

PhD Thesis

# **Endogenous p62 level is suitable to measure basal autophagy in *Drosophila* cells**

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## **Introduction**

Macroautophagy (simply referred as autophagy) is an evolutionarily conserved lysosomal degradation pathway which is present in a basal level in all eukaryotic cells and ensures steady-state homeostasis but also differentiation, survival and remodeling. It has an essential role in normal cytoplasmic turnover and in eliminating the damaged, dysfunctional, unused cellular components or whole organelles (Cuervo 2008).

During autophagy there are different morphological steps (Figure 1): first an isolation membrane forms that engulfs more or less randomly a part of the cytoplasm. Upon fusion of its edges, a double-membrane autophagosome is generated, which then fuses with a lysosome to deliver the contents for degradation. Monomers from the bulk cytoplasmic components are recycled for reuse in energy producing and synthetic pathways (Baehrecke 2003).

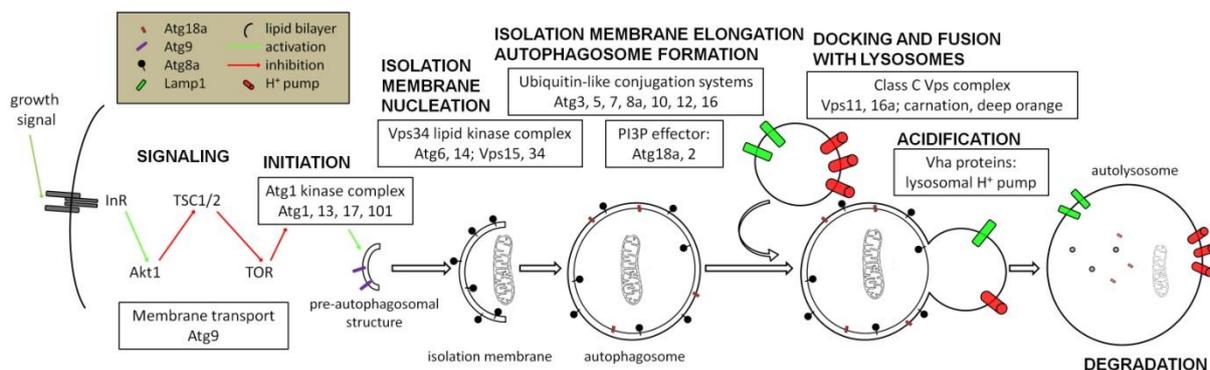
Autophagy has a function in several processes maintaining development and regulating lifespan. It has a significant role in avoiding stress during starvation, hypoxia or physical/chemical impacts by activating stress-induced autophagy to maintain cell survival and cell defense (Glinsmann and Ericsson 1966, Arstila and Trump 1969, de Duve 1969, Rez and Kovacs 1973, Mizushima 2007, Xie and Klionsky 2007). Its role was identified also during ontogenesis and named developmental autophagy (Clarke 1990, Edinger and Thompson 2004). Autophagy has a crucial role in many physiological processes such as aging, cell growth, adaptive immunity and cellular defense against invading pathogens (Melendez, Talloczy et al. 2003, Levine 2005, Scott, Juhasz et al. 2007, Rubinsztein, Marino et al. 2011). Reduced or enhanced autophagic activity can contribute to pathological conditions like neurodegenerative diseases (Huntington-, Alzheimer-, Parkinson-disease etc), tumors, dystrophy (Juhasz, Erdi et al. 2007, Mizushima, Levine et al. 2008, Simonsen, Cumming et al. 2008, Kongara and Karantza 2012). These examples indicate that autophagy is important for homeostatic balance, that's why the understanding of its molecular mechanism and genetic background is necessary in basic research.

The regulation of autophagic machinery and process is evolutionarily conserved (Melendez and Neufeld 2008, Pattingre, Espert et al. 2008). Autophagic protein complexes and related different morphological stages of the process can be separated into six distinct groups (Figure 1): 1. initiation; 2. isolation membrane nucleation; 3. isolation membrane elongation and autophagosome formation; 4. docking and fusion with lysosomes; 5. acidification and degradation of autolysosomes and at last 6. recycling.

Levels of the selective autophagy substrate p62 have been established in recent years as a specific readout for basal autophagic activity. Human p62/SQSTM1 (sequestosome-1) is a multidomain scaffold protein involved in various signaling pathways regulating a number of processes including apoptosis, stress responses, and cell growth (Moscat and Diaz-Meco 2009). Its single *Drosophila melanogaster* homolog Ref(2)P (refractory to sigma P) - for simplicity, hereafter referred as p62 - encoded protein product shows a similar domain structure to human p62. Both contain a PB1 domain required for self-oligomerization and binding to other PB1-domain proteins, a ZZ-type zinc finger domain, an LIR (LC3-interacting region) required for its interaction with Atg8/LC3 family members, and a C-terminal ubiquitin-binding UBA (ubiquitin-associated) domain (Nezis, Simonsen et al. 2008).

The Atg8/LC3 interaction enables selective degradation of p62 by autophagy, and by acting as a specific adaptor protein it also ensures the targeting of ubiquitinated proteins for lysosomal degradation. Numerous human degenerative disorders are accompanied by the formation of cytoplasmic aggregates containing p62 and ubiquitinated proteins (Bartlett, Isakson et al. 2011). These abnormal aggregates also form in response to impaired autophagy, so p62 level inversely correlate with dysregulation of basal autophagy.

In our laboratory we work with fruit fly (*Drosophila melanogaster*) which is one of the best model organisms for autophagic studies. Its genome contains two thirds of the human disease gene homologues and its short lifespan as well as the genetical and cell/developmental experimental methodology makes it a very efficient model for basic research.



**Figure 1. Regulation and mechanism of autophagy** (Made by Péter Nagy)

## **Methods**

An in vivo whole-genome RNAi screen for genes involved in autophagy in Drosophila larvae  
Identifying genes involved in autophagy in an *in vivo* RNA interference whole-genome screen in mosaic larval fat bodies and characterizing the positive hits.

### Molecular Cloning and Embryo Injections

Generation of an inducible dominant negative (DN) Atg4 transgenic fly strain containing a Cysteine to Alanine mutation.

### Generation of Polyclonal Anti-p62 and Anti-GFP Antibodies

For immunohistochemistry and Western blot analyses we generated polyclonal anti-p62 and anti-GFP antibodies in rabbits and rats respectively.

### Western blot

Anti-p62 and anti-GFP antibodies were tested with samples made from whole larvae and adult fly heads together with free GFP and p62 levels for measuring autophagic flux with p62-GFP reporter cleavage.

### Immunohistochemistry

The new anti-p62 antibody was tested with immunohistochemistry in different mutant animals and RNAi fat body cell clones.

### Microscopy

Electronmicroscopical analyses of Atg4DN expressing Drosophila larvae. Phenotypes of the fat body samples made from the whole-genome screen and immunohistochemistry were studied with fluorescent microscopy.

### Statistics

Results collected from Western blots and microscopical images were evaluated with ImageJ. Data were normalized to control animals or control cells. Two-tailed two-sample unequal Student's t test was used to estimate p values in all cases.

## **Results**

- ❖ First we carried out an in vivo whole-genome RNAi screen for genes involved in autophagy in *Drosophila* larvae. We tested the effect of knocking down 7397 RNAi strains in a primary screen. We used secondary tests to narrow down our 1047 hits to 224 phenotypically different genes and establish three different categories: AL (autolysosomal digestion block), AP (autophagosome maturation block) and ATG (ATG gene-like) groups.
- ❖ We identified most known regulators of autophagy in a blind fashion in the primary screen and also new, unpublished autophagy gene candidates (Myc, AtI, Syx17).
- ❖ We tested the specificity of our new anti-p62 antibody in Atg mutants, p62-RNAi and p62 overexpressing cells.
- ❖ We analyzed the accumulation of endogenous p62 in Atg and S6K mutants. The number and size of p62-positive dots were significantly increased in known autophagy mutant fat bodies that was verifying accelerated p62 amount during impaired basal autophagy. We have also found that loss of S6K significantly increased the number (but in this case not the size) of p62 aggregates in larval fat body cells, consistent with its suggested positive role in autophagy.
- ❖ We established the role of basal autophagy in mosaic fat body cell clones with immunohistochemistry for genes carrying a phenotypically lethal mutant/RNAi allele. After the Atg mutant experiments we tested the p62 level in fly strains with loss of known autophagy regulators by immunostaining and observed a similar p62 accumulation, thereby further confirming that autophagy negatively correlates with the autophagic activity.
- ❖ Housekeeping genes are often identified as putative hits in various genome-wide RNAi screens, although their effect is likely indirect in most cases. The p62 immunohistochemistry assay is potentially suitable for distinguishing specific regulators of autophagy (“hits”) from indirect effects (“noise”).

- ❖ We showed that the GFP-tagged p62 reporter, which was previously used successfully in *Drosophila* to detect basal autophagy defects, is only suitable for detecting impaired autophagy in a limited manner because it can substantially self-aggregate independently of autophagy. Still, comparing GFP signal intensities may yield qualitatively similar information to data obtained by looking at endogenous p62 levels in some but not all cases.
- ❖ The Atg16, Atg18a and Pten RNAi lines that showed differences in p62 accumulation in immunostainings also differed in starvation-induced autophagy phenotypes in L3 and L2 larval stages. The effect in L2 larval stage starvation-induced autophagy correlated with the immunohistochemistry results of the L3 stage p62 accumulation. Our results suggest that differences seen in starvation-induced autophagy in the L2 stage and the progressive formation of p62 aggregates (analyzed in the L3 stage) may both reflect the knockdown efficiency of the given RNAi line in earlier stages (that is, L2).
- ❖ Co-expression of p62-GFP and mCherry-Atg8a represented an artefact and not bona fide autophagic structures – these form large, colocalizing aggregates even in well-fed animals.
- ❖ On the basis of our observations p62-GFP conversion into free GFP in Western blot analysis of starvation-induced- and developmental-autophagy is a much more effective methodology than examination of p62-GFP expressing cells with fluorescent microscopy.
- ❖ Larvae from our transgenic dominant-negative Atg4a expressing strain strongly suppressed starvation-induced autophagy.
- ❖ Co-expression of mCherry-Atg8a with dominant-negative Atg4 restored both starvation-induced autophagy in starved animals and the normal turnover of p62. These findings supported our hypothesis that partial loss of Atg4a function can be rescued by overexpression of Atg8a.

## **Main points of the thesis**

1. In case of impaired autophagy our p62 antibody is suitable to detect the accumulated amount of p62. p62 levels negatively correlate with autophagy.
2. S6 kinase which is a physiological substrate of TOR kinase may positively regulate autophagy, as lack of it results in p62 accumulation in fat body cells.
3. Housekeeping genes presumably in many cases indirectly regulate the autophagic response.
4. Overexpressed p62-GFP reporter protein can self-aggregate irrespectively of proper or impaired autophagy.
5. The intensity of p62-GFP fluorescent reporter negatively correlates with autophagy.
6. p62 levels may remain normal despite a strong block of starvation-induced autophagy if the onset of efficient gene knockdown is delayed during larval *Drosophila* development. Based on these observations, the perdurance of maternally supplied gene products during development may also account for some of the differences seen in p62 levels in different Atg mutants.
7. Co-expression of p62-GFP and mCherry-Atg8a is not ideal because it can lead to false conclusions regarding autophagic response.
8. Expression of dominant-negative Atg4 blocks starvation-induced autophagy.
9. The partial loss of Atg4a function can be rescued by overexpression of Atg8a.

## **Outcome**

The putative hits identified in the *in vivo* whole-genome RNAi screen are excellent for further studies for our lab, providing continuous material for publications (Myc, Atl, Syx17). I would like to highlight that the characterization of the last gene was published recently.

We successfully generated and characterized a well functioning *Drosophila* p62 antibody, which is a widely known and frequently used selective autophagic marker in animal cells. Our article in this subject was published in PloS One on August 31st, 2012. 18 research groups has asked for this antibody as of June 10th, 2013.

Our work has important implications for other model systems as well: we show that I. statistical analysis of p62-positive aggregates in immunostained cells and tissues is similarly effective as western blots for the estimation of basal autophagy levels, II. immunostaining of mosaic fat bodies allows for testing the specific role of genes with lethal phenotypes in basal autophagy, III. endogenous p62 provides a much more sensitive measure of autophagy levels than a constitutively overexpressed GFP-tagged reporter in microscopy.

The p62 immunohistochemistry or other assays in basal autophagy is potentially suitable for distinguishing specific regulators of autophagy (“hits”) from indirect effects (“noise”).

We highly recommend verifying results with independent RNAi strains in every case because the RNAi efficiency substantially depends on the duration of RNAi, regarding when the interference effect „switches on”.

Although our experiments in this thesis involved fat body cells of *Drosophila* larvae, we are convinced that the results and limitations presented here will be applicable to most cells and organisms used in autophagy research.

## **Publications**

**Pircs K**, Nagy P, Varga A, Venkei Z, Erdi B, Hegedus K, Juhasz G.

Advantages and limitations of different p62-based assays for estimating autophagic activity in *Drosophila*

*PloS One*. 2012; **7**(8): e44214.

Erdi B\*, Nagy P\*, Zvara A, Varga A, **Pircs K**, Menesi D, Puskas LG, Juhasz G.

Loss of the starvation-induced gene *Rack1* leads to glycogen deficiency and impaired autophagic responses in *Drosophila*.

*Autophagy*. 2012; **8**(7): 1124-1135.

Takats Sz, Nagy P, Varga A, **Pircs K**, Karpati M, Varga K, Kovacs A, Hegedus K, Juhasz G. Autophagosomal Syntaxin17-dependent lysosomal degradation maintains neuronal function in *Drosophila*.

*J Cell Biol*. 2013; **201**(4): 531-9.

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