Introduction

The proper balance of anabolic and catabolic processes ensures cellular and organism-wide homeostasis under physiological conditions. Major intracellular catabolic pathways include the ubiquitin-proteasome system, responsible for the regulated degradation of selected proteins, and autophagy, capable of degrading all intracellular macromolecules and even whole organelles. The major pathway of autophagy utilizes double-membrane vesicles called autophagosomes to deliver cytoplasmic cargo for degradation in lyosomes. Basal levels of autophagy are required to prevent the accumulation of abnormal protein aggregates that are detrimental for cell function and survival, especially in long-lived cells such as neurons. Autophagic activity is tightly controlled in all cells: factors increasing the rate of cell growth usually decrease, while deprivation of nutrients or growth factors dramatically increases the levels of autophagy. A set of about 20 evolutionarily conserved genes required for autophagy has been identified in yeast. These Atg (Autophagy) gene products orchestrate the formation of early autophagic structures where autophagosome generation takes place. The Atg1 protein kinase complex is directly controlled by TOR (Target Of Rapamycin), a kinase that coordinates growth-promoting stimuli with the availability of nutrients, ATP and oxygen. Inactivation of TOR rapidly activates the Atg1 complex, resulting in autophagy induction. Autophagy also requires a specific phosphatidylinositol-3-kinase (PI3K) complex, the transmembrane protein Atg9 and factors involved in its cycling from its cellular reservoirs to pre-autophagosomal structures, and two ubiquitin-like protein conjugation systems that ensure lipidation and membrane association of the ubiquitin-like protein Atg8. Atg8 is associated with the surface of autophagosomes similar to a coat protein. *Drosophila* is an excellent model for autophagy, as polyploid tissues like the fat body grow rapidly during the larval stages, and store nutrients to support metamorphosis later on. Therefore, low levels of autophagy are observed during the feeding period in larval stages L1 (approximately 24-48 hours after egg laying – AEL), L2 (48-72h AEL), and most of the L3 (72-120h AEL). Larvae leave the food at around 108 h AEL (the onset of the wandering period) to find a proper, dry place for pupariation, which is observed around 120h AEL. Robust induction of autophagy is seen in the larval fat body and midgut in wandering larvae, or in response to starvation during earlier stages [1].

A small-scale screen to identify kinases and phosphatases involved in autophagy

According to the specific aims of the funded OTKA project, we selected a set of genes encoding kinases, phosphatases, or their direct binding partners for a small-scale screen, testing altogether 247 RNAi lines against such genes, and 21 potential hypomorphic mutant lines. As expected from previous studies, we found that *Atg1, Vps34* and *S6 kinase* were required for autophagy [2,3]. Knockdown of several additional genes also affected autophagy, so we decided to carry out another large-scale approach, and select a few candidates to focus on based on these two datasets.

Microarray analysis of starvation-induced autophagy in Drosophila larvae

We compared the genome-wide expression changes of 86h AEL control larvae subjected to a 4-hour complete starvation to their well-fed siblings. We have also analyzed previously described autophagy-deficient null mutants for *Atg7* (on an isogenic genetic background with control larvae) and *Atg1*, with the purpose of finding gene networks that are specifically regulated in starved autophagy mutants [3,4]. Genes that showed an at least two-fold induction or repression with a p value ≤ 0.05 were considered regulated. We found that the expression of 2,819 versus 3,690 and

3,971 of 13,613 genes (represented by unique FBgn identifiers) was starvation-regulated in controls, *Atg7* and *Atg1* mutants, respectively.

Gene Ontology (GO) analysis revealed that genes belonging to 10 enriched GO categories were specifically induced in autophagy mutants. These groups include genes involved in the metabolism of purine nucleotides and in various catabolic processes. Numerous genes encoding members of the ubiquitin-dependent protein catabolic process were also found here. Only 3 enriched GO terms were specific for autophagy mutants among repressed genes. Genes in these categories encode proteins involved in DNA metabolism, amplification, and replication, such as DNA polymerases and essential DNA replication factors.

Most autophagy genes are transcriptionally induced during starvation

GO terms that include *Atg* genes were found enriched among the upregulated genes in all three genotypes. To focus more on autophagy, we looked at the expression of all *Atg* gene homologs. Many of these genes showed increased induction in autophagy mutants compared to controls.

Of the genes encoding members of the Atg1 kinase complex, *Atg1*, *FIP200*, and *Atg101* showed a moderate induction, while the levels of *Atg13* did not change. This is in agreement with recent findings that Atg13 is continuously degraded by the ubiquitin-proteasome system, and it is stabilized by the binding of Atg101 during autophagy induction [5]. Therefore, Atg13 protein levels appear to be mainly regulated on the post-translational level. It is important to note that *Atg101* showed the highest upregulation in this group (on average 3.5-fold in the 3 different genotypes), suggesting that transcriptional induction of *Atg101* potentially contributes to stabilizing Atg13.

Genes encoding members of the autophagy-specific PI3K complex (Vps34, Vps15, Atg6, Atg14) showed an even milder response: only *Vps34* encoding the lipid kinase itself was upregulated in all 3 genotypes. To our surprise, *EDTP* (Egg-derived tyrosine phosphatase) showed the highest average induction in this lipid signaling group: 2.9-fold. This gene encodes the *Drosophila* homolog of the lipid phosphatase Jumpy, a potent antagonist of the autophagy-promoting activity of Vps34 in mammals [6]. The upregulation of a potential inhibitor of autophagy may represent a negative feedback loop that acts to prevent hyperactivation. Elucidation of this hypothetical mechanism needs further studies.

Interestingly, genes encoding members of the pre-autophagosomal cycling complex showed the strongest average induction, between 3.2- and 6.2-fold. Atg9 is the only transmembrane protein among *Atg* gene products, and it has been shown to cycle between early autophagic structures and potential intracellular membrane sources to ensure membrane transport to forming autophagosomes [7]. Atg2 and Atg18 are required for the cycling of Atg9 in yeast.

Half of the genes (5/10) encoding members of the ubiquitin-like protein conjugation systems did not even show an upregulation. *Atg4a*, *Atg7*, *Atg8b* and *Atg16* were moderately induced, while the highest upregulation of all the *Atg* genes was detected for *Atg8a*, 8.4-fold on average.

Next we sought to confirm our findings obtained by microarray analysis of starved whole larvae. We followed the expression of selected genes by quantitative real-time PCRs, comparing fat bodies dissected from starved versus fed control larvae. By focusing on a tissue that produces the strongest autophagic response to starvation, these QT-PCR experiments further supported our most important findings: *Atg8a* showed by far the highest upregulation (169-fold), all members of the membrane transport group were strongly induced (10.6- to 35.2-fold), and transcription of *EDTP* increased 6.5-fold.

EDTP is a potential physiological regulator of autophagy

Based on the results of our RNAi/mutant screen, we selected two genes to focus on. Candidate *EDTP* hypomorphic mutant lines seemed to show elevated autophagy in preliminary experiments. Overexpression of *EDTP* (using either a genomic insertion/promotor line or from a transgene that we established) strongly suppressed autophagy, confirming the evolutionarily conserved role of this gene in autophagy regulation. Interestingly, we found that *EDTP* is expressed in the larval fat body, midgut, salivary gland, and oenocytes, which are the tissues that undergo autophagy upon starvation, whereas *EDTP* is not expressed in brain and imaginal discs where no induction of autophagy is seen. These data suggested that EDTP is a physiological regulator of autophagy, and probably contributes to shaping the extent of the starvation response. We then decided to generate a null mutant. We carried out P element remobilization experiments, the first three of which failed to produce a null mutant. Finally after 18 months, in the fourth attempt, we managed to identify a potential null mutant that removes the first exon of *EDTP*, including the translational start site. We are still characterizing this line to confirm that it is indeed a genetic null, and we will continue working on this project in my ongoing OTKA grant K83509.

Rack1 is required for a full autophagic response to starvation

Rack1 transcription increased 4.1-, 5.5-, and 4.1-fold in controls, *Atg7* and *Atg1* mutants, respectively. QT-PCR analysis also showed that *Rack1* was upregulated 21.6-fold in the fat body upon starvation.

Silencing of *Rack1* expression reduced autophagy based on the mCherry-Atg8a reporter in 58.6% of the cases compared to surrounding control cells. Next we used a combination of Lysotracker Red staining and Lamp-GFP, a reporter for lysosomes. Numerous small, presumably inactive Lamp-GFP positive lysosomes were seen in fat body cells of well-fed control larvae, which were not strongly acidic as revealed by lack of Lysotracker Red labeling. In contrast, large Lamp-GFP and Lysotracker-positive autolysosomes were formed during starvation. Silencing of *Rack1* reduced the number and size of Lysotracker and Lamp-GFP positive autolysosomes formed during starvation in 51.7% of the cases.

To expand on these RNAi studies, we have also analyzed an existing null mutant for *Rack1* [8]. Using the same mCherry-Atg8a and Lysotracker assays, we observed again a strong reduction of autophagy in *Rack1* mutants. As a final proof for the role of Rack1 in autophagy, we have generated transgenic animals expressing Rack1. Heat shock-mediated expression of Rack1 in mutant larvae completely rescued their autophagy phenotype.

Similar to the above findings, ultrastructural analysis showed that while numerous autophagosomes and large autolyosomes were observed in controls, *Rack1* mutant fat body cells contained very few autophagosomes and mostly smaller autolysosomes. Quantitative evaluation of electron micrographs showed that the average size of individual autolysosome cross-sections decreased 6-fold in Rack1 mutant fat body cells compared to controls. Overall, we found a 76% decrease in the total area of the autolysosomal compartment relative to total cytoplasmic area. Strikingly, very few autophagosomes were found in *Rack1* mutant cells, suggesting that Rack1 is required for efficient autophagosome formation.

Endogenous Rack1 localizes to early autophagic structures

Immunofluorescence analysis revealed that Rack1 is present in numerous cytoplasmic particles in most tissues. We next carried out colocalization experiments to assess the potential role of Rack1

in autophagy. Atg8a fusion proteins are the best characterized fluorescent reporters for autophagosome generation. We found that endogenous Rack1 partially colocalized with GFP-Atg8a. In contrast, not a single case of colocalization was observed between endogenous Rack1 and the lysosome reporter Lamp-GFP. We next used immunogold labeling of endogenous Rack1 to localize it on the ultrastructural level. We found that Rack1 was associated with pre-autophagosomal isolation membranes and autophagosomes. Again, no labeling of lysosomes was seen.

Rack1 is involved in glycogen synthesis

Immuno-electron microscopy analysis also identified that endogenous Rack1 localized in the periphery of, or immediately next to glycogen particles in fat body cells, in addition to being present in unidentified cytoplasmic clusters. Taking advantage of a monoclonal antibody specific for glycogen, we were also able to show that most Rack1-positive dots localized next to or in the periphery of glycogen particles in laser scanning confocal microscopy.

These localization data suggested an important role for Rack1 in glycogen homeostasis. The activity of glycogen synthase is a major determinant of glycogen levels. Phosphorylation of glycogen synthase by Shaggy, the *Drosophila* homolog of glycogen synthase kinase 3B (GSK-3B) promotes glycogen synthesis [9]. A physical interaction between the human homologs of Rack1 and GSK-3B was already reported [10]. In line with that, we found that endogenous Rack1 frequently colocalized with endogenous Shaggy in *Drosophila* fat body cells.

Glycogen stores are present in the larval *Drosophila* fat body, midgut, and muscles. The fat body is the major storage organ with large lipid, glycogen and protein reservoirs, much like our liver and white fat tissues. Plenty of glycogen particles were present in wild-type larvae in the fed state, and also after 24 hours of starvation in a 20% sucrose solution. All glycogen particles disappeared during a 24-hour complete starvation. We found that in contrast to control larvae, most cells had either no glycogen stores or contained decreased amounts in *Rack1* mutants. Similarly, RNAi silencing of *Rack1* decreased the size and number of glycogen particles in a cell-autonomous manner.

Evaluation of the results

Taken together, we have carried out a small-scale RNAi screen and whole-genome microarray experiments to identify genes involved in starvation-induced autophagy in *Drosophila*, and also compared gene expression changes between control, *Atg7* and *Atg1* mutant larvae. Based on a Gene Ontology analysis, genes involved in catabolic processes such as proteasomal degradation and amino acid catabolism were more significantly upregulated in starved autophagy mutants, which is likely a compensatory reaction. On the other hand, genes required for DNA replication were specifically downregulated. Mitotic tissues continue to grow and divide even in starved *Drosophila* larvae [11]. The repression of these genes required for DNA replication in autophagy mutants suggest that energy-consuming DNA replication processes are strongly inhibited in starved autophagy mutants, since presumably polyploid cells are not able to supply nutrients to support diploid cell divisions in the absence of autophagy.

We detected increased transcription of most *Atg* genes upon starvation, and an even higher upregulation was seen in fat bodies dissected from starved versus fed animals. The most highly induced gene was *Atg8a* in all cases, in agreement with previous studies that showed the importance of *Atg8* induction during autophagy in various models. It is important to note that *Atg8b* is mostly expressed in adult testis, and its very low level larval expression is restricted to the fat body. In line with this, mutation of *Atg8a* completely blocks starvation-induced autophagy in the fat body,

confirming that *Atg8b* expression is negligible for autophagy in this setting. Induction of *Atg8a* is likely necessary to make up for the degradation of half of its protein products involved in each autophagosomal cycle. Members of the Atg9 cycling complex showed the strongest average upregulation of the functional groups of Atg proteins. We speculate that the sudden induction of autophagy in polyploid larval *Drosophila* tissues requires a lot of membrane to sustain the high level of autophagosome generation, explaining the increased transcription of genes whose products are required for membrane transport.

Based on preliminary experiments, the lipid phosphatase *EDTP* is a physiological inhibitor of autophagy. We are currently carrying out further studies to elucidate the mechanism and significance of this potential in vivo regulatory pathway.

Rack1 (Receptor of activated protein kinase C 1) was induced in whole animals and in dissected fat bodies during starvation, and loss of *Rack1* strongly inhibited autophagy, with a striking reduction of autophagosome number and autolysosome size compared to starved controls. Autophagosomes generally have a short half-life of 5-10 minutes, which is the most likely explanation why basal levels of autophagy are very difficult to visualize in most tissues. Autophagosomes appear in high numbers during strong induction of autophagy. Since these structures were rarely seen in *Rack1* mutants, and Rack1 was at least transiently associated with pre-autophagosomal structures and autophagosomes, these results altogether strongly suggest that Rack1 is required for efficient autophagosome formation. We speculate that autophagosomes generated at this lower level are rapidly cleared by fusing with lysosomes to give rise to the small autolysosomes abundant in *Rack1* mutants. As we observed no defects in cell size/cell growth, DNA polyploidization or lipid droplet accumulation in Rack1 loss of function cells, the autophagy defect seems to be a highly specific phenotype and not just a consequence of general problems with altered cell physiology.

Rack1 is an evolutionarily conserved guanine nucleotide-binding scaffold protein with a WD40-repeat: 77% (243/315) of the amino acid residues are identical and 87% (275/315) are similar between *Drosophila* Rack1 and human GNB2L1. A proteomic study of Atg complexes found that GNB2L1 interacts with human homologs of Atg1, Atg4, Atg14, and Atg18 [12]. Although GNB2L1 was not classified as a high-confidence interacting partner, these data support our hypothesis that Rack1 may act as a scaffold, transiently binding multiple Atg proteins at pre-autophagosomal structures to achieve maximal activity. In line with that, it is interesting to note that the potential interacting partners of human Rack1 include a member from all four Atg protein complexes. Further biochemical studies such as co-immunoprecipitation experiments are necessary to verify this mechanism.

In addition, half of the Rack1-positive dots localized to glycogen particles, and loss of Rack1 prevented the proper formation of glycogen stores in larval fat body cells. The high degree of colocalization with GSK-3B strongly suggests that Rack1 is associated with a pool of GSK-3B that promotes glycogen synthesis. The known interacting partners of Rack1 include three subunits of AMPK (AMP-activated protein kinase): PRKAA1, PRKAA2, and PRKAB2 [12]. The β-subunit of AMPK has a glycogen-binding domain that targets a pool of this kinase to bind the surface of the glycogen particle. Activated AMPK turns on catabolic processes to generate ATP, and it also inhibits glycogen synthesis through direct phosphorylation of glycogen synthase [13]. We hypothesize that a signaling complex containing Rack1, GSK-3B, and AMPK assembles on glycogen particles to regulate the rate of synthesis through phosphorylating different amino acid residues of glycogen synthase, with AMPK being responsible for targeting this complex to glycogen. In this scenario, the activity of multiple kinases including AMPK and GSK-3B would determine the rate of glycogen synthesis and breakdown.

Rack1 was originally described as a cytoplasmic receptor for activated protein kinase C (PKC). The structure of Rack1 resembles that of the β-subunit of heterotrimeric G proteins. The individual WD40 repeats can simultaneously bind to different proteins, making Rack1 a candidate platform for integrating several signaling pathways. Rack1 was shown to physically interact with β-integrin, various kinases including PKC, AMPK, GSK-3B and Src, protein phosphatase 2A, focal adhesion components, and even ribosomes [14]. Indeed, Rack1 plays a role in a wide range of processes including cell adhesion and migration, cell survival, and translation. Recent findings showed that Rack1 promotes and is required for progression of several types of cancers, and its increased expression predicts poor clinical outcome for breast cancer patients [15,16,17,18].

Here we have shown that *Drosophila* Rack1 is also involved in the autophagic response to starvation, potentially acting again as a scaffold protein during the formation of autophagosomes. In addition, Rack1 is necessary for the proper generation of glycogen particles in the larval fat body, likely through recruiting Shaggy/GSK-3B to promote glycogen synthesis. Taken together, we have demonstrated that novel roles in autophagy and glycogen synthesis must be added to the already diverse list of functions for Rack1.

I submitted the manuscript describing our microarray and *Rack1* data to the journal Autophagy, and it is currently under review.

A PhD student Balázs Érdi joined this OTKA-funded project in January 2009. As Balázs carried out many experiments concerning Rack1, he is listed as first author on the manuscript. Balázs will be able to submit his PhD thesis upon acceptance of this work.

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