Connections between autophagy and other degradative pathways: investigating the role of Rab11 protein

Main points of the Ph.D. thesis

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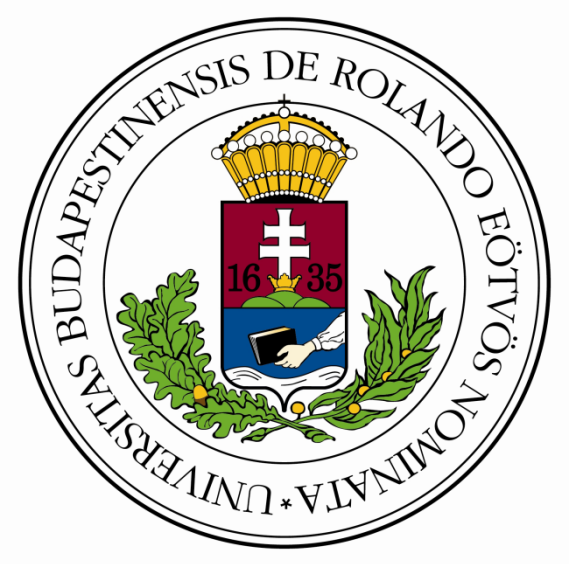

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Budapest, 2014

Introduction

Macroautophagy (hereafter autophagy) is an evolutionary conserved bulk degradation process of eukaryotic cells. During this process, damaged organelles and portions of cytoplasm are sequestered by double-membrane vesicles, called autophagosomes. Autophagosomes undergo fusion events with lysosomes producing autolysosomes, in which acidic hydrolases degrade their content, thereby providing monomers for biosynthetic and energy productive processes.

The regulation of autophagy was originally described in yeast (*Saccharomyces cerevisiae*) but even has a key role in multicellular organisms as a cytoprotective response to stress and pathological conditions. Recently, its role was revealed in several well-known human diseases as well (Mizushima et al., 2008). Due to autophagy impairment, old and damaged organelles and aggregate-prone proteins accumulate leading to neurodegenerative diseases, such as Alzheimer's or Parkinson's disease. Autophagy functions in the clearance of dysfunctional mitochondria, thereby it decreases the probability of mutations supporting tumor progression.

Considering these, the investigation of autophagy is timely and necessary: its function in physiological and pathological processes is required for the development of novel therapeutic strategies in the future.

During the maturation process, autophagosomes can fuse not only with lysosomes; there is growing evidence about the convergence of the autophagic route and different vesicle populations of the endosomal pathway in order to form hybrid organelles called amphisomes. Afterwards, amphisomes fuse with lysosomes and their content is degraded. While a lot of studies focus on the formation of autophagosomes and the early steps of autophagy, many questions remains open concerning the late stages of autophagy, amphisome formation and the exact molecular mechanisms of the fusion events. The spatial and temporal regulation of autophagosome maturation is a barely examined field of autophagy, therefore our knowledge about this has to be broadened. As a recent review discussed, a crosstalk may exist between autophagic and endosomal processes, likely playing

an important role in the coordinated regulation of both degradative pathways (Lamb et al., 2013). However, there is no experimental evidence for this.

Rab11 belongs to the Rab small GTPase protein family, members of which are the main regulators of membrane trafficking and fusion events (Stenmark, 2009). Rab11 has an important role in the endosomal pathway and it is a widely used marker of recycling endosomes (Hsu and Prekeris, 2010). In addition, Rab11 is implicated in various steps of autophagy as well. Previous studies performed in cultured mammalian cells showed that Rab11 may have a role in autophagosome maturation (Fader et al., 2008; Richards et al., 2011). Other results suggest that Rab11 is required for the earliest steps of autophagy: it may have a role in the isolation membrane (phagophore) expansion (Longatti et al., 2012; Knævelsrud et al., 2013).

Based on these, Rab11 may have a function in the coordination of autophagic and endosomal routes. However, its autophagic role is not clear, since it was implicated in many different steps of this process. Therefore, we aimed to investigate the role of Rab11 in an *in vivo* model, the larval fat body of fruit fly (*Drosophila melanogaster*), suitable for studying both autophagy and endocytosis.

Materials and methods

- **Mutants, transgenic flies and genetics.** Drosophila is a widely used model system, and the sophisticated methods and approaches allow quick and easy investigation of the genetic background of various cellular processes. For studying the autophagic role of Rab11 and that of its interaction partner, Hook, we used many combinations of different mutations and transgenic constructions enabling expression of fluorescent reporters and other proteins as well as gene silencing by RNA interference.
- **Recombinant DNA technology.** We developed plasmid constructions for studying protein-protein interactions and generating transgenic fly stocks.
- **Cell culture, co-immunoprecipitation.** For investigation of protein interactions and mapping protein binding sites we used cultured Drosophila D.Mel-2 cells and performed co-immunoprecipitation (co-IP) studies.
- **Western blot and localization studies.** Co-IP samples and lysates of whole larvae or fat bodies were analyzed by Western blot. For studying the localization of various proteins we used fluorescent reporter fusions or performed immunostaining experiments.
- **Other histological processes.** We used vital stains and endocytic tracers suitable for specific visualization of organelles.
- **Microscopy.** Fluorescent reporters and immunostainings were detected by fluorescent microscopy. We performed ultrastructural studies using electron microscope.
- **Statistical analysis.** Our data were quantified and analyzed using the appropriate statistical approach.

Results, thesis

1. Using three independent RNAi lines, a hypomorphic Rab11 mutation and by overexpression of the dominant negative form of the Rab11 protein, we showed that Rab11 participates in autophagy. We found that loss of Rab11 function results in autophagy impairment.

2. By studying the pattern of fluorescent reporters, performing immunostainings, Western blot analyses and ultrastructural studies, we found Rab11 to be required for autophagosome maturation. Defect in Rab11 function resulted in impaired autophagic degradation and accumulation of immature and abnormal autophagosomes.

3. Due to the lack of Rab11 in fat body cells, we detected an accumulation of acidic late endosomes, visualized by the altered pattern of several fluorescent reporters, endogenous proteins or vital stains.

4. By examining the colocalization of autophagic markers with different endosomal proteins and vital stains, we found that Rab11 is essential for the fusion of autophagosomes with late endosomes (that is, for amphisome formation).

5. Our colocalization studies showed that upon autophagy induction, Rab11 translocates from recycling endosomes to autophagosomes.

6. Using co-immunoprecipitation studies carried out in Drosophila cell culture and whole larvae, we found that Rab11 interacts physically with Hook protein, a well-known regulator of the endosomal maturation process.

7. In the fat body cells of *hook* mutant larvae, we studied the pattern of several autophagic and endosomal markers. We found that, similarly to Rab11, Hook is

required for amphisome formation. However, lack of Hook does not lead to the accumulation of late endosomes.

8. We showed that upon starvation-induced autophagy, Hook translocates from late endosomes to autophagic structures in a Rab11-dependent manner, thereby allowing the maturation process of late endosomes.

9. We mapped the Rab11 binding site to the central coiled-coil domain of Hook, previously found to be responsible for Hook homodimerization. Furthermore, we found that presence of Rab11 upon starvation-induced autophagy resulted in a decrease in the amount of homodimer-forming Hook protein, suggesting that, in this condition, Hook rather forms heterodimers with Rab11.

10. Our co-immunoprecipitation studies revealed that the N-terminus of Hook is responsible for binding to α-tubulin, component of microtubules.

11. While overexpression of full length Hook resulted in impairment of autophagy and of endosome maturation, overexpression of the N-terminally deleted Hook did not lead to detectable changes in these processes.

12. Our results indicate that upon autophagy induction, Rab11 removes Hook from late endosomes. Likely, through their physical interaction, Rab11 prevents Hook to fulfill its negative regulatory role, thereby facilitating endosome maturation and amphisome formation.

13. Furthermore, we showed that in larval fat body cells, impairment in proteasomal degradation leads to a decreased cell size and an enhanced autophagic activity.

Discussion

As it was demonstrated in previous works, late endosomes can undergo fusion with nascent autophagosomes and promote their maturation (Köchl et al., 2006; Filimonenko et al., 2007). In compliance with two earlier studies (Fader et al., 2008; Richards et al., 2011), our results show that Rab11 is required for this process. Lack of Rab11 resulted in the accumulation of abnormal autophagosomes and late endosomes, probably due to the failure of amphisome formation.

Furthermore, we observed that upon autophagy induction Rab11 translocates from recycling endosomes (RE) to autophagosomes. This is in line with recent findings suggesting that recycling endosomes provide membrane source for autophagosome formation, and that Rab11 is required for this membrane trafficking process - both in cultured mammalian cells and Drosophila (Longatti et al., 2012; Puri et al., 2013; Knævelsrud et al., 2013). However, while these studies found that Rab11 is necessary for autophagosome formation, we did not detect any impairment in the early steps of autophagy. A possible explanation (supported by numerous findings) may be that in different cell types autophagosome membrane derives from distinct membrane sources (Mari et al., 2011). Conceivably, although REs provide membrane for autophagosome formation in Drosophila as well, they are not the main membrane source for autophagy.

In addition to these, our results also provide mechanistic insights into the maturation of endosomes and autophagosomes: that is, the interaction between Rab11 and Hook is crucial for the maturation of these structures. Previously, Hook was found to be a negative regulator of endosome maturation in Drosophila (Narayanan *et al.*, 2000). In line with this, we found that overexpression of full length Hook protein mimics the effects of Rab11 depletion on the maturation of autophagosomes and endosomes. Moreover, we showed that the N-terminal microtubule binding domain of Hook is required for its negative regulatory role.

Our results reveal a key mechanistic role for Rab11 in the removal of Hook from the late endosomes, thereby allowing termination of endosome maturation and subsequent fusion

with lysosomes. Based on these findings, we developed a model representing a mode of crosstalk between autophagic and endosomal pathways (Figure 1). Upon starvation, the enhanced autophagic activity requires an increased input from the endo-lysosomal system. For this purpose, Rab11 removes Hook from the late endosomes, allowing subsequent fusion of these compartments with immature autophagosomes.

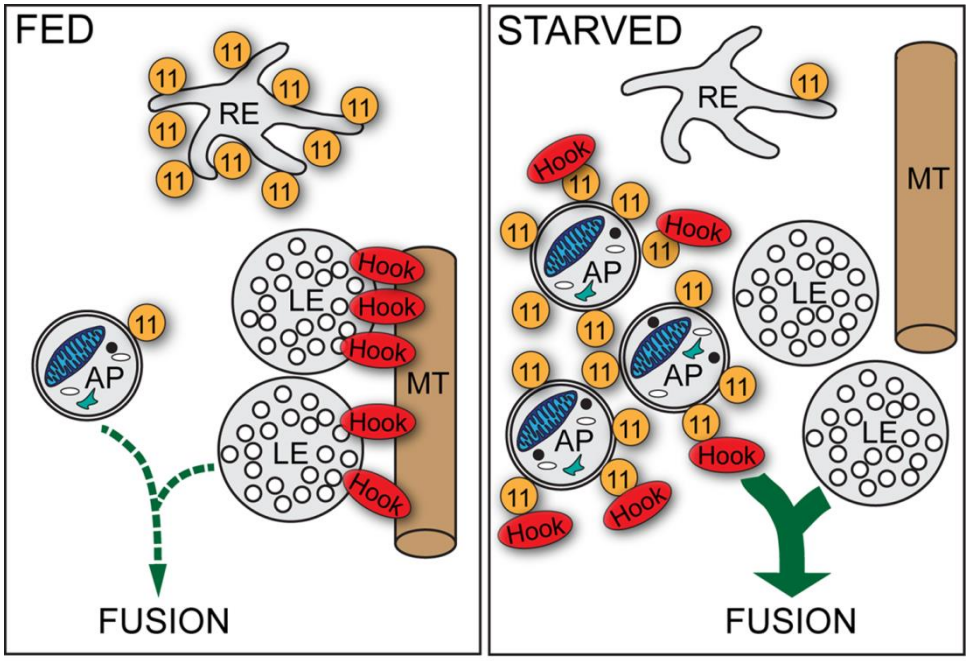

Figure 1. Model for the role of Rab11 and Hook in the process of autophagy. RE: recycling endosome, LE: late endosome, AP: autophagosome, MT: microtubule, 11: Rab11.

In summary, our results suggest that Rab11 functions in a molecular crosstalk mechanism between autophagic and endosomal pathways, so it can be responsible for the coordinated regulation of both processes. As this phenomenon has important medical relevance, further investigations revealing new participants and mechanisms are crucial.

Publications

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