Final report

Introduction

Macroautophagy (hereafter: autophagy) is an evolutionary conserved self-degrading and self-renewal process of eukaryotic cells during which damaged or dispensable proteins and organelles are getting wrapped into double membraned autophagosomes. These transport vesicles eventually fuse with lysosomes where the cargo get degraded and the resulting monomers could be utilized by anabolic or energy producing processes of the cell[1].

Although, the genetic regulation of early steps of autophagy was extensively studied in the last two and a half decades, our knowledge is very limited about the later steps of the process, like autophagosome-lysosome fusion, autolysosome formation and maturation. The proteins and their interactions involved in autophagosome fusion was discovered only in the last decade. Fusion of endo-membranes requires three main factors, small GTPases, tethering complexes and SNARE proteins. The most common GTPases involved in membrane fusion are the members of the Rab family. Rab2 and Rab7 are the best characterized small GTPases involved in autophagosome-lysosome fusion so far[2-4]. These Rabs promotes fusion through their interaction with the HOPS tethering complex. HOPS not only tethers the two membranes in close proximity, but also promotes the formation of the Syx17-Snap29-Vamp7/8 SNARE complex which eventually performs the autophagosome-lysosome fusion[5-8].

The self-renewal function of autophagy is essential for the proper function of terminally differentiated cell types, like muscle fibers or neurons. Accordingly, constantly growing evidence suggest that loss of autophagy can contribute to the occurrence of neuromuscular disorders[1]. Warburg Micro syndrome (WMS) is a rare, neuromotor disorder which results in gradual paralysis of limbs, early onset of cataract and mental retardation [9]. WMS is caused by the heritable mutations of Rab3GAP1, Rab3GAP2, Rab18 and TBC1D20 genes [10,11]. Rab3GAP1 and Rab3GAP2 are forming the heterodimeric Rab3GAP complex which functions as an activator of the small GTPase Rab18[12]. Thus, Rab3GAP complex and Rab18 are acting together in the same functional module. Although the genetic background of WMS is well known, the alterations in cell physiology which eventually results in the onset of the disease are still not understood. Additionally, the role of autophagy in the occurrence of WMS has been poorly characterized.

We used Drosophila melanogaster as model system. As 70% of disease associated human genes have orthologs in the Drosophila genome, flies are excellent for establishing invertebrate models of human diseases. Thus, transgenic or mutant adult flies are frequently used for modelling neuromuscular disorders. Additionally, fat tissue of starved Drosophila larvae is popular and established experimental system for studying autophagy.

Rab3GAP-Rab18 module is required for autolysosome formation and maturation

According to the work plan, we characterized the phenotype of Rab18 and Rab3GAP1 or 2 loss of function Drosophila fat cells. By using 3xmCherry-Atg8a autophagy, and Lamp-3xmCherry lysosomal reporters we showed that knocking down Rab18 or Rab3GAP complex subunits results in pathologic tubulo-vesicular morphology of autolysosomes, compared to control cells in which large, round, punctate autolysosomes are detectable. To assay whether autophagosome-lysosome fusion is affected in Rab3GAP-Rab18 loss of function cells, we carried out colocalization studies by using mCherry-Atg8a autophagic and Lamp1-GFP lysosome markers. Although we observed moderate reduction in the colocalization rate in loss of function cells, the lysosomal and autophagic markers still showed

remarkable overlap. This result suggests that Rab3GAP-Rab18 module has only a minor role in regulating autophagosome- lysosome fusion. We further confirmed these findings by transmission electron microscopy, as we observed autolysosomes with abnormal morphology and accumulation of unfused autophagosomes and in Rab3GAP2 mutant cells.

We supposed that the perturbed autolysosome morphology in cells lacking Rab3GAP-Rab18 module could be occurred due to inhibited lysosome/autolysosome maturation. To understand which step of the lysosome maturation process is perturbed in these loss of function cells, we carried out immunolabeling of early (anti-Rab5) or late endosomes (anti-Rab7), and lysosomes (anti-Rab7, anti-Arl8, anti-Cathepsin L). We found that the morphology of both the late endosomal and lysosomal, but not the early endosomal compartment was seriously damaged in RNAi or mutant cells. Finally, we assayed the integrity of the lysosomal biosynthetic transport by testing the delivery of lysosomal hydrolase Cathepsin L to Rab7 positive late endosomes and lysosomes. We found that Rab7 almost completely colocalized with Cathepsin L positive organelles in controls. This was reduced in Rab3GAP2 mutant cells, however some overlap between these two markers was remained. In summary, our findings suggest that cells lacking Rab3GAP-Rab18 module are both ineffective in autophagosome-lysosome fusion and lysosomal biosynthetic transport which eventually results in defective maturation and morphology of lysosomes /autolysosomes.

Rab3GAP-Rab18 module regulates autolysosome maturation together with the Vps34 Complex1

According to the work plan we supposed that Rab3GAP complex regulate lysosome/autolysosome maturation through inactivating the endosomal Rab5[12]. This hypothesis suggests that hyperactivation of Rab5 should show similar phenotype to loss of Rab3GAP. To test this, we overexpressed the GTP locked, hyperactive point mutant form of Rab5 in fat cell clones, and we observed the appearance of fewer and fainter, but large autolysosomes with rounded morphology. As our findings were contradictory to our hypothesis: the autophagic phenotype of hyperactive Rab5 was obviously different from the tubulo-vesicular autolysosomes found in Rab3GAP loss of function cells, we concluded that Rab3GAP likely regulates autophagy not through inactivation of Rab5.

With regard to a previously published large scale protein-protein interaction study[13] we raised an alternative hypothesis that Rab3GAP-Rab18 module may regulates autophagy through interaction with the Atg14 containing Vps34 lipid kinase complex (hereafter Vps34 Complex I)[14]. To elucidate whether the Rab3GAP-Rab18 module and Vps34 Complex I regulate the same step of autophagy, we carried out loss of function experiments. For these studies we knocked down the Complex I specific subunit Atg14, or overexpressed UVRAG protein to displace Atg14. Both strategies resulted in the same phenotype: fluorescent lysosomal or autophagic reporters showed tubulo-vesicular pattern in the Vps34 Complex I loss of function cells. We further confirmed these findings by electron microscopy experiments: we observed immature autolysosomes with defective morphology and unfused autophagosomes, in Complex I loss of function cells. These phenotypes were reminiscent that we observed in cells lacking Rab3GAP-Rab18 module.

To find out if Rab18 interacts with any of the Vps34 complex, and whether this interaction is GTP dependent we carried out yeast two hybrid experiments. For these studies we cloned three constitutive (Vps34, Vps15, Atg6) and two alternative subunits (Atg14, UVRAG) of the Vps34 complex and the GTP and GDP locked point mutant forms of Rab18. As a result, we found interaction between Atg6 and the GTP but not the GDP bound form of Rab18. To further confirm that this interaction is GTP dependent, we expressed the GTP and GDP locked mutant Rab18 proteins in bacteria and performed GST pull down

experiments. By incubating the beads together with lysates of HA-Atg6 expressing flies, we observed that HA-Atg6 bound to the GTP but not the GDP locked Rab18 form. Our finding that the interaction between the Rab18 and the Vps34 complex subunit was GTP dependent, suggest that Vps34 complex can be considered as a novel Rab18 effector. Due to the elevated amount of molecular biology experiments, a new PhD student, Andras Rubics joined to the group.

In addition to interaction studies, by using GFP and HA tagged reporter constructs, we found that Rab3GAP-Rab18 module members and subunits of the Vps34 Complex I can coexist on Rab7 positive late endosomal/lysosomal compartments. Furthermore, we also detected partial colocalization between Rab18 and Atg14 or Atg6 reporters. Finally, we observed that Rab3GAP2 and Rab18 can localize to Atg8a positive autophagosomes. Our results suggest that Rab3GAP-Rab18 module act together with Vps34 Complex I to facilitate autolysosome formation and maturation and it is likely that this cooperation takes place at the surface autophagosomes and late endosomes.

Rab3GAP2 mutant flies serve as a novel in vivo model of WMS

According to the research plan by utilizing a Rab3GAP2 mutant Drosophila line, we also aimed to establish a novel in vivo model of WMS. To assay whether Rab3GAP2 mutants are good candidates for modelling this syndrome, we tested the motility of young and aged control and mutant flies. We found that even the young Rab3GAP2 mutant flies show motility defects compared to controls, which was progressively increased by age, reminiscent of the progressive loss of motoric function visible at WMS patients. To understand whether this motility defect occurs due to the perturbation of autophagy in neurons or muscles, we assayed the autophagic degradation both in both tissue. Interestingly, we observed extensive accumulation of specific autophagy cargo proteins, p62 and lipidated Atg8a only in muscle, but not in nervous tissue lysates. By immunofluorescence we further confirmed this finding: in thoracic muscles of Rab3GAP2 mutants we found accumulation of p62 aggregates. Our findings suggest that defective autophagy in muscles upon the lack of Rab3GAP2 can lead the motility defects and highlight that perturbation of autophagy can be a critical contributor to the onset of WMS. We summarized our data (discussed above) about the role of Rab3GAP-Rab18 module in autophagy and the maintenance of motoric functions in a manuscript, which had been accepted for publication in FEBS Journal during the preparation of this report. Additionally, my undergraduate student Luca Lévay wrote her Master thesis about her work related to this project and she was awarded by the First Prizes both in the university and the national level of Scientific Student Council Conference (TDK 2018 and OTDK 2019).

Ykt6 is an unconventional SNARE mediating autophagosome-lysosome fusion

In addition to the Rab3GAP-Rab18 story, we also worked on a parallel project, aiming to understand the role of Ykt6 in lysosomal fusion. Ykt6 was interesting for us not only because its loss of function inhibited lysosome fusion and maturation, but the protein itself contains a putatively Rab GTPase interacting Longin domain on its N terminus. Therefore, we carried out several protein-protein interaction studies, but unfortunately, we could not observe interaction between Ykt6 and any of the tested Rab proteins. In parallel, we could demonstrate and prove that Ykt6 localizes to Cathepsin L positive lysosomes and plays essential role in autophagosome-lysosome fusion. By performing co-immunoprecipitation and pull-down experiments we found that Ykt6 forms a previously unknown SNARE complex with two of the three SNARE-s executing autophagosome-lysosome fusion: Syx17 and Snap29, but not with Vamp7[5]. Importantly we also observed, that Ykt6 is outcompeted from this complex

by Vamp7, and overexpression of Vamp7 can rescue the autophagosome-lysosome fusion in Ykt6 loss of function cells. By further pull down experiments we showed that Ykt6 binds to the HOPS tethering complex. Additionally, we found that the Longin and the SNARE domains of Ykt6 shows different binding preference to different HOPS subunits. Based on our data we suggested a model that Ykt6 containing SNARE complex stabilizes the membrane fusion machinery then transforms to the Vamp7 containing complex performing the membrane fusion. This model was supported, by several molecular biology methods including expressing recombinant Ykt6 protein for pull-down experiments, and a fly line expressing HA-tagged Ykt6 reporter was also generated along with a novel polyclonal antibody specific for Ykt6. We published this data in PLOS Genetics[15] a D1 rated journal in SCIMAGO ranking. After finishing his work on this project, my undergraduate student Győző Szenci wrote his Master thesis, defended his degree, and was awarded by the First Prize in the Scientific Student Council Conference (TDK 2017) at Eötvös Loránd University.

Small GTPase Arl8 promotes all lysosomal fusion events

In course of our research aiming to identify small GTPases that regulate lysosomal/autolysosomal fusion and maturation we got interested in understanding the function of the lysosomal resident small GTPase, Arl8. Arl8 was known to be involved in lysosome positioning, but it also interacts with lysosome fusion factors, like the HOPS complex subunits[16]. In course of our work we carried out loss of function studies by using a mutant allele and RNAi mediated knock down of Arl8 in fat bodies of starved Drosophila larvae. As a result of these experiments we observed fragmentation and perinuclear accumulation of autophagic and lysosomal markers mCherry-Atg8a and Lamp-mCherry respectively. We performed colocalization experiments by immunolabeling autophagic and lysosomal markers and we found that both autophagosome-lysosome fusion was highly perturbed in Arl8 loss of function cells. Additionally, we showed that Arl8 localized only to autolysosomes but not autophagosomes, suggesting that Arl8 resides on lysosomal membranes while facilitating the fusion process. Furthermore, we demonstrated that Arl8 function and lysosomal localization and function was dependent of Blos1, Blos2 and Snapin proteins which are common subunits of BLOC1 and BORC complexes, which are known as regulators of biogenesis of lysosome related organelles and lysosome positioning[17].

To analyse whether Arl8 is a general lysosomal fusion factor, we tested other non-autophagy related lysosomal fusion events. For these studies we used different tissue types: Garland nephrocytes or the larval salivary gland to study the endosome-lysosome (endocytosis) or secretory granule-lysosome (crinophagy) fusion respectively. By knocking down Arl8 in both tissues, we observed that lysosomes could not fuse with the vesicles derived from the endocytic or the secretory pathways. These studies showed that Arl8 is a general regulator of lysosomal fusion events. As project members did not have the expertise for carrying out crinophagy studies, an expert of the field: Tamas Csizmadia joined to the project. We published our results about Arl8 function in lysosomal fusion events in the Biochimica et Biophysica Acta – Molecular Cell Research[18], a Q1 rated journal in SCIMAGO ranking.

Articles published in international peer-reviewed journals:

1. The Warburg Micro Syndrome associated Rab3GAP-Rab18 module promotes autolysosome maturation through the Vps34 Complex I
Takáts S, Lévay L, Boda A, Tóth S, Simon-Vecsei Z, Rubics A, Varga Á, Lippai M, Lőrincz P, Glatz G, Juhász G.
FEBS JOURNAL, 2020, Accepted manuscript

2. Drosophila Arl8 is a general positive regulator of lysosomal fusion events.
Boda A, Lőrincz P, Takáts S, Csizmadia T, Tóth S, Kovács AL, Juhász G.
BIOCHIM BIOPHYS ACTA MOL CELL RES, 2019 Apr;1866(4):533-544

3. Non-canonical role of the SNARE protein Ykt6 in autophagosome-lysosome fusion.
Takats S, Glatz G, Szenci G, Boda A, Horvath GV, Hegedus K, Kovacs AL, Juhasz G.
PLOS GENETICS, 2018 Apr 25;14(4):e1007359.

Posters and Talks on Hungarian and international scientific conferences:

1. TALK: SNARE protein Ykt6 as swiss army knife in vesicular fusion events
POSTERS: Rab3GAP-Rab18 module promotes autolysosome maturation through Atg14-PI3K complex; Ykt6 acts as a non-canonical SNARE in autophagosome-lysosome fusion
Hungarian Molecular Life Sciences 2019 Conference
29-31 March 2019. Eger, Hungary

2. POSTER: Rab3GAP-Rab18 module: a novel regulator of autolysosome maturation
Keystone Symposia - Autophagy: From Model Systems to Therapeutic Opportunities
17-21 February 2019. Santa Fe, USA

3. TALK: Analysis of the Rab3GAP-Rab18 module function in Drosophila
Transautophagy COST Workshop on Autophagy in model organisms 2018
6-8 September 2018. Budapest, Hungary

4. POSTERS: Rab3GAP-Rab18 module: a novel regulator of autolysosome maturation; Non-canonical role of the SNARE protein Ykt6 in autophagosome-lysosome fusion
43th FEBS Congress.
07-12 July 2018. Prague, Czech Republic
The first poster (number P.14-029-Tue) won the FEBS Letters Poster Prize (https://2018.febscongress.org/congratulations-to-the-febs-press-poster-prizewinners-2094).

5. TALKS: Role of Small GTPases in Autophagy. The Rab3GAP-Rab18 Module; Ykt6 Acts as a Non-canonical SNARE in Autophagosome-lysosome Fusion
13th COINS 2018 International Conference of Life Sciences
28 February - 02 March 2018. Vilnius, Lithuania

6. TALKS: Kis GTPázok szerepe az autofagoszóma-lizoszóma fúzióban; Az Ykt6, egy atipikus SNARE fehérje szerepe az autofágia során
SFB I. Sejt-, Fejlődés- és Őssejt-Biológusok évi találkozója
28 October 2017. Debrecen, Hungary

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