The role of Atg6/UVRAG/Vps34 containing lipid kinase complex in autophagy and endocytosis

Main points of the PhD. thesis

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Introduction

Macroautophagy (hereafter simply autophagy) is a eukaryotic cell biological process in which cells utilize the lysosomal system to degrade intracellular components. At the beginning of the process a double membrane cistern, the so-called phagophore emerges and engulfs a portion of the cytoplasm (e.g. malfunctioning organelles, pathogens, even aggregates of misfolded proteins). The enclosure of the phagophore creates an autophagosome, which then fuses with the components of the endolysosomal system, giving rise to an autolysosome. The autophagic cargo then becomes degraded in the acidic lumen of the autolysosome, and the resulting monomers are recycled back to the cytoplasm (Boya et al., 2013; Mizushima et al., 2008). Autophagy is important for maintaining the homeodynamic balance of the cell - therefore its perturbation is known to contribute to various diseases (Mizushima et al., 2008).

Receptor mediated endocytosis (hereafter endocytosis) is an important cell biological process, in which cells take up receptor-bound extracellular material, forming intracellular vesicles (endosomes), which either can be recycled back to the plasma membrane or can be degraded in the lysosome. In the latter case the early endosomal membrane invaginates in order to be able to digest selected transmembrane proteins and - due to other changes as well - transforms into a late endosome, also called multivesicular body. The late endosome then fuses with an existing lysosome or with Golgi-derived lysosomal enzyme transporting vesicles, forming a sufficiently acidic compartment (a new lysosome) in which the intraluminal content can be degraded.

Thus endocytosis is also a key process in the regulation of many signal transduction pathways: receptor molecules are taken up and sequestered in order to silence signaling; or in various situations (such as in the case of Notch signalization) early steps of endocytosis are required for the activation of the receptors. Moreover, endocytosis is also needed for establishing normal cell polarity by transporting plasma membrane components to their destination (Babst, 2011).

Phosphoinositide 3-kinase III (hereafter PI3K(III)) is responsible for phosphorylating phosphatidylinositol (PI), generating phosphatidylinositol 3-phosphate (PI3P). This
membrane lipid localizes to both early endosomes and the phagophore, and FYVE or PROPPIN domain containing effector proteins - mediating the maturation of endosomes or autophagosomes - are able to bind it (Backer, 2008; Baskaran et al., 2012).

The core of the PI3K(III) is composed of three subunits: the catalytic subunit Vps34 and the two regulatory subunits Vps15 and Atg6 (Vps30 in yeast, Beclin 1 in mammals). In most eukaryotic cells this complex exists in two forms with different additional subunits. Atg14 and UVRAG forms a functional complex with the core proteins in the PI3K(III) complex I and complex II, respectively (Itakura et al., 2008). Based on yeast and mammalian cell line studies, complex I is required for autophagosome generation, whilst complex II is needed during endosome maturation (Itakura et al., 2008). However, Atg14 has recently been suggested to promote endocytic traffic and UVRAG - by mediating autophagosome-lysosome fusions as well – was found to be required for autophagy (Jiang et al., 2014; Kim et al., 2012; Liang et al., 2006; Liang et al., 2008). Although the role of Atg6 in autophagy is undoubted, its function in other processes such as endocytosis is less clear; moreover, many studies suggest Atg6 to be an autophagy specific protein (Burman and Ktistakis, 2010; Furuya et al., 2005; Liang et al., 1999; Yu et al., 2015; Zeng et al., 2006). These findings raise the possibility that roles of the different PI3K(III) complexes and even that of Atg6 and may differ in distinct cell types.

Homologues of all PI3K(III) components can be found in the fruit fly Drosophila melanogaster and - as expected from other model systems - they are important for autophagy and endocytosis (Abe et al., 2009; Bánréti et al., 2012; Juhász et al., 2008; Lee et al., 2011; Shravage et al., 2013). However, our current knowledge regarding the role of the different PI3(K) complex types in similar conditions is poor. Therefore our aim was to analyze the role of the core component Atg6 and the subtype specific components Atg14 and UVRAG in the developing Drosophila imaginal wing disc and pupal wing.
Methods

- **Genetics.** The fruit fly *Drosophila melanogaster* is a widely used model for studying vesicular traffic and offers the opportunity to alter gene expression in a tissue-specific, spatially controlled manner. We used an RNAi approach on several transgenic flies expressing fluorescent endosomal or autophagy markers to examine the effect of Atg6, Atg14 or UVRAG depletion. We chose two different Gal4 lines to drive the expression of the RNAi constructs in the wing epithelia. MsGal4 and enGal4 was used to trigger transgene expression in the whole wing or exclusively in the posterior compartment of the wing, respectively. In the latter case the anterior half of the wing served as control. As we dispose of an Atg6 null mutant line, most of our results obtained with RNAi flies were also confirmed by studying mutant clones using the mitotic recombination technique. Also, in some cases we generated whole wings and eyes exclusively composed of null mutant cells.

- **Western blot and immunolocalization studies.** For studying the localization of various proteins we used fluorescent reporter proteins or performed immunostaining experiments. The amount of Notch, p62 or Cathepsin L protein in Atg6 mutants were examined using Western-blot.

- **Other histological processes.** To follow the fate of the uptaken Notch receptor we used anti-Notch antibody on living wing cells as an endocytic tracer.

- **Microscopy.** Fluorescent samples were examined by fluorescent microscopy and we performed ultrastructural studies by electron microscopy. We used enzyme cytochemistry and immunocytochemistry to examine the ultrastructural localization of endosomal/lysosomal markers.

- **Statistical analysis.** Our data were quantified and analyzed using the appropriate statistical approach.
Results, theses

1. Using GFP-2xFYVE (a fluorescent PI3P binding reporter protein) we showed that in the absence of Atg6 or UVRAG, no PI3P can be detected in the pupal wings. In contrast, in Atg14 depleted wings PI3P production seems to be normal.

2. Using fluorescent endosomal and lysosomal markers we showed that in the absence of Atg6 or UVRAG (and in contrast to Atg14 depletion), abnormal endosomes and lysosomes accumulate.

3. By immunohistochemistry using mCherry-tagged Atg8 (an autophagic vacuole-specific fluorescent reporter) we showed that unlike UVRAG, Atg6 and Atg14 are both required for autophagy.

4. By electron microscopy we showed that the apical cytoplasm of Atg6 RNAi and mutant cells accumulates abnormal multivesicular and multilamellar bodies, similarly to UVRAG RNAi cells. Using enzyme cytochemistry and immunocytochemistry we demonstrated that these organelles are defective endolysosomes. Ultrastructure of Atg14 RNAi cells appeared to be normal.

5. Using immunohistochemistry we showed that upon the loss of Atg6 or UVRAG (but not in the absence of Atg14), defects in endosomal traffic leads to the accumulation of Notch, Delta and Wingless in intracellular puncta.

6. We performed an ex vivo Notch traffic assay and we found that cell surface-localized Notch was internalized normally but later became trapped in vesicular structures in Atg6 depleted cells.

7. Using Western blot we could show that in Atg6 mutant larvae maturation of the lysosomal Cathepsin L is perturbed and the signaling domain of Notch is accumulated.
8. Using a fluorescent Notch signaling reporter we could show that depletion of Atg6 or UVRAG leads to the enhancement of Notch signaling, unlike in the case of Atg14 depletion.

9. We showed that wing-specific silencing of Atg6 (either by RNAi or by mutant wing generation) and wing-specific UVRAG RNAi leads to the malformation of wings: they become blistered or creased, indicating cell polarity problems. In contrast, Atg14 RNAi wings are vestigial, due to excessive cell death (based on TUNEL assay and anti-activated caspase immunostainings). No cell death could be detected in Atg6 or UVRAG RNAi wings.

10. Using rhodamin-phalloidin staining for detection of wing hairs in pupal wings and antisera against different cell adhesion proteins, we showed that both basolateral and planar cell polarity and also the localization of cell adhesion proteins are severely altered in Atg6 or UVRAG RNAi cells, in contrast to Atg14 RNAi cells.

11. We generated compound eyes composed exclusively of Atg6 null mutant cells by mitotic recombination. We observed that - compared to controls - the eye color is slightly darker, and the hexagonal arrangement of ommatidia is lost, resulting in a rough eye phenotype in mutants. It is indicates that Atg6 is required for the generation of lysosome-related organelles and proper eye development.
Discussion

Atg6 is a key player in mediating autophagy, as in the absence of Atg6 no autophagosomes can be formed (Burman and Ktistakis, 2010; Kang et al., 2011; Scott et al., 2004). Atg6, along with Vps34 and Vps15 forms two distinct PI3K(III) complexes: either with Atg14, forming PI3K(III) complex I; or with UVRAG, forming PI3K(III) complex II. Based on yeast and mammalian cell line studies, PI3K(III) complex I is required for autophagosome generation, whilst complex II is required for endosome maturation (Itakura et al., 2008). As the core member Vps34 and Vps15 has been shown to regulate both autophagy and endocytosis (Abe et al., 2009; Backer, 2008; Bechtel et al., 2013; Itakura et al., 2008; Jean and Kiger, 2014; Juhász et al., 2008; Kihara et al., 2001), it can be speculated that Atg6 is also an important regulator of endocytosis. However, the role of Atg6 in endocytosis seems to be controversial: several papers provide evidence that Atg6 may be dispensable for endocytosis, whilst others take a stand on the opposite (Furuya et al., 2005; Liang et al., 1999; Shravage et al., 2013; Thoresen et al., 2010; Zeng et al., 2006). Moreover, the PI3K(III) complex I-specific Atg14 has recently been suggested to promote endocytic traffic while the complex II-specific UVRAG has been suggested - by mediating autophagosome-lysosome fusions - to be required for autophagy (Jiang et al., 2014; Kim et al., 2012; Liang et al., 2006; Liang et al., 2008).

It is worthy of note that most of these studies were carried out on cultured cells; thus the direct evidence for the participation of Atg6 in other processes than autophagy remained to be demonstrated in vivo (Funderburk et al., 2010). Therefore, our aim was to elucidate the different in vivo roles of Atg6 and the two PI3K(III) complexes. For this purpose, we used RNAi-silenced and mutant developing Drosophila wing disc and pupal wing cells and performed detailed light and electron microscopic analyses in order to examine the resulted phenotypes.

We could demonstrate that - similarly to Vps15 and Vps34 (Abe et al., 2009; Juhász et al., 2008) - Atg6 is essential for endosome maturation. In the absence of Atg6, cells fail to produce PI3P, therefore the maturation of endosomes stalls; similarly to Vps15 RNAi cells (Abe et al., 2009). Atg6 depleted cells show altered cell polarity, leading to wing or eye malformations. As most cell adhesion proteins are transported (and are also degraded) via
vesicular transport (Burdick et al., 1996; Devenport, 2014; Lee and Streuli, 2014; Mellman and Yarden, 2013; Sebagh and Borg, 2014), it can be assumed that the disturbance of cell polarity in Atg6 depleted cells has resulted from the failure of endosomal traffic. As Atg6 is suggested to be a tumor suppressor as well (Furuya et al., 2005; Kang et al., 2011; Liang et al., 1999; Qu et al., 2003), and many tumor cells show altered cell polarity (Lee and Streuli, 2014; Sebagh and Borg, 2014; Sever and Brugge, 2015; Tellkamp et al., 2014), it can be hypothesized that the tumor suppressive ability of Atg6 - at least partially - is due to its requirement for the transportation of cell adhesion molecules to their normal destination.

Notch signaling is a very extensively studied pathway, which is essential for normal organ development, and its disturbance often leads to cancer (Andersson et al., 2011; Ayaz and Osborne, 2014; Chillakuri et al., 2012; Hu et al., 2012). In this process, endocytosis is a key step for both receptor activation and downregulation (Barth and Kohler, 2014; Estella and Baonza, 2015; Fortini and Bilder, 2009; Vaccari et al., 2010; Vaccari et al., 2008). Importantly, we found that due to the failure of endosomal degradation, Atg6 mutant and RNAi cells accumulate Notch (and other signaling proteins, such as Wingless) leading to signaling enhancement. This phenotype is remarkably similar to that of Vps34 or UVRAG mutant fruit fly cells (Juhász et al., 2008; Lee et al., 2011).

UVRAG is a component of PI3K(III) complex II involved in endocytosis, but it has been suggested to regulate autophagy as well (Itakura et al., 2008; Liang et al., 2006; Liang et al., 2008). Since besides participating in PI3K(III) complex II, UVRAG has been speculated to form other complexes in mammals - such as with class C Vps required for autophagosome-lysosome fusion (Liang et al., 2008) -, the question remained open: which UVRAG-mediated processes are independent from PI3K(III) complex II. Phenotypes of UVRAG RNAi cells in all cases were almost identical to that of Atg6 RNAi cells except the autophagy failure. As no autophagy defect could be detected in UVRAG depleted cells, it can be assumed that at least in fruit flies - UVRAG does not interact with class C Vps proteins. A similar observation was made on fruit fly larval fat cells (Takáts et al., 2014), confirming our results.

Atg14 was identified as the mammalian autophagy-specific factor for Beclin 1 and is also a component of the PI3K(III) complex I (Itakura et al., 2008; Matsunaga et al., 2010; Sun et al., 2008). Although Atg14 is considered to be autophagy-specific, there are some results
suggesting that Atg14 may also participate in the regulation of endocytosis (Kim et al., 2012). The *Drosophila* orthologue of Atg14 was shown to be also essential for autophagy in the fat body (Bánréti et al., 2012; Pircs et al., 2012), but the participation of Atg14 in other PI3K(III)-mediated processes remained unclear. We found that although depletion of Atg14 causes very serious malformations in the wing, this effect is neither the consequence of altered cell polarity nor resulted from endocytosis defects. In wing epithelium, Atg14 seems to be required exclusively for autophagy. Although we observed elevated cell death in Atg14 depleted wings, this is most likely due to an ‘off-target’ effect has been observed in many other RNAi flies obtained from the VDRC KK RNAi library (Green et al., 2014).

Our data presented here suggest that the UVRAG-containing PI3K(III) complex II acts as an essential regulator of endocytosis, required for membrane trafficking and downregulation of several signaling pathways in Drosophila (Figure 1). Due to these functions, Atg6 cannot be considered as an exclusive autophagy-related protein: it is indispensable for proper organ development and - as a key component of PI3K(III) complex II – it has several tumor suppressive functions as well. In contrast, the Atg14-containing PI3K(III) I complex is involved exclusively in autophagy.

![Figure 1: Schematic representation summarizing the multiple roles of the two PI3K(III) complexes in *Drosophila*.](image)

Figure 1: Schematic representation summarizing the multiple roles of the two PI3K(III) complexes in *Drosophila*. 
PhD. thesis is based on the following publications


Other publications of the author


References


