Autophagy in endosomal mutants

Desperately seeking to survive

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The endosomal and autophagic pathways are essential for the degradation and renewal of cellular components. After a complex maturation process, both pathways converge to their final destination, the lysosome. A close link between these two pathways was described along the last decade, notably through the analysis of ESCRT mutants. Although in mammals ESCRT mutants are unable to complete autophagic maturation due to the lack of fusion with the endolysosomal system, the role of ESCRT in the autophagic process still remains an open issue. Using *C. elegans*, we recently showed that blockage of the endosomal maturation triggers the induction of autophagic activity in ESCRT mutant.[1] This increase of autophagic flux is an attempt to correct cellular defects and promote the survival of mutant animals.

Autophagosomes and Endosomes: Brother in Arms?

Macro-autophagy, here referred as autophagy, is essential for the degradation and the renewal of intracellular components. Although it is constitutively active at a basal level to maintain cellular homeostasis, notably by recycling damaged organelles, this process was initially described as an inductive mechanism in response to a cellular stress like nutrient deprivation.[2-4] During the last decade, induction of autophagic functions was reported in a large variety of physiological and pathological mechanisms such as development, cell death, aging, antigen presentation, neurodegeneration and cancer progression.[5-9] Generally described as a protective mechanism for the cell, autophagy was also demonstrated, in particular situations, to contribute to cell death, highlighting its double-edged behavior.[6,9]

Under induction, such as starvation, the autophagic process starts by the elongation of the phagophore, also named the phagophore. This membrane proceeds to the sequestration of cytoplasmic material, which ends by its complete closure to form a double membrane vesicle called the autophagosome. Finally, in yeast, the autophagosome fuses with the vacuole where sequestered materials are degraded (**Fig. 1A**). In contrast to yeast, studies in mammalian cells revealed that before fusion with the lysosome, the autophagosome can fuse with endosomes to generate an intermediate compartment called the amphisome.[10-13]

The other vesicular degradative pathway, the endosomal system, allows the sorting of ubiquitinated plasma membrane proteins to the lysosome.[14] After endocytosis, the endosomal maturation internalizes ubiquitinated proteins to the intraluminal vesicles (ILVs) of the multivesicular body (MVB). Similarly to autophagosomes, MVBs generally fuse with the lysosome where ILVs and their content are degraded.[15] The budding of ILVs and concomitant formation of degradative cargoes in the MVB are achieved by the successive recruitment of ESCRTs complexes (endosomal sorting complex required for transport) (**Fig. 1A**).[16-18] The four ESCRT and the VPS-4 ATPase complexes are evolutionarily well conserved and are composed by the VPS-E proteins (class E vacuolar protein sorting).
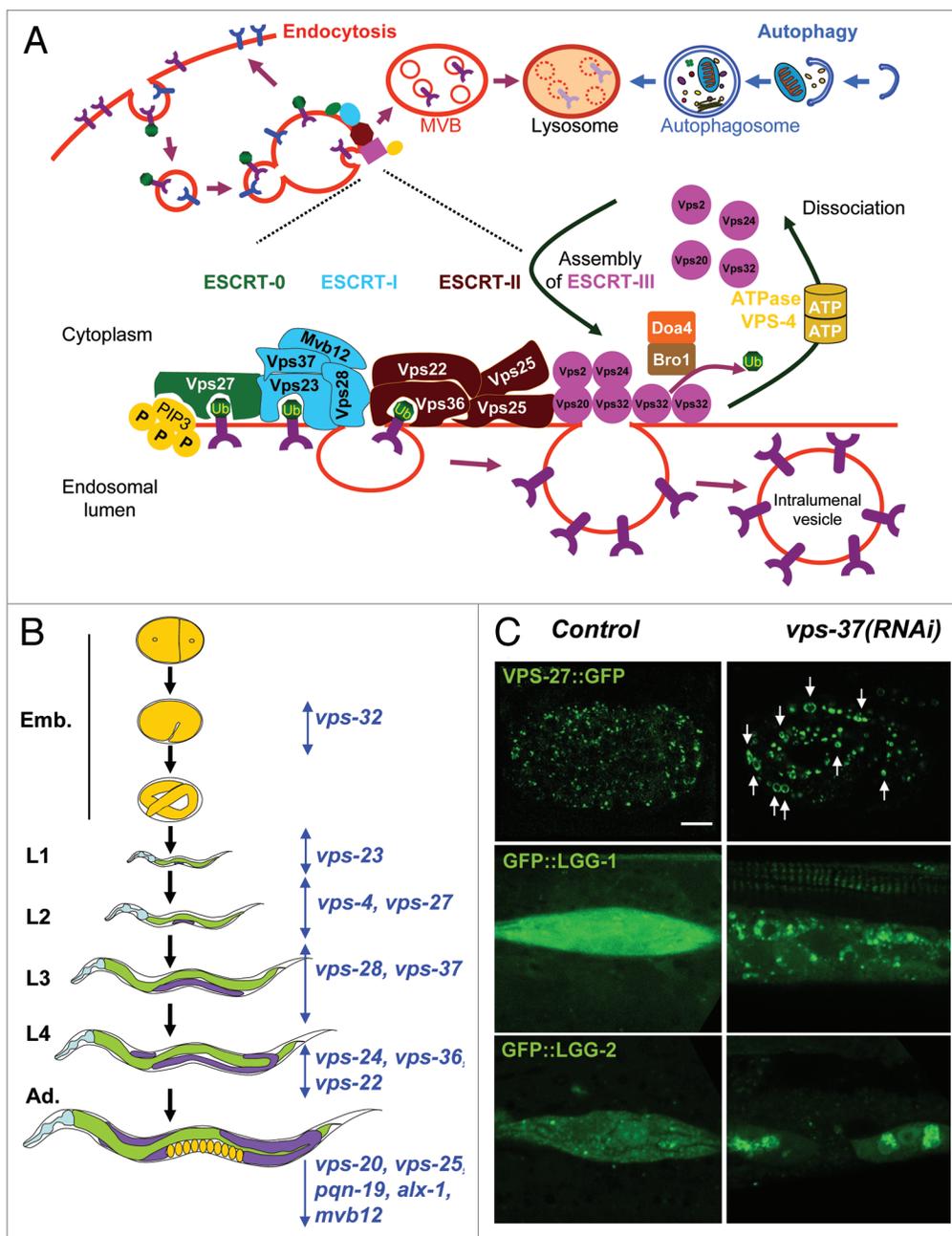
Figure 1. ESCRT mutations result in endosomal maturation and autophagy defects in *C. elegans*. (**A**) Schematic representation of the ESCRT machinery and endosomal degradative pathway. Membrane proteins (Y) are internalized by endocytosis and addressed to early endosomes. They may either be recycled back to the plasma membrane or further directed to late endosomes when monoubiquitylated (green octagon). During endosomal maturation, the cargoes specified for degradation are directed to the intra-luminal vesicles (ILV) formed by invagination of the limiting membrane of the multivesicular body (MVB) compartment, which finally fuses with the lysosome. The formation of ILV with the protein cargoes is achieved by complex machinery located on the cytosolic face of the endosomal membrane. The four endosomal sorting complexes required for transport (ESCRT-0 to -III) are recruited sequentially to the membrane via specific interactions involving protein-lipid and protein-protein domains. Ubiquitylated cargoes are directed to specific sub-domains, deubiquitylated by the de-ubiquitinase Doa4 and addressed to the forming ILVs. ESCRT-0 is dedicated to the formation of cargoes of ubiquitylated proteins at the endosomal membrane. ESCRT-I and II are crucial for the formation of the ILVs while ESCRT-III is required for their scission. The AAA+ ATPase complex is involved in disassembly of ESCRT machinery. To simplify the scheme, proteins associated with ESCRT have not been represented. Macro-autophagy (indicated in blue) allows the degradation of cytoplasmic constituents by nucleation, elongation then closure of a double-membrane autophagosome, which fuses with the lysosome. (**B**) Schematic representation of *C. elegans* development and stages of arrest of ESCRT mutants. After embryonic development (Emb. yellow) and hatching, animals proceed through four larval stages (L1-L4) and molts to reach adulthood (Ad.). The adult animal is hermaphrodite and reproduces by self-fertilization. Only main internal organs are represented (pharynx in blue, intestine in green and gonad in purple). The length of the embryo and the adult is respectively 50 μm and 1.5 mm. Stages of arrest of ESCRT mutants or RNAi are indicated by blue arrows. (**C**) Confocal images of the endosomal protein VPS-27::GFP and the autophagosomal proteins GFP::LGG-1 and GFP::LGG-2 in the epidermis of control and *vps-37(RNAi)* animals. Enlarged endosomes (white arrows) are visible during embryonic development while an increase of autophagosomes (dotted structures) is only detected at larval stages. Scale bar is 10 μm. Data taken from Djeddi et al.[1]

Several studies in nematode, fly and mammals showed that in addition to the characteristic endosomal maturation defect, mutations in ESCRT components lead to an increase in the number of autophagosomes.[19-22] Surprisingly, this autophagic phenotype was not identified in yeast ESCRT mutants. These studies highlight a close link between the endosomal and autophagic pathways, which can be explained by three main hypotheses. First, a defective maturation of autophagosomes (e.g., fusion with the lysosome) could result from the endosomal defect and lead to the accumulation of autophagic structures. Second, because ESCRTs complexes are able to modify membrane surfaces, they could play a direct role in autophagosome formation, notably during the closure of the phagophore. Third, a deregulation of cellular homeostasis due to an endocytosis defect could trigger an autophagic response.

To address this question we have used *C. elegans* which is a powerful model to study autophagy in the context of aging, stress response, development, cell survival and cell death.[5,8,23-25] We combined genetic, biochemical and microscopy analyses to study the interactions between these two vesicular pathways in vivo and during the whole life cycle.[1]

Do all ESCRT Mutants Display Identical Phenotypes?

Using RNA interference approach and knock out mutants, we inactivated 11 different ESCRT genes and analyzed their developmental as well as their cellular phenotype. All those genes encode for different proteins that compose the ESCRT and VPS-4 ATPase complexes. Although all those genes are supposed to be involved in the same cellular mechanism, the biogenesis of the MVB, we noticed that the depletion of ESCRT genes led to an heterogeneity in developmental phenotypes ranging from an embryonic or larval lethality to an absence of obvious phenotype (**Fig. 1B**). On the basis of this observation we sorted *vpsE* genes in three categories related to the strength of the developmental phenotype. The first one is only composed of *vps-32* (ESCRT III), which inactivation led to an embryonic

lethality, and could reflect some particularities in its functions.[21] The second group, composed of various ESCRT-0, I, II, III components and the VPS-4 ATPase, presented a larval arrest phenotype, which stage can differ between genes. The inactivation of genes from the third group did not lead to developmental lethality. Even though these results contrast with studies in yeast, where all ESCRT mutants are viable, they have also been described in drosophila and mouse.[26,27]

We therefore analyzed the cellular phenotypes of ESCRT mutants and observed several similarities despite their developmental heterogeneity. In particular, larval lethal animals recurrently presented a molting defect. As we reported previously for *vps-27* mutant, this phenotype could be correlated with a defect in cholesterol trafficking.[22] For most of the mutants, we also observed the emergence of vacuoles mainly in epithelial tissues, which are positives for the endosomal marker Hrs/VPS-27 (**Fig. 1C**). This defect corresponds to the blockage of the endosomal maturation in ESCRT mutants, originally characterized in yeast but also observed in metazoans.[21] Because several studies have previously reported an increase of autophagic vesicles in ESCRT mutants, we also analyzed this aspect.[19,21,28,29] To investigate the autophagic pathway in wild-type or ESCRT mutant context, we used GFP fusion proteins of LGG-1 and LGG-2 (LC3 GABARAP GATE16), the worm homologs of the LC3 human autophagic marker.[24] We observed a strong increase of the number of autophagosomes in all ESCRT mutants analyzed (**Fig. 1C**).

In summary, it appears that despite their developmental heterogeneity, ESCRT mutants in *C. elegans* present three similarities; (1) a molting defect; (2) a blockage of endosomal maturation (3) an increase of the number of autophagosomes.

Is Autophagy Blocked or Induced in ESCRT Mutants?

The increase of the number of autophagic vesicles in ESCRT mutants raises the question of their nature. Does it reflect an accumulation of unresolved autophagosomes due to their inability to achieve

their maturation and fusion with the lysosome? Alternatively, can it reflect an increase of a functional autophagic flux? Because autophagy is a highly dynamic mechanism, monitoring an increase of autophagic flux vs. a blockage of autophagosomal maturation is still a challenge.[30] Indeed, the simple observation of an increase of GFP::LGG-1 or GFP::LGG-2 puncta is inconclusive. Therefore, to address this question we used the fusion proteins GFP::LGG-1 and T12G3.1::GFP to monitor the autophagic flux by western blotting (**Fig. 2**).

Under autophagy induction, the LC3 proteins are post-translationally modified during autophagosomes formation; their C-terminus is cleaved and then conjugated to a phosphatidyl-ethanolamine (PE) group. This LC3 lipidated form, called LC3II, allows its specific binding to autophagic membranes. By electrophoresis migration, this form migrates faster than the original one (LC3I) and is used to quantify the formation of autophagosomes.[25] Moreover, the use of GFP fusion proteins allowed us to analyze the formation of the autophagolysosomes. When the autophagosome fuses with the lysosome, GFP::LGG-1 protein is degraded by lysosomal enzymes, releasing a GFP fragment that is less sensitive to lysosomal proteolysis. The detection of this GFP fragment indicates that autophagosomes have efficiently fused with the lysosome. In our western blot experiments, we observed an increase of both the lipidated form of GFP::LGG-1 and the GFP cleaved fragment (**Fig. 2**). These results indicated that autophagosomes are formed and still able to complete fusion with the lysosome. To confirm these results we performed a similar analysis using another *C. elegans* autophagic marker, T12G3.1::GFP.[31] T12G3.1 is an homolog of P62/SQSTM, a cargo adaptor protein, which is incorporated in the complete autophagosome and degraded in lysosome. As for GFP::LGG-1, we observed both a strong increase of the dotted pattern of T12G3.1::GFP in ESCRTs mutants by microscopy and an increase of the GFP cleaved protein by western blot. Therefore the strong increase of the number of autophagosomes in ESCRT mutant is likely linked
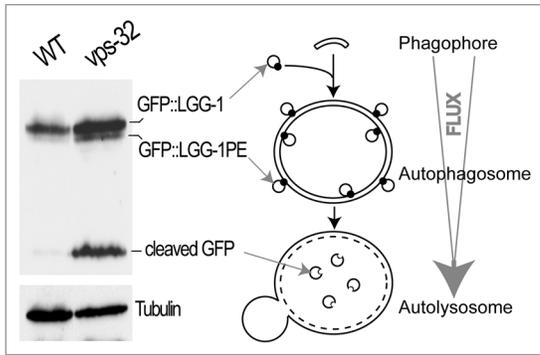
Figure 2. Analysis of the autophagic flux in *C. elegans*. (Left) western blot of total protein extracts of synchronized GFP::LGG-1 embryos incubated with anti-GFP or tubulin antibodies. (Right) Schematic representation of autophagic flux. GFP::LGG-1 is present in the cytosol and after conjugation with phosphatidylethanolamine (GFP::LGG-1PE) is associated with the membrane of the autophagosome. Then, the fusion with the lysosome to form an autolysosome results in an acidification and the release of a GFP fragment (cleaved GFP has a molecular weight of 28 kDa). To analyze the autophagic flux, the ratios of GFP::LGG-1PE to tubulin and cleaved GFP to tubulin are quantified and normalized to the control. For *vps-32(RNAi)* embryos, which were analyzed close to their developmental arrest, the quantification indicates an increase in the autophagic flux. Data taken from Djeddi et al.[1]

to an increased flux rather than a simple maturation blockage.

In yeast, the autophagosome fuses directly to the lysosome but in mammals it can previously fuse with endosomes and MVBs to generate an amphisome. It has been reported in fly and mammals that ESCRT mutants present an accumulation of autophagosomes because of their inability to fuse with the endo-lysosomal system.[28,32] To test this hypothesis, we first needed to assess the existence of the amphisome in *C. elegans*. Rusten et al. showed in the fly that this intermediate compartment is positive either for the autophagic marker Atg-8 (the LC3 drosophila homolog) and the endosomal marker Hrs/vps-27.[28] Therefore, we performed co-localization analysis by immunofluorescence confocal imaging using GFP::LGG-1 and VPS-27 or VPS-32. In both cases, such a compartment presenting both kind of markers was detectable albeit at a very low level. We also inactivated the small GTPase *rab-7,* which is essential for the fusion of both the endosome and the autophagosome with the lysosome. In this context, we observed a 5-fold increase in double positive structures. We then performed the same co-localization analysis in ESCRT mutant context. We noticed that neither *vps-4* nor *vps-32* inactivation increased the number of amphisomes. These results indicate that in *C. elegans*

embryo, amphisomes can be detected but are very infrequent in basal autophagic conditions or in ESCRT mutants. This also suggests that in *C. elegans*, a direct fusion between the autophagosome and the lysosome could be preferential.

Altogether our results led us to conclude that the increase of autophagic structures in an ESCRT mutant background is related to an induction of autophagic flux rather than an accumulation of autophagosomes.

Is Autophagy Deleterious or Beneficial for ESCRT Endosomal Mutants?

Another argument supports the idea that the increased number of autophagosomes is not simply the result of a blockage of their maturation. Our analysis of ESCRT mutants revealed that, except for *vps-32* mutant, enlarged endosomes appeared much earlier than the stage of lethality, which contrasts with autophagic structures whose accumulation precedes the death. This correlation in the emergence of autophagic structures and developmental arrest therefore raises the possibility that the increase of the autophagic activity in ESCRT mutants is responsible for the lethality. Indeed, depending on the pathophysiological context, autophagy can either have a beneficial or a detrimental

effect on the cellular survival. For example, autophagy may be increased to resolve polyglutamine protein aggregates, which are at the origin of many neurodegenerative diseases. However, excessive autophagy can be just as deleterious as a defective autophagy since both can promote apoptosis.[7,9]

To characterize whether autophagy is responsible for lethality, we performed a genetic approach to modify the level of autophagy in ESCRT mutants. We first decreased the basal level of autophagy by silencing *lgg-1*, *lgg-2* or *atg-7* in ESCRT knock out mutants for *vps-27*, *vps-36* or *vps-32*. Interestingly, our results did not show any improvement in the viability of ESCRT mutants and we even observed that *vps-27* mutants died at an earlier stage when autophagy was blocked. This suggests that the increase of the autophagic process is not responsible for the lethality but may be a protective mechanism. To go further in the analysis, we then increased the basal level of autophagy by depleting Tor protein, which is the main nutrient sensor and negative regulator of autophagy in the cell. In this condition we noticed a delay in their stage of lethality even if we did not observe a total rescue of the ESCRT mutants. These results indicate that autophagy is induced as a consequence of the blockage maturation and is beneficial for the survival of endosomal mutant animals.

We finally analyzed whether autophagy induction is able to correct the cellular defects of ESCRT mutants. As previously described, we performed a series of experiments in which we modified the level of autophagy in ESCRT mutants and then analyzed by microscopy the level of vacuolization in the epidermis and the size and number of VPS-27 endosomes. Interestingly, the impairment of autophagy led to an increase of the endosomal defect (abnormal enlarged endosomes) accompanied by a massive vacuolization of the epidermis. In contrast, a higher autophagic basal level strongly reduced epidermis defects together with the size and number of enlarged endosomes. These results led us to conclude that the induction of the autophagy in ESCRT mutants is an adaptive response trying to block the formation and/or degrade abnormal enlarged endosomes.

Conclusion

Beyond its role in the renewal of intracellular components, the role of autophagy in cell survival vs. cell death is ambiguous. Autophagy was shown to have both a beneficial effect by promoting cell survival and a detrimental effect by promoting cell death. Using *C. elegans* ESCRT mutants, we have shown that the induction of autophagy is likely an adaptive response to the blockage of endosomal maturation. Despite the induction of this cell survival mechanism, ESCRT mutants accumulate cellular defects and finally die. By increasing autophagy, cells try desperately to correct cellular defects but not enough efficiently to promote the survival of ESCRT mutant animals.

Depending on the species and possibly the cell type, it appears that ESCRT mutations could differentially affect the interaction between the endosomal and autophagic pathways. A blockage of autophagosomal maturation was described in ESCRT mutants in flies and mammals whereas we showed an induction of the autophagy in *C. elegans*. Specificities in the mechanisms of autophagosomal maturation and fusion are one possible explanation of this differential observation.

Our data raise still unanswered questions on the signaling pathways, which are involved in this response, on the autophagic mechanism itself and on the selectivity of this process. Nevertheless, our study on *C. elegans* provides a new paradigm to investigate the physiology of autophagy in cellular stress and also new insights on the interaction between the endosomal and the autophagic pathway.

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