# How do ESCRT proteins control autophagy?

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## Summary

**Autophagy, a conserved mechanism for lysosomal degradation of cytoplasmic components, has received much attention recently owing to its importance in tissue remodelling and innate immunity, and because it has been proposed that autophagy protects against cancer and neurodegenerative diseases. Although much of the molecular machinery that mediates autophagy has been identified, there are still aspects of this pathway that remain enigmatic. One open issue is the involvement of endosomal sorting complex required for transport (ESCRT) proteins, which were originally identified for their role in sorting ubiquitylated membrane proteins into multivesicular bodies. In this Opinion article, we discuss four possible models that could explain the observation that autophagosomes accumulate in ESCRT-depleted cells. We propose that the involvement of ESCRT proteins in the fusion of autophagosomes with the endolysosomal system is the most plausible model.**

This article is part of a Minifocus on the ESCRT machinery. For further reading, please see related articles: 'The ESCRT machinery at a glance' by Thomas Wollert et al. (*J. Cell Sci*. **122**, 2163-2166) and 'No strings attached: the ESCRT machinery in viral budding and cytokinesis' by Bethan McDonald and Juan Martin-Serrano (*J. Cell Sci*. **122**, 2167-2177).

Key words: Autophagy, Endosome, ESCRT, Lysosome, Multivesicular body, Ubiquitin

## Introduction

The degradation of cytoplasmic material is an essential function of eukaryotic cells and serves multiple purposes, including the removal of potentially toxic organelle remnants and protein aggregates, the elimination of invading pathogens, and the recycling of macromolecules under conditions of starvation or tissue remodelling. Such degradation is achieved by an evolutionarily conserved process known as macroautophagy, often referred to simply as autophagy (Mizushima et al., 2008). The autophagic process begins with a signalling event (Fig. 1A) that triggers the engulfment of portions of cytoplasm by a double membrane, the phagophore (Fig. 1B). The exact origin of this organelle remains controversial (Reggiori et al., 2004; Axe et al., 2008). The phagophore eventually seals around the sequestered cytoplasmic contents to form an autophagosome. When the autophagosome fuses with a lysosome that contains high concentrations of hydrolytic enzymes, the engulfed material is degraded in the resulting autolysosome (Fig. 1C2). Autophagosomes can alternatively fuse with endosomes to form amphisomes (Fig. 1C1), which also are thought to have some degradative ability.

Genetic screens in yeast have identified some 30 Atg proteins, which are the components of the core autophagic machinery (Suzuki and Ohsumi, 2007). The finding that Atg proteins are also conserved in higher eukaryotes has facilitated a molecular dissection of autophagy in these organisms as well. Biochemical analyses of the Atg machinery have revealed that its core consists of two ubiquitin-like conjugation systems, the ultimate function of which is to cause the lipidation and membrane association of the Atg8 component (Suzuki and Ohsumi, 2007), an event that causes phagophore elongation in vitro (Nakatogawa et al., 2007).

Studies of nematodes, flies and mammals have revealed that the endosomal sorting complex required for transport (ESCRT) machinery has a role in autophagy (Table 1). This was an unexpected finding, given that ESCRT components failed to be identified in screens for *atg* mutant in yeast, although an ESCRT regulator was identified in a yeast screen for mutants that induce autophagy under nonstarved conditions (Shirahama et al., 1997). The fact that ESCRT-mediated control of autophagy is physiologically important is illustrated by the finding that genetic disruption of the ESCRT machinery results in autophagosome accumulation and neurodegenerative disease (Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007).

The ESCRT machinery consists of three complexes, ESCRT-I, ESCRT-II and ESCRT-III, which were originally identified because of their function in the biogenesis of intralumenal vesicles (ILVs) in multivesicular bodies (MVBs) and lysosome-like vacuoles in yeast, and for the sorting of ubiquitylated membrane proteins into these vesicles (Katzmann et al., 2001; Babst et al., 2002b; Babst et al., 2002a). A fourth complex has been found to function upstream of ESCRT-I and has consequently been named ESCRT-0 (Raiborg and Stenmark, 2009). ESCRT-0, ESCRT-I and ESCRT-II contain ubiquitin-binding subunits and mediate the sorting of ubiquitylated cargo, whereas ESCRT-III is the principal machinery for the formation of ILVs and also for mediating two topologically related membrane abscission reactions – virus budding and cytokinesis (Raiborg and Stenmark, 2009). Even though there are intersections between the endocytic and the autophagic pathways (see Fig. 1), it is not clear why the inactivation of the ESCRT machinery causes an accumulation of autophagosomes. In this Opinion article, we discuss four alternative models that could explain this observation; namely, that the ESCRT machinery could be involved in the induction of autophagy, the closure of the phagophore, autophagosome fusion or lysosome biogenesis.
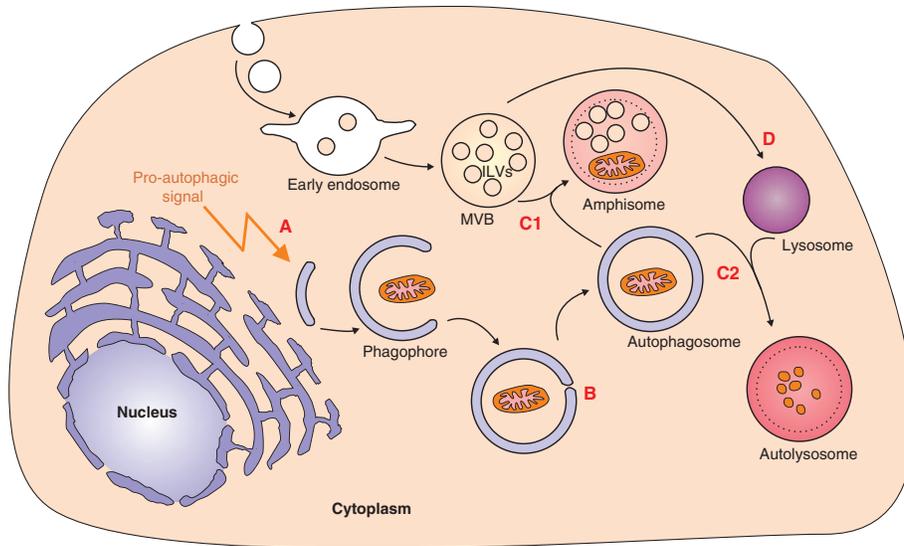
**Fig. 1.** The ESCRT machinery could be involved at one or more of four key stages of autophagy. The ESCRT machinery is required for the biogenesis of MVBs and the sorting of ubiquitylated membrane proteins into their intralumenal vesicles (ILVs). When the ESCRT machinery is inactivated, this could cause cellular stress that results in pro-autophagic signalling, in addition to preventing MVB biogenesis and degradative receptor sorting (A). Alternatively, ESCRT inactivation might prevent closure of the phagophore (B), which is topologically equivalent to the abscission of ILVs from the limiting membrane of the MVB. A third possibility is that the ESCRT machinery is required for the fusion of autophagosomes with MVBs to make amphisomes (C1) or with lysosomes to make autolysosomes (C2). Finally, it is possible that the ESCRT machinery is required for lysosome biogenesis (D).

## Alternative mechanisms to explain the involvement of ESCRTs in autophagy

The increased number of autophagosomes that are observed when the ESCRT machinery is inactivated can, in theory, be explained by either an enhanced initiation of autophagy (Fig. 1A) or a decreased autophagic flux (Fig. 1B-D). In the sections below, we discuss several alternative mechanisms that might underlie the observed phenotype.

### Induction of autophagy

Autophagy is tightly controlled by cellular signalling mechanisms, particularly those that are activated in response to metabolic state and cellular stress (Mizushima et al., 2008). Classically, autophagy has been characterized as a cellular response to amino acid starvation, generating free amino acids through degradation of cytoplasmic proteins. The protein kinase TOR (target of rapamycin) is central in suppressing autophagy under nutrient-rich conditions, in part by phosphorylating the autophagy initiator kinase Atg1 (ULK1 in mammals) and its regulator Atg13 (Jung et al., 2009; Hosokawa et al., 2009; Chang and Neufeld, 2009; Chan et al., 2009; Ganley et al., 2009). The mechanism by which TOR senses the nutritional status via L-glutamine uptake and efflux has been elucidated recently. L-glutamine is transported into cells in a $Na^+$-dependent manner through the transporter SLC1A5 (solute carrier family 1, member 5) and is, in turn, used to import L-leucine via the antiporter SLC7A5-SLC3A2, a heterodimeric protein complex. Intracellular L-leucine is sensed by an unknown factor that activates TOR complex 1 (Nicklin et al., 2009). When the expression of SLC1A5 is knocked down, autophagy is induced. Even though the intracellular trafficking of SLC1A5 and SLC7A5-SLC3A2 is incompletely understood, it is clear that these transporters are not constitutively associated with the plasma membrane but are internalized in response to starvation (Avissar et al., 2008; Edinger et al., 2003; Nicklin et al., 2009). Consistent with this, the small GTPase Rab7, which controls late endosomal trafficking, regulates the surface expression of SLC7A5, and its functional inactivation causes increased levels of amino acid transporters at the plasma membrane (Edinger et al., 2003). Because ESCRT proteins, similarly to Rab7, regulate degradative trafficking of endocytosed membrane proteins, this raises the possibility that their depletion might influence the surface expression of amino acid transporters, thereby providing a signal for autophagy (Fig. 1A). However, the finding that ESCRT depletion causes increased recycling of endocytosed growth-factor receptors argues against this possibility (Raiborg et al., 2008). If amino acid transporters are regulated in a similar manner as growth-factor receptors, ESCRT depletion would be predicted to increase rather than decrease the levels of amino acid transporters at the cell surface, thereby preventing the induction of autophagy.

In addition to sensing nutrient status, TOR complex 1 is also activated by growth factors via class I phosphoinositide 3-kinase and the downstream protein kinase Akt, and inhibition of this pathway can also stimulate autophagy. However, ESCRT inactivation enhances rather than inhibits growth-factor signalling, which rules out the possibility that the inactivation of the ESCRT machinery activates autophagy via inhibition of the Akt pathway (Rodahl et al., 2009b). A more plausible activator of autophagy downstream of ESCRT proteins is Jun N-terminal kinase (JNK), which becomes activated when the ESCRT machinery is inhibited (Herz et al., 2006; Rodahl et al., 2009a). JNK has been reported to mediate Fas-induced autophagy, although it remains to be investigated whether its activation in the absence of ESCRT function is responsible for inducing autophagy (Zhang et al., 2008). Interestingly, a recent study in *Drosophila* suggests that JNK-mediated induction of autophagy is independent of Atg13 and nutrient signalling (Chang and Neufeld, 2009). This raises the exciting possibility that multicellular organisms might have evolved additional pathways to control autophagy that bypass the need for nutrient signalling, thereby serving multiple purposes that are specific for animal life and development.

### Phagophore closure

One of the least well-characterized events in autophagy is the final closure of the phagophore cup, which results in the formation of a completely sealed autophagosome. Besides representing a fusion between advancing protrusions of the phagophore, phagophore closure can be regarded as the fission of a narrow membrane tube that is filled with cytosol (Fig. 1B). The topology of this fission is the same as that involving the abscission of a vesicle budding inwards into an endosome, the membrane stalk of a virus budding from the plasma membrane or the final abscission of two

**Table 1. Evidence that ESCRT proteins are required for the completion of autophagy**

| Protein | Organism (cell type) | Type of experiment | Autophagy-related phenotype | Reference |
|---|---|---|---|---|
| **ESCRT-0 complex** | | | | |
| Hrs | *C.e.* | LOF mutant | Accumulation of autophagosomes | (Roudier et al., 2005) |
| Hrs | *M.m.* | Neuron-specific KO | Neuronal death; accumulation of ubiquitylated protein aggregates | (Tamai et al., 2008) |
| Hrs | *H.s* (HeLa cells), *M.m.* (MEFs) | KO, shRNA | Increased number of autophagosomes; decreased number of autolysosomes | (Tamai et al., 2007) |
| **ESCRT-I complex** | | | | |
| Tsg101 | *H.s.* (HeLa cells) | siRNA | Accumulation of autophagosomes | (Doyotte et al., 2005) |
| Tsg101 | *H.s.* (HeLa cells) | siRNA | Accumulation of autophagosomes and amphisomes | (Filimonenko et al., 2007) |
| Tsg101,Vps28 | *D.m.* | LOF mutant | Accumulation of autophagosomes; lack of autolysosomes and amphisomes; neuronal death | (Rusten et al., 2007) |
| **ESCRT-II complex** | | | | |
| Vps22 | *H.s.* (HeLa cells) | siRNA | Accumulation of autophagosomes and amphisomes | (Filimonenko et al., 2007) |
| Vps25 | *D.m.* | LOF mutant | Accumulation of autophagosomes; lack of autolysosomes and amphisomes; neuronal death | (Rusten et al., 2007) |
| **ESCRT-III complex** | | | | |
| Vps32 | *M.m.* (neurons) | siRNA | Accumulation of autophagosomes; decreased number of autolysosomes; neuronal death | (Lee et al., 2007) |
| Vps32 | *D.m.* | LOF mutant | Accumulation of autophagosomes; neuronal death | (Rusten et al., 2007) |
| Vps24 | *H.s.* (HeLa cells) | siRNA | Accumulation of autophagosomes and amphisomes | (Filimonenko et al., 2007) |
| Vps2B | *H.s.* (HeLa cells) | DN mutant | Accumulation of autophagosomes and protein aggregates | (Filimonenko et al., 2007) |
| Vps2B | *M.m.* (neurons) | DN mutant | Accumulation of autophagosomes; neuronal death | (Lee et al., 2007) |
| **Other** | | | | |
| Vps4 | *S.c.* | GOF mutant | Increased autophagy (measured as the accumulation of processed soluble alkaline phosphatase) | (Shirahama et al., 1997) |
| Vps4 | *H.s.* (HeLa cells) | DN mutant | Accumulation of autophagosomes and autolysosomes | (Nara et al., 2002) |
| Vps4 | *D.m.* | DN mutant | Accumulation of autophagosomes | (Rusten et al., 2007) |

*C.e.*, *Caenorhabditis elegans*; *D.m.*, *Drosophila melanogaster*; DN, dominant-negative; GOF, gain of function; *H.s.*, *Homo sapiens*; LOF, loss of function; KO, knockout; MEFs, mouse embryonic fibroblasts; *M.m.*, *Mus musculus*; *S.c.*, *Saccharomyces cerevisiae*; shRNA, short hairpin RNA; siRNA, small interfering RNA.

cells during cytokinesis. Because these three processes all require the ESCRT machinery (Raiborg and Stenmark, 2009), one can hypothesize that the same is true for phagophore closure. It would be very difficult to detect incomplete closure by standard methods of electron and confocal microscopy, and incompletely sealed phagophores could easily be mistaken for intact autophagosomes. If an unsealed phagophore fused with a lysosome, degradation of the sequestered material and the inner phagophore membrane would be inefficient owing to the leakage of lysosomal enzymes out of the fusion product. This might explain some of the abnormal-looking autophagic structures that are observed in ESCRT-mutant cells (Rusten et al., 2007).

Autophagosome fusion

A key event in autophagic flux is the fusion of the autophagosome with endosomes and lysosomes (Fig. 1C1,2). Inhibition of such fusion would cause an accumulation of autophagosomes and a corresponding decrease in amphisomes and autolysosomes, respectively. Indeed, this idea is in line with experimental observations of cells that have inactivated ESCRT functions (see Table 1). The typical phenotype observed in such cells is a markedly elevated number of autophagosomes, which is sometimes accompanied by increased numbers of large protein aggregates that contain the autophagy substrate p62. The observations that autolysosomes containing lysosomal-associated membrane protein 1 (LAMP1) decrease in number in ESCRT-III-deficient mouse cortical neurons, and in *Drosophila* larvae that are deficient for ESCRT-I and ESCRT-II, provide compelling evidence that autophagosome-lysosome fusion requires the ESCRT machinery (Lee et al., 2007; Rusten et al., 2007). However, increased numbers of amphisomes and autolysosomes in the absence of normal ESCRT function have also been reported in some studies (Filimonenko et al., 2007; Nara et al., 2002), which could reflect either that there was incomplete ESCRT downregulation in these models, or that there are alternative mechanisms of autophagosome accumulation.

Similarly to other membrane-fusion events that occur in eukaryotic cells, autophagosome fusion is thought to involve the formation of SNARE complexes, which mediate the fusion of transport vesicles with target membranes. Tethering between the autophagosome and endolysosomal membranes prior to SNARE-complex assembly appears to be mediated by Rab7 and its effector/activator complex HOPS (homotypic fusion and vacuole

protein sorting) (Yorimitsu and Klionsky, 2005; Lindmo et al., 2006; Jager et al., 2004; Gutierrez et al., 2004; Akbar et al., 2009; Pulipparacharuvil et al., 2005; Seals et al., 2000). Because ESCRT proteins are involved in MVB biogenesis but have not been demonstrated to mediate membrane fusion, it remains an open question how ESCRTs mediate autophagosome fusion. To address this, it will be interesting to explore possible interactions between ESCRT components and the HOPS machinery.

### Lysosome biogenesis

An alternative model to explain the decreased turnover of autophagosomes in ESCRT-depleted cells is that lysosome biogenesis might be perturbed. Indeed, lysosomes receive cargos from endosomes (Luzio et al., 2003) and, in yeast ESCRT mutants, the delivery of certain membrane-bound hydrolases to the lumen of the lysosome-like vacuole requires the ESCRT machinery (Katzmann et al., 2001). However, studies of mammalian cells have shown that the trafficking of mannose 6-phosphate receptors (which freight the majority of lysosomal enzymes from the biosynthetic pathway to the endolysosomal system) is mostly unperturbed in the absence of ESCRT functions (Raiborg et al., 2008). Moreover, no striking differences in lysosome numbers have been detected in ESCRT-defective cells (Rusten et al., 2007; Doyotte et al., 2005; Filimonenko et al., 2007; Razi and Futter, 2006). It thus seems unlikely that the increased number of autophagosomes observed in ESCRT-defective cells would be due to a lack of lysosomes.

### Conclusion

None of the four scenarios discussed above can yet be ruled out as an explanation for the observed accumulation of autophagosomes in the absence of normal ESCRT function. However, on the basis of the available evidence, we propose the following reverse-order ranking of their plausible roles in this process. In our opinion, the least likely scenario is that ESCRT depletion inhibits autophagy by preventing lysosome biogenesis (Fig. 1D), as there is no evidence that ESCRT proteins are required for lysosome biogenesis. A more plausible scenario is that the ESCRT machinery is required for phagophore closure (Fig. 1B), especially as this process is considered to be a membrane-fission event that is topologically equivalent to other ESCRT-dependent fission events. However, according to this model, one would expect to observe more fusion products between lysosomes and (unsealed) autophagosomes, which has not been reported. The second most plausible explanation is that ESCRT depletion causes metabolic stress, which thereby triggers signalling pathways that stimulate increased autophagy initiation (Fig. 1A). If this were the case, one would expect to observe not only an increased number of autophagosomes but also of autolysosomes in cells in which the ESCRT machinery is nonfunctional. This is indeed the case on expression of dominant-negative Vps4, the ATPase that disassembles and recycles ESCRT-III subunits (Nara et al., 2002). However, under more direct conditions of ESCRT inactivation, such as inhibiting the expression of ESCRT subunits with short-interfering RNA (siRNA) or genetic mutation, such an increase in autolysosome number has not been observed. This argues against the possibility that autophagy induction is a general mechanism that accounts for the effects of ESCRT inactivation. Therefore, this leads us to propose that the most plausible model to explain the requirement of ESCRT proteins in the completion of autophagy is the involvement of ESCRTs in autophagosome fusion (Fig. 1C1,2). Inhibited fusion of autophagosomes with endosomes and lysosomes would cause an accumulation of autophagosomes and would leave only low numbers

of amphisomes and autolysosomes, which is consistent with most observations (Roudier et al., 2005; Rusten et al., 2007; Lee et al., 2007).

Although we favour the autophagosome-fusion model to explain the requirement of ESCRTs for the completion of autophagy, we cannot exclude the possibility that alternative explanations exist – either those described by the three other models discussed here or other scenarios that we did not imagine. It is important to emphasize that the autophagic phenotypes observed in cells in which the ESCRT machinery is inactivated in different systems are pleiotropic. This raises the possibility that the phenotype could be caused by a combination of several mechanisms; for example, a combination of increased autophagy induction and decreased autophagosome fusion. Therefore, it will now be important to test the different models experimentally. This is technically challenging and will probably require in vitro reconstitution of autophagosome biogenesis and fusion. If these approaches are successful, these studies will be of great interest, as they might illuminate the possibility that there is an interplay between two degradative processes of high physiological and pharmacological interest – MVB sorting and autophagy.

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