The role for *Drosophila Atg9* in regulation of actin cytoskeleton

PhD thesis

Written by

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2020

Introduction

The cellular self-degradative pathway known as autophagy plays a crucial roles in a variety of physiological and pathological processes, including differentiation, development, aging, neurodegeneration, and tumorigenesis [1][2]. Atg9 is the only known evolutionarily conserved transmembrane protein among Atg gene products, and is likely responsible for membrane transport and recycling of membranes during autophagosome biogenesis [3]. Autophagy was suggested to be involved in oogenesis in *Drosophila*, potentially by affecting the communication between somatic and germline cells in the ovary. Knockdown or mutation of various *Atg* genes in somatic follicle cells interfered with proper development of oocytes in mosaic animals. Interestingly, oogenesis could still proceed when both follicle cells and germline cells were mutant for Atg1 or Atg7 [4][5]. However, unlike other viable *Atg null* mutants including *Atg3/Aut1, Atg5, Atg7*, and *Atg16* [6][7], *Atg9* knock-out female flies are almost completely infertile (this study [8], and [9], raising the possibility that Atg9 plays an autophagy-independent role in oocyte development.

The oogenesis of *Drosophila* is a well-characterized developmental process. The ovary is composed by ovarioles, and an ovariole consists of egg chambers. One oocyte and 15 nurse cells compose an egg chamber, that is covered by somatic follicle cells. The egg goes through 14 developmental stages, that are distinguishable by different molecular- and cell biological features [10]. One of the most investigated cellular processes of *Drosophila* oogenesis is the actin cytoskeleton organisation. Failures in nurse cell actin organisation can impair the actin-dependent processes, for instance the nurse cell dumping. From the 10B stage, during dumping nurse cells contract to expel their cytoplasmic contents into the oocyte to nourish and enlarge it. Incorrect dumping likely leads to smaller and rounded eggs that are mainly infertile. There are numerous known regulatory factors in nurse cell actin organisation: Ena/VASP, profilin, capping proteins, etc.) [11][12][13]. It is known, these actin regulators have commonly emergence in many cell types with similar functions (e.g. nervous system). In my thesis, I have investigated the role of *Drosophila Atg9* in actin organisation. Results have suggested an autophagy-independent role for Atg9, regarding ovary, larval tissues, and embryonic nervous system.

Specific aims

We aimed at to generate and characterize a *null* mutant *Drosophila* strain for *Atg9* gene using CRISPR/Cas9. We tested mutant flies focusing autophagic defects, the ovary morphology, and egg development. Genetic and biochemical tests were carried out to identify two new interacting partners for *Atg9*. Genetic interactions and effects of $Atg9^{B5}$ were investigated in *Drosophila* ovary and nervous system. We have accomplished these aims throughout these steps:

1. Generation *null* mutant for *Atg9* using CRISPR/Cas9.
2. Identification and characterization of *Atg9 null* mutant ($Atg9^{B5}$).
3. Studying morphology of ovary of $Atg9^{B5}$ females.
4. Investigation of role of *Atg9* in actin organization:
    - immunohistochemistry
    - subcellular localisation for Atg9 using *Atg9*-3xmCherry transgenic flies
    - interaction tests: yeast two-hybrid, GST pull-down and anti-tag coimmunoprecipitation

Methods

1. Using CRISPR/Cas9 for generating the $Atg9^{B5}$ null allele.
2. Sequencing and PCR for validating the deletion in *Atg9* gene.
3. Western blot analysis.
4. Generating somatic clones in larval fat body.
5. Lysotracker staining of larval fat body.
6. Testing autophagy defects using Paraquat poisoning, *Pseudomonas aeruginosa* infection (oxidative stress tolerance), and locomotion test.
7. Light- epifluorescence, and electron microscopy.
8. Recombinant DNA techniques.
9. Generating transgenic *Drosophila* lines for *Atg9*.
10. Immunohistochemistry of embryonic-, larval-, and adult tissues and organs.
11. Yeast two-hybrid technique.
12. Anti-tag coimmunoprecipitation.
13. GST pull-down.
14. Preparation of primer embryonic neural cells.

15. Measurement of cytoplasmic streaming.
16. Statistical analysis.

Results

1. We generated a *null* mutant *Drosophila* line for *Atg9* gene using CRISPR/Cas9. The resulted $Atg9^{B5}$ allele caused obvious autophagic defects, moreover, led to almost complete infertility of mutant females, but these phenotypes were rescued by our *Atg9* transgenes validated the observed phenotypes derived from lack of *Atg9*.
2. *Atg9 null* flies had shorter lifespan and were less resistance against oxidative stress and infections (*Pseudomonas aeruginosa*) compared to wild controls and a rescued flies.
3. However, *Atg9 null* females had seemingly normal ovaries, lack of *Atg9* led to less and deformed, smaller eggs (~30%). These abnormal eggs seemed like "dumpless" eggs, suggesting failed dumping. The dumpless phenotype was confirmed by measuring of cytoplasmic streaming of oocyte: cytoplacmic streaming was significantly slower in $Atg9^{B5}$ oocytes, than in wild or rescued (*Atg9*-3xHA) oocytes. These together have suggested depletion of Atg9 has resulted in defective actin cytoskeleton formation.
4. Using CLEM and confocal microscopy of nurse cells, we have investigated the subcellular localization of Atg9. Atg9 localized to membrane curves at the tip of actin cables. Lack of *Atg9* caused a delay of actin cable formation after 10B stage compared to wild control and the *Atg16* autophagy control ($Atg16^{d129}$), suggested an autophagy-independent role for Atg9 in actin organization.
5. We showed *Atg9* had interactions with the actin regulators, Ena/VASP and profilin. Lack of *Atg9* altered the localisation of Ena in nurse cells, as well as Atg9 colocalized with eighter Ena or profilin in larval salivary gland. Due to triple colocalization was never detected, we have concluded Atg9, Ena, and profilin did not function in the same complex during actin regulation.
6. We have confirmed interaction between Atg9 and profilin using anti-tag coimmunoprecipitation and GST pull-down, showing Atg9 and profilin are in the same protein complex in *Drosophila*. In addition, we have observed some further actin-related phenotypes in *Atg9 null* egg chambers: fusion of nurse cells and ring canal loss. However, in $Atg9^{B5}$ these phenotypes occurred rarely, but loss of one copy of *ena* and/or

chic (profilin coding gene) (*ena*^{23} and *chic*^{221}) was able to aggravate them, suggesting there have been genetic interactions between *Atg9* and the actin regulator *ena* and *chic*.

7. Using yeast two-hybrid test, we have confirmed bindings between the first cytosolic domain of Atg9 (CTD1) and Ena, and the fourth cytosolic domain (CTD4) and profilin. It was known, profilin preferred to bind proline-rich regions, thus we studied the amino acid sequence of CTD4. A PPRPPAAP motif was found as a potential binding sequence for profilin, therefor we changed prolines of the proline-rich motif to alanine (CTD4mut_P). The CTD4mut_P domain has been seemingly less preferred by profilin suggesting prolines of CTD4 were involved in binding to profilin.
8. We have investigated the role of *Atg9* in embryonic nervous system regarding actin cytoskeleton. Using *ex vivo* primer embryonic neural cell culture, *Atg9*^{B5} caused enhanced filopodia- and axon growth, moreover, in later embryonic stages lack of *Atg9* led to midline crosses of ventral nerve chords. Due to *null* alleles of *ena* or *chic* resulted in similar defects to *Atg9* [14], these observations suggested, *Atg9* played a role in neurogenesis through cooperation with actin regulators.

Summary

In our study, the *Atg9*^{B5} *null* allele was generated using CRISPR/Cas9. In *Drosophila*, lack of *Atg9* led to typical autophagy defects regarding shorter lifespan, neuromuscular defects and reduces stress tolerancy, moreover, *Atg9*^{B5} mutation led to reduced fertility of females. *Atg9*^{B5} females often laid smaller, so-called “dumpless” eggs, that were the result of defective actin organisation in nurse cells. The Atg9 localized to the plasma membrane closed to tip of actin cables, and lack of *Atg9* caused abnormal actin cable formation. Genetic interactions were identified between *Atg9*, and two of actin regulatory gene, *ena*, and *chic*. Due to the actin organization takes place on a similar way organism-wide, it may explain why *Atg9*^{B5} led to defective neuron growth, similarly to some of *ena* or *chic* mutant alleles. *Atg9* homologs in other organism, e.g. in mammals, thus our results raise a similar actin-regulatory role for Atg9 in higher order organisms, as well.

Publication for the PhD thesis

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

Vps8 overexpression inhibits HOPS-dependent trafficking routes by outcompeting Vps41/Lt

Lőrincz, Péter ; Kenéz, Lili Anna ; Tóth, Sarolta ; Kiss, Viktória ; Varga, Ágnes ; Csizmadia, Tamás ; Simon-Vecsei, Zsófia ; Juhász, Gábor

ELIFE 8 Paper: e45631 (2019) (DOI: 10.7554/eLife.45631)

On the Fly: Recent Progress on Autophagy and Aging in *Drosophila*

Maruzs, Tamas ; Simon-Vecsei, Zsofia ; Kiss, Viktoria ; Csizmadia, Tamas ; Juhasz, Gábor

FRONTIERS IN CELL AND DEVELOPMENTAL BIOLOGY 7 Paper: 140 , 15 p. (2019) (DOI: 10.3389/fcell.2019.00140)

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