rab GTPases regulate autophagy [17,18], the impact of their recruitment by autophagy components on the other cellular processes that they affect, as well as the coordination of the use of these regulators for autophagy and cellular membrane trafficking events, remain to be elucidated.

**Atg8–GAP interaction as a regulatory signalling platform.** The TBC (Tre2, Bub2 and Cdc16) domain-containing family of proteins affect membrane trafficking through their action as Rab–GAPs to inhibit Rab–GTPases. A screen for TBC proteins that inhibit

starvation-induced autophagy identified 11 candidates that are likely to have an effect on autophagosome formation indirectly through a membrane trafficking step [19]. At least 14 Rab–GAPs of the TBC domain-containing family interact through LIRs with LC3 [15]. One such member, TBC1D5, interacts with Vps29—a component of the retromer complex on endosomes that modulates the recycling of transmembrane receptors from endosomes to the trans-Golgi network [15]. TBC1D5 also interacts with LC3 through two LIRs, but the amino-terminal LIR of TBC1D5 has a dual role by interacting directly with Vps29 in a step that is necessary for the transport of cargo in retromer vesicles. A second carboxy-terminal LIR of TBC1D5 interacts with LC3 on autophagosomes and LC3 binding displaces Vps29, indicating that the redirection of TBC1D5 from endosomal trafficking to autophagosomes is a form of cellular reprogramming.

**LC3 as a regulator of GEF activity.** LC3 interacts with a GEF called AKAP-Lbc, a member of the A-kinase anchoring protein (AKAP) family [16]. This interaction inhibits the Rho–GEF activity of AKAP-Lbc in HEK293 cells, thus preventing it from activating RhoA, a GTP-binding protein that controls many cellular processes, including actin cytoskeleton remodelling. LC3 overexpression strongly reduces the ability of AKAP-Lbc to interact with RhoA and impairs the Rho–GEF activity of the anchoring protein. Consequently, the ability of RhoA to promote cytoskeletal rearrangements associated with the formation of actin stress fibres is impaired. Conversely, AKAP-Lbc mutants that do not interact with LC3 have a higher

basal Rho–GEF activity compared with the wild-type protein. This suggests that LC3 binding maintains AKAP-Lbc in an inactive state, thereby impairing its ability to promote downstream signalling.

*Non-lipidated LC3 as a regulator of viral replication.* Coronaviruses (CoVs), including SARS and mouse hepatitis virus (MHV), are enveloped RNA viruses that trigger the formation of DMVs used for viral replication and transcription. MHV uses the host cell machinery for COPII-independent vesicular export, from the endoplasmic reticulum, of a protein called EDEM1 to produce cellular membranes for its replication. MHV infection results in the accumulation of EDEM1 and OS9, an endoplasmic reticulum chaperone, in the DMVs coated with the non-lipidated LC3-I and Atg8 autophagy marker. The downregulation of LC3, but not the inactivation of host cell autophagy, inhibits CoV infection [20]. Nidoviruses and rotaviruses also replicate in DMVs decorated with LC3 [21].

*Non-lipidated Atg8 as a regulator of yeast vacuole fusion.* In the yeast *Pichia pastoris*, the non-lipidated form of Atg8 is important in fusion of the vacuole [22]. Septated vacuoles fuse to become a single spherical vacuole during adaptation from glucose to methanol. Atg8 is necessary for vacuolar fusion during adaptation of *P. pastoris* cells to methanol. Although vacuole fusion requires the processing of an inhibitory C-terminal peptide of Atg8 by Atg4, it does not require the lipidation of Atg8 to phosphatidylethanolamine on membranes. Neither Atg7, nor the Gly116 residue of Atg8 involved in conjugation to phosphatidylethanolamine, are necessary for this process. Atg8 localizes to vacuolar membranes and its previously described tethering and fusion activities are necessary for vacuole fusion [23]. Vacuole fusion has been studied *in vivo* and recapitulated *in vitro* from yeast. It requires multiple SNAREs, but Atg8 was not identified in this analysis [24]. It is therefore probable that Atg8 localized at the vacuolar membrane recruits SNAREs and/or other fusion components through its ability to interact with AIMs on many yeast proteins [11], rather than serving as the direct fusogen [25].

### Non-autophagic roles of Atg5 and Atg12–Atg5 conjugates

*Atg12–Atg5 as a suppressor of innate antiviral immune signalling.* Atg12 and its conjugation partner, Atg5, are also involved in non-autophagic signalling processes. The infection of mammalian cells by many RNA viruses produces virus-derived immunostimulatory RNA (isRNA) structures. In cells infected by vesicular stomatitis virus (VSV), Newcastle disease virus, influenza or Japanese encephalitis virus, 5'-triphosphorylated RNA produced during the virus life cycle is recognized by a cytoplasmic RNA helicase protein, RIG1. Other RNA viruses, such as encephalomyocarditis virus, generate dsRNA that is recognized by an alternative RNA helicase, MDA5. These helicases have N-terminal caspase recruitment domains (CARDs) that interact with other CARD proteins, such as the signalling protein interferon- $\beta$  promoter stimulator 1 (IPS1). The homotypic interaction between the CARDs of IPS1 and either RIG1 or MDA5 is crucial for subsequent signal transmission, which in turn activates NF $\kappa$ B, interferon regulatory factor 3 (IRF3) and IRF7, leading to their translocation into the nucleus and transcriptional activation of type I interferon (IFN) and other pro-inflammatory genes. Secreted type I IFNs act in an autocrine or paracrine manner to induce viral RNA degradation by activating RNase L activity and also by blocking viral protein synthesis by inhibiting eukaryotic translation initiation factor 2 activity [26].

### Glossary

AMBRA1	activating molecule in beclin 1-regulated autophagy 1
AP3	adaptor protein complex 3
Bcl-xL	B-cell lymphoma extra large
BLOC1	biogenesis of lysosome-related organelles complex 1
CatK	cathepsin K
COPII	coat protein complex II
dsRNA	double-stranded RNA
EDEM1	endoplasmic reticulum degradation enhancer, mannosidase alpha-like 1
FADD	Fas-associated death domain
Grh1	Grasp homolog 1
HEK	human embryonic kidney
keap1	kelch-like ECH associated protein 1
LC3	light chain 3
MEKK	mitogen-activated protein/ERK kinase
NF $\kappa$ B	nuclear factor kappa B
Nrf2	NF-E2 related factor 2
RIG1	retinoic acid-inducible gene 1
Sar1	secretion-associated RAS-related protein 1
SARS	severe acute respiratory syndrome
SNARE	soluble NSF-attachment protein receptor
TRAF	tumour necrosis factor receptor-associated factor
ULK1	Unc-51-like kinase 1
VAMP7	vesicle-associated membrane protein7
Vps23	Vacuolar protein sorting 23

The Atg12–Atg5 conjugate negatively regulates isRNA-generated type I IFN production (Table 1; [26,27]). The conjugate interacts with the RNA helicases RIG1 and MDA5 thereby interfering with the ability of the helicases to engage in their normal CARD interactions with IPS1, consequently impairing type I IFN production. This is reflected by the increase in type I IFN production in VSV-infected Atg5-knockout fibroblasts and a reduction in viral replication. Overexpression of Atg5 or Atg12 alone in HEK293 cells increases the formation of the Atg12–Atg5 conjugates and suppresses RIG1 or MDA5-mediated signals, leading to the reduced production of type I IFNs. Atg7-knockout fibroblasts, in which the formation of the Atg12–Atg5 conjugate is impaired, also show hyper-responsiveness to isRNA stimulation.

Atg5 also interacts with the CARDs of interleukin- $\beta$  converting enzyme (also known as caspase 1, a key regulator of apoptosis) and the nucleotide-binding oligomerization domain protein 1 (an intracellular receptor for peptidoglycan), suggesting additional unexplored roles of Atg5 and the Atg12–Atg5 conjugate.

In summary, as with ubiquitin-signalling pathways, ubiquitin-like Atg proteins in their conjugated and unconjugated forms function as signalling scaffolds to modulate many cellular pathways in addition to their traditional roles in autophagy.

### Non-autophagic roles of other Atg proteins

Several modulators of the ubiquitin-like Atg proteins, as well as other non-ubiquitin-like Atg proteins, are crucial for a variety of cellular processes (listed in Table 1).

*Axon guidance.* In *Caenorhabditis elegans*, UNC-51, the homologue of yeast Atg1 and human ULK1, regulates axon guidance in many neurons. As such, worms with mutated UNC-51 have uncoordinated movement [28]. This serine/threonine kinase modulates the subcellular transportation of UNC-5, a growth

Non-autophagic roles of Atg proteins					
Cell survival/ Apoptosis	Modulation of cellular transport	Secretory processes	Signalling	Transcriptional/ Translational responses	Membrane reorganization
<ul style="list-style-type: none"> <li>Autophagy inhibition and/or Apoptosis activation by caspases e.g. Calpain cleavage of Atg5, Atg4 or Beclin 1 cleavage by caspase 3</li> </ul>	<ul style="list-style-type: none"> <li>Redirection of membrane compartments e.g. LC3 rerouting of endosomes via binding TBC1D5</li> <li>Redirection of receptor transport e.g. UNC-5 regulation by UNC-51 in <i>C. elegans</i></li> </ul>	<ul style="list-style-type: none"> <li>Regulated secretion e.g. Atg16L1 role in hormone secretion in PC12 cells</li> <li>Exocytosis e.g. Atg16L1 and Atg5 in antimicrobial peptide secretion in Paneth cells</li> <li>Polarized secretion e.g. Cathepsin K secretion in osteoclasts</li> <li>Conventional secretion e.g. Otoconin secretion in vestibular sensory cells</li> <li>Unconventional secretion e.g. Yeast Acb1, IL1-<math>\alpha</math>, IL1-<math>\beta</math></li> </ul>	<ul style="list-style-type: none"> <li>Cytoskeletal remodelling e.g. LC3 inhibition of AKAP-Lbc</li> <li>Modulation of type 1 IFN production by sequestration of CARD proteins by Atg12-Atg5</li> </ul>	<ul style="list-style-type: none"> <li>Regulation of p21<sup>CDK1N1A</sup></li> <li>Translation of HCV RNA</li> </ul>	<ul style="list-style-type: none"> <li>Viral replication centres e.g. CoV, MHV, MNV</li> <li>Bacterial replication e.g. <i>Brucella vacuole</i></li> <li>Vacuolar fusion in yeast e.g. Non-lipidated Atg8 in <i>P. pastoris</i></li> </ul>

**Fig 2** | Summary of non-autophagic roles of autophagy proteins. See text for details. *C. elegans*, *Caenorhabditis elegans*; CARD, caspase recruitment domain; CoV, coronavirus; HCV, hepatitis C virus; IFN, interferon; MHV, mouse hepatitis virus; MNV, murine norovirus; *P. pastoris*, *Pichia pastoris*.

cone receptor for the dorsoventral axon guidance protein UNC-6/Netrin. UNC-51 also interacts with LET-92, the catalytic subunit of protein phosphatase 2A (PP2A), which is important for cellular functions and the dephosphorylation of UNC-51 targets. Thus, the PP2A-UNC-51 complex regulates axon guidance.

**Regulated secretion.** In neuroendocrine PC12 cells, Atg16L1 is localized on hormone-containing dense-core vesicles by interaction with Rab33A. Knockdown of Atg16L1 causes a marked reduction in the level of hormone secretion in PC12 cells, independently of autophagic activity [29].

**Secretion granule exocytosis.** Interestingly, coding variants of ATG16L1 are genetically linked to susceptibility to Crohn disease—a chronic inflammatory condition of the intestinal tract [30]. Atg16L1- and Atg5-deficient Paneth cells of the mouse ileal epithelium have significant abnormalities in the exocytosis of granules containing antimicrobial peptides [31]. Similarly, Paneth cell abnormalities are apparent in Crohn disease patients homozygous for an ATG16L1 risk allele that predisposes these patients to the disease [32].

**Polarized cathepsin secretion in osteoclasts.** In osteoclasts, autophagy proteins, such as Atg5, Atg7, Atg4B and LC3, participate in the polarized secretion of lysosomal contents [33]. Osteoclasts secrete lysosomal enzymes such as cathepsin K (CatK) and hydrochloric acid into an extracellular resorptive space (resorptive lacuna). Upon attachment to the bone, osteoclasts become polarized, the ruffled border is formed and an actin ring confines and seals the contact site between the osteoclasts and the bone. The secretion of demineralizing and degradative enzymes

is confined to the resorption lacuna [33]. Bone resorption causes the formation of bone pits or cavities. Atg5 and Atg7 are required for the localization of secretory lysosomes within the actin ring and for bone resorption, but not for osteoclast development or actin ring formation. These processes require LC3 and its conversion to LC3-II. Whilst it is clear that these autophagy proteins are important in CatK secretion at the ruffled border membrane in osteoclasts, the mechanism of secretion is not understood. Lysosomes containing the degradative enzymes were proposed to fuse with the plasma membrane, in which LC3 is localized in an Atg5-dependent manner.

LC3 localizes to many cellular membranes to orchestrate autophagosome formation [34], but how its localization to the plasma membrane in osteoclasts allows lysosome fusion with the plasma membrane is unclear. LC3 might assemble SNAREs that allow this selective fusion event to occur [35]. Indeed, SNAREs such as the v-SNARE VAMP7 are commonly required for lysosome exocytosis by fusion with the plasma membrane [36], as well as for autophagosome formation from membranes derived from the cell surface [35]. Previously, Ca<sup>2+</sup>-triggered lysosomal exocytosis events involving a subset of vesicles containing VAMP7 and synaptotagmin VII were implicated in plasma membrane wound healing and neurite outgrowth [37,38]. Interestingly, synaptotagmin VII and VAMP7 are also important in osteoclast function, CatK secretion and bone resorption [39], suggesting that LC3 isoforms and other Atg proteins [33] might be involved more broadly in processes such as neurite outgrowth and plasma membrane wound healing.

**Balance disorders.** Additional evidence for the role of Atg proteins in protein secretion comes from the analysis of Atg4 isoforms in mice,



**Table 1** | Non-autophagic roles of autophagy proteins

Atg proteins	Non-autophagic role	References
Atg8/LC3-PE	GTPases—balancing autophagy with other cellular processes GAPs—many TBC-family proteins (signalling platforms, redirection of endosomal components to autophagosomes, recruitment of autophagy regulators) GEF—LC3 regulates Rho–GEF activity of AKAP-Lbc	[17–19] [15] [16]
Non-lipidated Atg8	Component of double-membrane vesicles involved in replication of certain viruses Vacuole fusion in yeast	[20, 21] [22]
Atg5 or Atg12–Atg5	Regulation of isRNA-generated type I IFN production through Atg12–Atg5 interactions with CARDs of helicases RIG1, MDA5 Unknown functions—Atg5 interactions with other CARD proteins	[26,27] [26,27]
Calpain-cleaved Atg5	Apoptosis—mitochondrial insertion, Bcl-xL activation, cytochrome c release	[54]
Worm Unc-51 (Atg1)	Axon guidance	[28]
Atg16L	Interaction with Rab33A is required for hormone secretion in PC12 neuroendocrine cells; required for granule exocytosis pathway in intestinal Paneth cells	[29,31,32]
Atg5, Atg7, Atg4B and LC3	Required for cathepsin K secretion in bone osteoclasts	[33]
Atg4B and Atg5	Required for maintenance of balance (equilibrioception) and indirectly for secretion of otoconins by vestibular sensory epithelial cells of the inner ear in mice	[40,41]
Many Atg proteins but not vacuolar hydrolases or proteins necessary for autophagosome fusion with vacuole	Necessary for unconventional secretion of certain proteins, such as Acb1, lacking classical signal peptides	[44–48]
Atg5, LC3, Atg16L and autophagosomal membranes	Caspase-8-dependent apoptosis	[56]
Atg4, beclin 1	Cleavage by caspases suppresses autophagy and induces apoptosis	[55,57,58]
Atg5, Atg7	Needed for adipogenesis in mice	[59,60]
LC3, Atg2	Lipid droplet formation in mice	[62,63]
Atg12–Atg5, Atg7 and Atg16L1	Required for IFN $\gamma$ -mediated host defence against murine norovirus replication	[65]
Beclin 1, LC3, Atg4B, Atg5, Atg7 and Atg12	Crucial for HepC viral RNA translation, virus replication and egress from cells	[64]
ULK1, beclin 1, Atg14L and PI3K activity	Membrane compartment needed for <i>Brucella abortus</i> replication	[66]
Atg7	Cell cycle arrest via p53 interaction and expression of p21 <sup>CDKIN1A</sup>	[68]

AKAP, A-kinase anchoring protein; Bcl-xL, B-cell lymphoma extra large; CARD, caspase recruitment domain; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; HepC, hepatitis C; IFN, interferon; isRNA, immunostimulatory RNA; MDA5, melanoma differentiation-associated protein 5; PI3K, phosphatidylinositol 3 kinase; RIG1, retinoic acid inducible gene 1; TBC, Tre2, Bub2 and Cdc16; ULK1, Unc-51-like kinase 1.

which have four orthologues of yeast Atg4, the protease that activates LC3 orthologues and also deconjugates LC3-II to free LC3-I. A significant proportion—approximately 25%—of *Atg4B*<sup>-/-</sup> mice have a motor coordination defect manifested by abnormal tilting of the head, a tendency to walk backwards and a marked inability to swim, which are all characteristics of balance-related disorders caused by inner ear otoconial abnormalities [40,41]. These otoconia consist of organic calcium carbonate crystals embedded in a proteinaceous matrix composed of proteins called otoconins. The defects in the *Atg4B*<sup>-/-</sup> and *Atg5*<sup>-/-</sup> mice have been traced to a deficiency in the secretion of otoconins by vestibular sensory cells of the inner ear. This process depends on appropriate mRNA and protein levels of BLOC1 and AP3 subunits, both of which are adaptors in the coat-mediated vesicular transport of otoconial proteins from the Golgi apparatus to the plasma membrane. Similar balance defects have also been noted in mice deficient in BLOC1 or AP3 subunits.

How Atg protein defects impair the mRNA and protein levels of BLOC1 and AP3 is unclear, although p62, a key signalling regulator that coordinates cell-fate decisions and transcriptional programmes

that allow cells to cope with redox insults [42], might be involved. A block in autophagy causes levels of p62 to increase. The functions of p62 are mediated through its interaction with partners such as keap1, TRAF6, MEKK3 and caspase 8. When p62 protein levels increase in cells as a consequence of a block in autophagy, other cellular pathways are dysregulated. In particular, p62 sequesters keap1, an E3 ligase for the transcriptional activator Nrf2. Keap1 itself is downregulated by autophagy. Thus, as p62 levels increase, free keap1 might decrease, allowing Nrf2 levels to increase, perhaps indirectly having an impact on BLOC1 and AP3 mRNA and protein expression [43].

*Role of Atg proteins in unconventional protein secretion.* Eukaryotic cells release proteins into the extracellular space by two main routes. The first is the conventional secretion pathway for proteins that are synthesized at the endoplasmic reticulum and contain a signal for translocation into the endoplasmic reticulum, followed by their vesicular transport to the Golgi membranes and subsequent export from the cell. Examples of molecules that follow this pathway include insulin, neurotransmitters, hormones,

**Sidebar A | In need of answers**

- (i) Is the non-autophagic effect caused directly or indirectly by an impact on a canonical or non-canonical autophagy-related pathway? There should be one or more ATG genes that block canonical and non-canonical autophagy that do not affect the non-autophagic pathway being studied. As illustrated in several of the examples cited, it is often necessary to assess the roles of several early- and late-acting ATG genes to understand what part of the autophagy machinery is being diverted to the non-autophagic function.
- (ii) As with ubiquitin, can the ubiquitin-like proteins Atg8 and Atg12 serve as a general lipid/protein modification for non-autophagic functions? This area needs to be explored further in yeast and mammalian models to define the variety of roles.
- (iii) What are the proteins that recognize Atg8/LC3 and Atg12 as protein modifiers involved in non-autophagic roles? This would require studying the cellular functions of more Atg8/LC3-interacting partners, as well as Atg12-interacting partners. The effect of mutating or knocking down the genes encoding these interacting partners on autophagy-related pathways should be studied directly, and hopefully there would be little or no effect, but some non-autophagic pathway must be impaired. In general, deeper mechanistic insights are necessary to appreciate the non-autophagic roles of Atg proteins in signalling, membrane transport, cell cycle arrest, and in cell survival, death and differentiation.

mucins and collagens. The second pathway is the unconventional secretion process of proteins synthesized in the cytoplasm that lack a signal for entry into the endoplasmic reticulum–Golgi membrane pathway. Examples of proteins that use this pathway include insulin-degrading enzyme, acyl-CoA-binding protein (Acb1), fibroblast growth factor 2, interleukin 1- $\beta$  (IL-1 $\beta$ ) and many galectins. The mechanism of unconventional protein secretion remains poorly understood, but there is good evidence that Acb1, and probably IL-1 $\beta$ , are secreted by an autophagosome-like vesicular intermediate, requiring many but not all proteins of the normal degradative autophagic pathway [44–48]. Unlike autophagy, this vesicular intermediate captures cytosolic cargo and delivers it to the exterior of the cell, bypassing the normal process of autophagosome and lysosome fusion [49]. Data from studies of *Saccharomyces cerevisiae* suggests that this process begins at a new compartment for unconventional protein secretion (CUPS) that contains the proteins Grh1, Vps23, Atg8 and Atg9, as well as phosphatidylinositol-3-phosphate [50]. The mechanism of formation of the CUPS and secretory autophagosome-like vesicles from this compartment are unknown. Several autophagy proteins that produce degradative autophagosomes are probably involved in the formation of secretory autophagosomes, but how these vesicles avoid fusion with the lysosome and vacuole is not understood. Intriguingly, this unconventional secretory pathway might be used by many pathogens to evade destruction in the lysosomes and instead used for their release from the cells. New evidence suggests that caspases, particularly caspase 3, the death regulator caspase, regulate unconventional protein secretion. A significant proportion of the caspase-dependent secretome of nutrient-starved human apoptotic endothelial cells comprises proteins lacking conventional secretion signals, suggesting an association between caspase activation and unconventional secretion pathways [51].

*Cleavage of Atg proteins by caspases and regulation of apoptosis.* There is both *in vitro* and *in vivo* evidence that caspases cleave Atg

proteins and regulate autophagy, and that this also activates apoptosis [52,53]. A calpain-mediated (calpains 1 and 2) N-terminal cleavage product of Atg5 makes human neutrophils and other cell types more responsive to apoptotic stimuli. Apoptosis is associated with the translocation of this Atg5 fragment from the cytosol to mitochondria, in which it associates with the anti-apoptotic molecule Bcl-xL and triggers cytochrome c release and caspase activation, without activating autophagy [54]. Similar results have also been observed in several other cell types [55].

*Autophagosomal membrane as a platform for apoptosis.* During apoptosis triggered by the extrinsic pathway, extracellular signals activate plasma membrane-localized death receptors of the tumour necrosis factor receptor (TNFR) family and cause them to multimerize. This multimerization recruits FADD and the initiator caspase 8 to form a death-inducing signalling complex (DISC), which in turn causes caspase 8 oligomerization and autoactivation through self-cleavage. Interestingly, there is complex cross-talk between autophagy and apoptosis pathways through both caspase-dependent and caspase-independent mechanisms [56]. For example, human Atg4 processing by caspase 3 [55], and beclin 1 cleavage by caspase 3 or caspase 8, can stimulate apoptosis whilst inhibiting autophagy [57,58].

The anti-tumour compound SKI-1 is a sphingosine kinase inhibitor that induces apoptosis in SV40 T-antigen transformed mouse embryonic fibroblasts (MEFs) and also triggers autophagy [56]. In these cells, the formation of the apoptotic DISC depends on the presence of Atg5 and the autophagosomal membrane containing LC3 and Atg16L. Caspase 8 is recruited to the autophagosomal membrane through the association of Atg5 with FADD, and its activation within the DISC requires these Atg proteins and the autophagosomal membrane as a platform.

*Atg proteins required for adipogenesis.* Atg5 and Atg7, in their autophagic roles, are necessary for adipogenesis in mice [59,60]. In mutant animals lacking one of these proteins, adipose tissue deposits are smaller in mass than those observed in wild-type mice, and mutant adipocytes have unusual morphological characteristics including compartmentalized lipid droplets and many more mitochondria. The animals are also resistant to high-fat diet-induced obesity. The requirement of Atg5 and Atg7 in adipogenesis might be an indirect consequence of their roles in autophagy. One reflection of this is the fact that a form of autophagy, lipophagy and lipid-droplet-associated lipases are important in the lysosomal degradation of lipid droplets, and defective autophagy causes the accumulation of lipid droplets in hepatocytes [61]. However, there is evidence for the direct involvement of Atg proteins in lipid droplet formation. As examples, Atg2 and LC3 are involved in lipid droplet formation in mouse hepatocytes and cardiac myocytes [62,63].

*Host–pathogen responses.* The Atg12–Atg5/Atg16L1 protein complex, along with other autophagy proteins, is actively involved in host defence mechanisms against pathogens such as bacteria and viruses. Both host cells and viruses exploit subsets of the autophagy machinery, as well as autophagy-related membranous structures, to either restrict or enhance viral replication, independent of the conventional autophagy pathways [64]. For example, a subset of the autophagy machinery is required, in a

non-degradative capacity, for IFN $\gamma$ -mediated host defence against murine norovirus (MNV—a plus strand, single-stranded RNA virus responsible for human epidemics of gastroenteritis). Essentially, IFN $\gamma$ -activated macrophages co-opt some of the cellular Atg proteins to block norovirus infection by inhibiting formation of the replication complex. Importantly, the direct antiviral activity of IFN $\gamma$  against MNV in macrophages required Atg5–Atg12, Atg7 and Atg16L1, but not the induction of autophagy, the degradative activity of lysosomal proteases, the fusion of autophagosomes with lysosomes or Atg4B [65]. The mechanism by which the autophagy proteins described above interfere with membrane alterations and membrane-associated viral replication is not known.

**Viral protein translation.** In contrast to the example above, is the case of hepatitis C (HepC) virus, another subset of autophagy proteins, specifically beclin 1, LC3, Atg4B, Atg5, Atg7 and Atg12, which are crucial for the translation of HepC RNA, virus replication and egress from cells, rather than interfering with the viral life cycle [64].

**Membrane reorganization events in bacterial replication and egress.** A variation on this theme of membrane sequestration by pathogens comes from studies on the intracellular bacterium *Brucella abortus* that causes brucellosis [66]. This bacterium resides in a vacuole called the brucella-containing vacuole (BCV), which moves in a Sar1- and Rab2-dependent manner from an endocytic compartment to fuse with the endoplasmic reticulum, where the bacterium replicates (rBCV). During the post-replication stages of *B. abortus* infection in macrophages and epithelioid cells, the rBCV is transformed into a compartment with autophagic properties (aBCV). The aBCV formation and transport of *Brucella* to the endoplasmic reticulum is unaffected in autophagy-deficient cells obtained either by knockdown of specific autophagy components in HeLa cells or by using macrophages carrying null mutations in Atg proteins. However, BCV formation is dependent on autophagy proteins, such as ULK1, beclin 1, Atg14L and PI3K activity, but independent of other factors, for example Atg5, Atg16L1, Atg4B, Atg7 and LC3B that are required for membrane expansion steps in autophagosome formation. Although the mechanisms involved are unclear, some parallels have been drawn to other ‘non-canonical’ pathways of autophagy that are dependent on beclin 1 and ULK1, but independent of Atg5 and Atg7, as well as LC3 recruitment [67]. However, whilst the pathway described in reference [67] also depends on the small GTPase Rab9, aBCV formation does not require Rab9 in HeLa cells [66]. The authors suggest the possibility that aBCVs are important in bacterial release and cell-to-cell spread by an egress mechanism. Whether this egress mechanism is related to the role of Atg proteins in unconventional protein secretion as described earlier [45,47,48] remains unexplored.

**Gene transcriptional control.** A recent study shows how autophagy and cell cycle arrest are coordinated during nutrient deprivation. In starved MEFs, Atg7—dependent of its E1 activity—Atg5 and Atg6 are required for interaction with the tumour suppressor p53 and for transcriptional activation of the cell cycle inhibitor p21<sup>CDK1N1A</sup> [68].

## Summary

It is evident that the autophagic machinery is far more versatile in coordinating cellular activities than was previously appreciated. Indeed, attention has been drawn to other processes possibly

involving non-autophagic roles of the Atg machinery [69], such as the exencephaly caused in mice lacking AMBRA1, a protein that interacts with beclin 1. These new roles point to a complex interplay between autophagy, cell proliferation and cell death during neural development in mammals [70]. Additionally, the knockout of ATG genes in mice yields distinct phenotypes ranging from early embryonic to perinatal lethality, or an increased incidence of tumours in adulthood, which are phenomena we do not yet understand, but which also suggest roles for Atg proteins in tumour suppression [71]. The emerging themes that hold great promise for future exploration are the roles played by the autophagy machinery in the reprogramming of signalling, membrane transport, cell cycle arrest and in cell survival, death and differentiation (Sidebar A).

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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