UNIVERSITY OF SZEGED

Faculty of Science and Informatics

PhD School in Biology

# Analysis of *Drosophila* Atg8 proteins reveals multiple lipidation-independent roles

Abstract of PhD thesis

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## Introduction and background

Autophagy is a highly conserved intracellular degradation process in eukaryotic organisms. During the main pathway a membrane cistern, the so-called isolation membrane or phagophore is generated in the cytoplasm. This membrane then surrounds a part of the cytoplasm and encloses as a double-membrane vesicle, the autophagosome. Ultimately, the autophagosome fuses with a lysosome giving rise to an autolysosome, in which the content of the autophagosome can be decomposed by hydrolytic enzymes.

Autophagy is regulated by AMP and TOR kinase and the large complexes of Atg proteins. This catabolic pathway plays a very important role in eukaryotic cells: the turnover of the macromolecules and organelles has an anti-aging effect, and it is essential in the adaptation to nutrient-poor conditions during starvation. Besides these functions, autophagy has many other physiological and pathological functions. Therefore, the study of the Atg genes has a high significance.

One of the most highlighted Atg genes is Atg8, which is an ubiquitin-like protein that is conjugated to the phosphatidylethanolamine (PE) lipid of the phagophore membrane

during autophagy. The Atg8 protein is bound to the phagophore through lipidation of its C-terminal glycine residue. This process is mediated by a complex that is analogous with the ubiquitylation complex as the Atg8 conjugation system also has members with E1, E2, and E3-like functions. The process plays crucial roles during autophagy, including phagophore elongation, sealing, cargo recognition, autophagosome movement, and autophagosomal tethering to lysosome.

In mammals, seven Atg8 homologs were identified with a high degree of redundancy. In contrast, only two Atg8 paralogs were found in *Drosophila melanogaster*: the *Atg8a* and *Atg8b* genes. Yet, their role has remained unclear. During my PhD period, I studied and characterized these genes.

## Specific aims

1. Generation of an insect phylogenetic tree, which shows the copy number evolution of the Atg8 gene family.
2. Generation of null mutants for *Atg8a* and *Atg8b* genes, and a lipidation-defective *Atg8a* mis-sense mutant in *Drosophila melanogaster* using *in vivo* mutagenesis methods.
3. Characterization of these new Atg8 alleles in autophagy and development.
4. Generation of transgenic lines for *Atg8a* and *Atg8b* genes to rescue the new alleles.

5. Investigation of the expression pattern of the *Atg8a* and *Atg8b* genes, using endogenous promoter-driven transgenic reporters.
6. Investigation of the Atg8b mutant male-sterile phenotype.

## Materials and methods

1. Bioinformatics methods for reconstruction of the Atg8 copy number evolution in the class *Insecta*.
2. Using Plug-and-Play for generating Atg8a null allele ($Atg8a^{Tro\text{-}Gal4}$).
3. Using CRISPR/Cas9 mediated homologous recombination, for generating an Atg8a non-lipidable mutant ($Atg8a^{G116*}$).
4. Using CRISPR/Cas9 genome editing for generation of an Atg8b deletion null allele ($Atg8b^{16}$).
5. Sequencing and PCR for validating the new alleles in *Atg8a* and *Atg8b* genes.
6. Western blot analysis using anti-Atg8 and anti-Ref(2)P antibodies.
7. Generating and investigating somatic clones in larval fat body.
8. Lysotracker staining of larval fat body.
9. Using transgenic reporters and immunostaining in larval fat body.

10. Light, epifluorescent, and electron microscopy.
11. Recombinant DNA techniques.
12. Generating transgenic *Drosophila* lines for Atg8a and Atg8b.
13. Immunohistochemistry of testis samples.
14. Statistical analyses.

## Results

1. We reconstructed the copy number evolution of Atg8 family proteins in the insect species tree. We found that insects initially had two Atg8 family proteins, but the second Atg8 was lost in most species belonging to the order *Diptera*. *Drosophilidae* secondarily duplicated their *Atg8a* gene to give rise to a new paralog by retrotransposition duplication: *Atg8b*.
2. We generated a null mutant *Drosophila melanogaster* line for Atg8a gene using Plug-and-Play *in vivo* mutagenesis methods, through a Trojan (T2A)-Gal4 cassette inserted into the MI13726 transposon element. The generated mutant was validated by anti-Atg8 western blot analysis, where no protein expression was seen.
3. We generated a lipidation-deficient allele, using an ssDNA-template for CRISPR/Cas9-mediated homologous recombination. The generated mutant was validated, both by anti-Atg8 based western blot

approaches, where we observed the lack of lipidated Atg8a form and using DNA sequencing of the mutant locus.

4. Furthermore, the *Atg8b*$^{16}$ deletion null mutant was generated using a double gRNA strategy to remove the entire Atg8b coding region by CRISPR/Cas9. The deletion was detected by PCR.
5. Based on somatic clone analyses in the fat body model, we observed the strongly decreased level of acidic structures (mainly autolysosomes) and the increased level of the Ref(2)P (specific cargo of autophagy) in the Atg8a mutant cells, but not in the Atg8b mutant cells. These results suggest that Atg8b plays no role in autophagy, in contrast with Atg8a.
6. Using larval gastric caeca as a developmentally programmed tissue regression model, we observed strongly impaired degradation of gastric caeca in the Atg8a null mutant, but not in the Atg8a lipidation-deficient allele and in the Atg8b mutant.
7. Based on endogenous promoter-driven Atg8a and Atg8b reporters, we found that Atg8a shows a general expression pattern, and Atg8b shows expression only in the testis. In the testis Atg8a shows a moderate and general expression, while Atg8b expresses only in germline cells.

8. *Atg8b* null mutant shows a male-sterile phenotype, however, all other viable Atg mutants are fertile. This male-sterile phenotype is caused by Atg8b mutant sperm motility defects, but it is not associated with any obvious ultrastructural lesions in the testis.
9. Using microscopy and western blot analysis, we have also shown that Atg8b does not play a role in autophagy in the testis. In addition, an Atg8b transgene lacking a C-terminal glycine is also able to rescue the Atg8b mutant male-sterile phenotype.
10. Atg8a promoter-driven Atg8b coding transgenes are able to rescue Atg8a mutants. In the same way, the Atg8b promoter-driven Atg8a coding transgenes are able to rescue Atg8b mutant phenotypes.

## Summary

Atg8 proteins play important roles in autophagosome biogenesis in all eukaryotes. These are the most widely used markers for autophagy thanks to the association of their lipidated forms with autophagic membranes. The Atg8 protein family expanded in animals and plants, with most *Drosophila* species having two Atg8 homologs. In my thesis we use clear-cut genetic analysis in *Drosophila melanogaster* to show that lipidated Atg8a is required for autophagy, while its non-lipidated form is essential for developmentally programmed larval midgut and salivary gland

elimination and viability. However, expression of *Atg8b* is restricted to the male germline and its loss causes male sterility, via loss of motility of the sperms without affecting autophagy. We find that high expression of non-lipidated Atg8b in the male germline is required for fertility. The specific role of Atg8b relative to Atg8a in male fertility is thought to depend primarily on differences in gene expression rather than amino acid sequence differences. Consistent with these non-canonical functions of Atg8 proteins, loss of Atg genes required for Atg8 lipidation lead to autophagy defects but do not cause developmental failure or male sterility.

## Publications related to the PhD thesis

Analysis of Drosophila Atg8 proteins reveals multiple lipidation-independent roles

András, Jipa ; Viktor, Vedelek ; Zsolt, Merényi ; Adél, Ürmösi ; Szabolcs, Takáts ; Attila, L., Kovács ; Gábor, V., Horváth ; Rita, Sinka ; Gábor, Juhász

AUTOPHAGY, In press (2020)

(DOI: 10.1080/15548627.2020.1856494.)

Drosophila Atg9 regulates the actin cytoskeleton via interactions with profilin and Ena

Kiss, Viktória ; Jipa, András ; Varga, Kata ; Takáts, Szabolcs ; Maruzs, Tamás ; Lőrincz, Péter ; Simon-Vecsei, Zsófia ; Szikora, Szilárd ; Földi, István ; Bajusz, Csaba ; Tóth, Dávid ; Vilmos, Péter ; Gáspár, Imre ; Ronchi, Paolo ; Mihály, József ; Juhász, Gábor

CELL DEATH AND DIFFERENTIATION 27 : 5 pp. 1677-1692. , 16 p. (2020) (DOI:10.1038/s41418-019-0452-0)

## Other publications

Mutation in ATG5 reduces autophagy and leads to ataxia with developmental delay

Myungjin, Kim ; Erin, Sandford ; Damian, Gatica ; Yu, Qiu ; Xu, Liu ; Yumei, Zheng ; Brenda, A., Schulman ; Jishu, Xu ; Ian, Semple ; … Andras, Jipa ; Szabolcs, Takats ; Gabor, Juhasz ; Margit, Burmeister.

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(DOI:http://dx.doi.org/10.7554/eLife.12245.001)

The Ccz1-Mon1-Rab7 module and Rab5 control distinct steps of autophagy Burmeister

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MOLECULAR BIOLOGY OF THE CELL 27 (20), 3132-3142 (2016) (10.1091/mbc.E16-03-0205)

**Oral Conferences:**

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   Atg gének tanulmányozása ecetmuslicában
   PEME XV. PhD Budapest, Magyarország, (2017).

**Poster conferences:**

1. Jipa, A ; Takats, S ; Varga, A ; Vedelek, V ; Urmosi, A ; Mukami, M ; Sinka, R ; Horvath, VG ; Juhasz, G
   Characterization of mutant alleles of Atg8 genes in *Drosophila melanogaster*
   V4 Society for Developmental Biology, Brno, Csehország.
2. Jipa A, Takáts Sz, Varga A, Vedelek V, Sinka R, Juhasz G

Atg8 genes in *Drosophila melanogaster*
Straub-Napok, 2017. május 24-25. Szeged,
poszter Magyarország (2017).