

# The function of the *Drosophila* Atg7 and Rack1 proteins

Theses of Doctoral Dissertation

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## **1 Introduction**

During the process of macroautophagy part of the cell's cytoplasm gets engulfed by double-membrane vesicles which later fuse with lysosomes to break down its contents. The degradation products are reused later for energy production and resynthesis of macromolecules. This cycle provides a continuous turn over of cellular components.

Autophagy can be strongly induced during developmental changes and certain pathologic conditions or as a response for various types of stresses

Deeper understanding of the functions of autophagy can be crucial in the case of certain chronic diseases, such as Danon's disease, Huntington's disease, Alzheimer's disease and certain types of cancer just as well as in aging.

The genes involved in the process were originally isolated in the yeast *Saccharomyces cerevisiae*. The homologues of this set of genes, -called the Atg genes-, can be found in all eukaryotes. Genetic studies based on these genes proved the involvement of autophagy in an ever-growing list of processes and reveal more and more of the molecular mechanisms of its regulation.

The level of autophagy in *Drosophila* larval tissues rises steeply under starvation and during certain periods of development. This and the great variety of genetic tools available makes *Drosophila* an optimal subject of autophagy research

## **2 Objectives**

### **2.1 Characterization of the physiological role of Atg7-dependent autophagy**

Characterization of the effects of Atg7 loss of function on starvation-induced- and developmental autophagy, ontogenesis and viability.

### **2.2 Gene expression profiling of the starvation response in control and in Atg mutants**

Comparison of starved and well-fed wild type and autophagy mutant larvae with a Microarray and QPCR based approach in order to gain insight into the regulatory and possible compensatory mechanisms underlying gene expression pattern changes and to discover genes involved with novel function in autophagy.

### **2.3 Functional characterization of one or more selected genes**

Selection and functional characterization of genes which showed significant changes during starvation, for which experimental reagents are available (mutants, antisera, etc...) and based on the literature are deemed to be good candidates.

## **3 Methods**

### **3.1 Genetic mosaic analysis**

For the mosaic analysis we used the genotype *hsFLP, Act<sup>></sup> CD2<sup>></sup> Gal4, UASGFP/UAS-X*. Spontaneous activation of the Flippase reconstituted the Actin promoter which in turn controlled the expression of desired transgenes or dsRNA constructs in the gain of function cell clones. The surrounding cells were used as controls.

### **3.2 Generation of deletion mutants**

The Atg7, Sec6 and CG5335 deletion alleles were generated from existing P-element insertion bearing lines via transposase induced improper excision events.

### **3.3 Microarray analysis**

We compared two states (starved/well fed) on every microarray. The isolated total RNA were transformed to amino allyl cRNA in a multistep process. These probes were fluorescently labeled and hybridized onto the microarrays.

### **3.4 Bioinformatical analysis**

The genes which showed relevant changes were split into two groups based on whether they were induced or repressed. Functional clustering of the selected gene lists was performed on-line with the DAVID freely available software package.

### **3.5 TUNEL assay**

The TUNEL staining was performed using the TMR Red In Situ Cell Death Detection Kit (Roche) on both the larval midgut and on paraffin embedded brain slices.

### **3.6 Electron microscopic immunocytochemistry**

100 nm ultra-thin slices were processed for Rack1 Ubiquitin immunostaining. The three-step immunostaining was performed with rabbit anti-ubiquitin or anti-Rack1, followed by biotinylated anti-rabbit and 20nm gold-conjugated anti-biotin.

## **4 Results**

### **4.1 Generation of the Atg7-of-function mutant**

Transposase induced improper excision of the d06996 and EY10058 P-elements resulted in deletions which overlap at the catalytic region of Atg7 when crossed to each other. We used these heterozygotes as bona fide loss of function mutants.

### **4.2 Atg7 is essential for starvation- induced autophagy**

Atg8 :: GFP positive dots failed to accumulate in mutant cell clones in the fat body of larvae starved for a three hour period. Mutant cell clones inhabited severely reduced levels of autophagy shown by LysoTracker staining. The electron micrographs provide further evidence. We found that the number and size of autophagosomes and autolysosomes were severely reduced.

### **4.3 Atg7 is required for the developmental autophagy of the larval midgut**

We investigated the role of autophagy in the gastric caeca of GFP-Atg8 expressing wandering larvae. We found a strong decrease of GFP-Atg8 dots compared to the high levels observed in control animals. The electron micrographs showed that the cytoplasmic area occupied by autophagosomes and autolysosomes are 0,17 - and. 0.42% respectively. Which is a highly significant decrease compared to 0,95 and 3,22% values measured in controls.

### **4.4 Atg7 mutant flies have a shorter lifespan, are more sensitive to oxidative stress and nutrient deficiency**

Loss of function of the Atg7 shortened average lifespan by about 11 days under standard conditions. Complete nutrient deprivation killed mutant flies before the age of two days whereas the majority of control individuals survived this time point. Next we exposed mutants and controls to oxidative stress (Paraquat treatment) and measured survival times. Mutants lived approximately half as long as controls.

### **4.5 Progressive neuronal degeneration in mutants**

Inclusion bodies can be readily seen in neurons of 3 days old mutant brains revealed by electron microscopy. We also observed dead neurons in 30 days old mutants adults. None of these were found in control samples. The number of the inclusions increased with age. Semi-thick slices of entire brains showed small lesions in various brain regions of 30 days old mutants. We found signs of DNA degradation in a high number of neurons in 30 days old mutants revealed by TUNEL reaction.

### **4.6 Microarray analysis of starvation-induced autophagy in wild type and mutant Drosophila larvae.**

We compared gene expression profiles of 86-hour larvae exposed to 4-hour starvation to non-starved larvae with the same age. Among the most strongly induced genes are a large number of genes involved in the immune response in both groups.

Based on their functional annotation, we found 10 GO categories that were specifically enriched in the ATG mutants. One of these categories: the "ubiquitin-dependent protein degradation process" might imply a compensatory mechanism.

### **4.7 Most autophagy genes are transcriptionally induced during starvation**

The gene activated Atg8a the strongest among all Atg gene. Members of the Atg1 complex were only slightly induced. Genes of the membrane transport complex showed the strongest over all response to starvation.

#### **4.8 Quantification of gene expression levels of selected genes in the larval fat body**

The results of quantitative real-time PCR recapitulated the findings of the microarray experiments, although the extent of changes were generally much larger.

#### **4.9 Rack1 is required for a full autophagic response to starvation**

Rack1 (Receptor for Activated C Kinase 1) was selected for further functional analysis. Its transcription level showed a 4-fold increase in whole larvae, and an even higher 21.6-fold induction in isolated fat bodies. In mCherry-Atg8 expressing starving larvae, the number of mCherry positive dots rises considerably less in Rack1-specific dsRNA expressing fat body cell mosaics than in adjacent control cells. Results from Rack1 null mutants (Rack1 [1.8]) larvae were consistent with the above mentioned genetic mosaic experiments. Electron microscopy on the mutant further confirmed these findings. Detailed morphometric analysis revealed a striking reduction in the cytoplasmic area of autolyso- and autophagosomes (76% and 99% (!) reduction respectively). Additionally we also found that the area ratio of glycogen granules is also suppressed. Extending the studies to wandering larvae revealed that the rate of developmental autophagy is not affected by the Rack1 loss of function.

#### **4.10 Rack1 plays a role in glycogen synthesis**

Having observed that the glycogen stores are in fact building up in the sucrose bath (the media generally used for starvation experiments) we came to the conclusion that reduced glycogen levels in the Rack1 mutant may be due to a defect in glycogen synthesis rather than excessive glycogen breakdown.

With the aid of glycogen specific antibodies we were able to follow changes in the glycogen content of the cells also on a light microscopic level. On this basis, regarding the impact of Rack1 on glycogen levels we made two observations. First: Consistently with the electron microscopic results we found dramatically reduced numbers of glycogen particles in Rack1 mutant fat bodies. Secondly: Based on our mosaic RNAi experiments this effect is cell-autonomous.

#### **4.11 Endogenous Rack1 localizes to early autophagic structures and glycogen**

To further assess the function of Rack1 in glycogen synthesis and autophagy we carried out co-localization experiments with laser scanning confocal microscopy. In the fat body of GFP-Atg8-expressing animals Rack1 particles showed 5.8% colocalization to GFP Atg8 positive dots, and conversely GFP Atg8 positive dots colocalized with 6.1% of Rack1 particles. We found Rack1 positive dots adjacent to 77% of glycogen particles

## 5 Discussion

### 5.1 Atg7

Loss of function of the ATG genes in yeast causes lethality much earlier than normal under low nutrient conditions (Tsukada and Ohsumi, 1993). In *Drosophila* previously studied Atg genes have proved to be essential for normal development (Scott, Schuldiner et al. 2004). To account for the unexpected viability of our Atg7 null allele we can hypothesize the following:

- a) The fruit fly has another protein that can at least partially replace the function of Atg7,
- b) another mechanism is able to replace the function of macroautophagy at least to some extent (for example, the chaperone-mediated autophagy), or
- c) the process of macroautophagy itself is not essential. From this conclusion it would follow that the other Atg genes studied so far possess other essential, non autophagic functions.

After the publication of our paper on which this dissertation is partly based, others also observed progressive accumulation of ubiquitinated proteins and protein aggregates in the case of another Atg gene: Atg8 (Simonsen, Cumming et al. 2008). They have also shown a decline in the expression levels of autophagy genes in aging fruit flies, and that overexpression of Atg8 increases lifespan. On the question of whether there was a causal relationship between aging and autophagy, and how these processes are linked to each other future research must provide the answers (Cuervo, Bergamini et al. 2005).

Research on the role of autophagy in cell death is a very active and controversial field. The role of Atg7 in *Drosophila* development is ambivalent. On one hand, in the larval gut epithelium it contributes to the proper execution of processes leading to developmental cell death (in the absence of its function inhibition of DNA degradation occurs), on the other hand, in the central nervous system it promotes survival of neurons (in the absence of its function aggregates form and cells die). These results contribute to creating a more accurate picture of autophagy's role in aging and cell death, and later can help to solve the apparent contradictions inherent to this field.

## 5.2 Microarray

Genes with function in catabolic processes such as proteasomal protein degradation or amino acid metabolism were expressed to a higher degree in the autophagy mutants than in the control. These changes in catabolic processes can be interpreted as a compensatory reaction to the shortage of energy and nutrients caused by deficient autophagic function.

Since the mRNA levels of Atg genes in Atg1 and Atg7 mutants responded similarly to control, we can assume that the Atg1 and Atg7 has no significant role in the negative feedback regulation likely to control the gene expression of Atg genes.

## 5.3 Rack1

Half of the Rack1 positive granules associate to glycogen, furthermore Rack1 loss of function inhibits. Colocalization with GSK-3B may indicate that Rack1 can functionally interact with a pool of GSK-3B active in synthesis. Rack1 is known to bind three different subunits of AMPK. The  $\beta$  subunit of AMPK has a glycogen binding region which is responsible for its localization to glycogen granules. AMPK activated by high levels of AMP induces glycogen breakdown and inhibits glycogen synthase simultaneously (Hardie, 2011).

We assume that Rack1 as a scaffolding protein helps AMPK and GSK-3B to form a functional complex, while AMPK is responsible for the glycogen binding of the complex. This putative complex (along with other kinases and phosphatases), might regulate glycogen synthase activity via its phosphorylation pattern depending on cellular context.

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