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A genetic model with specifically impaired autophagosome–lysosome fusion

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Yeast studies identified the evolutionarily conserved core *ATG* genes responsible for autophagosome formation. However, the SNARE-dependent machinery involved in autophagosome fusion with the vacuole in yeast is not conserved. We recently reported that the SNARE complex consisting of Syx17 (Syntaxin 17), ubisnap (SNAP-29) and Vamp7 is required for the fusion of autophagosomes with late endosomes and lysosomes in Drosophila. Syx17 mutant flies are viable but exhibit neuronal dysfunction, locomotion defects and premature death. These data point to the critical role of autophagosome clearance in organismal homeodynamics.

Keywords: autophagy, autophagosome, Drosophila, lysosome, neurodegeneration, SNARE, Syntaxin 17, ubisnap/SNAP-29, Vamp7

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The multistep process of autophagy starts with the formation of autophagosomes, accomplished by the action of evolutionarily conserved *ATG* genes that were discovered in yeast almost 20 years ago. These double-membrane vesicles deliver sequestered cytoplasmic material for lysosomal degradation. Despite much progress in dissecting the initial steps of autophagy during the past decade, the identity of a specific molecular machinery involved in fusion of autophagosomes with late endosomes and lysosomes remained unknown until very recently in metazoan cells, partly because the SNARE-dependent fusion process described in yeast is not conserved in animals.

We recently reported the results of our genetic screen for SNAREs involved in autophagy. After expressing transgenic RNAi constructs in genetic mosaics of Drosophila, we evaluated starvation-induced autophagy using an mCherry-Atg8a reporter, which labels both autophagosomes and autolysosomes. Knockdown of three genes (*Syx17*, *ubisnap* and *Vamp7*/*CG1599*) produces a similar, unique phenotype: small mCherry-positive puncta accumulate in the perinuclear region of cells, unlike the fewer, bigger structures observed in adjacent control cells. Silencing of these genes completely blocks starvation-induced punctate LysoTracker staining, a widely used marker of digesting autolysosomes in the larval fat body, a functional analog of our liver and adipose tissues. Further genetic tests of Syx17 and Vamp7 mutants confirmed our findings obtained from the RNAi studies. Using a combination of various biochemical and microscopy-based assays including ultrastructural analysis, we found large-scale accumulation of double-membrane autophagosomes and a block of autolysosome formation in fat body cells upon loss of any of these three genes.

These results perfectly match the well-established rules of SNARE action, according to which a complex required for vesicle fusion is assembled from three Q and one R SNARE domains (Q and R refer to the amino acids in a specific position in the complex). Syx17 is a Qa SNARE, ubisnap has both Qb and Qc domains, and Vamp7 belongs to the group of R SNAREs. According to our expectations, we found that these three proteins bind to each other when overexpressed in cultured cells. We were also able to confirm the interaction of endogenous Syx17 and ubisnap in co-immunoprecipitation experiments with the help of our novel antibodies for these proteins. Syx17

appears on autophagosomes after their formation, as the endogenous protein colocalizes with Atg8a-positive autophagosomes, but it does not localize to Atg5-positive phagophores or to Atg8a-positive dots in Atg2 mutants, in which stalled phagophores accumulate that already contain Atg8a. Immuno-electron microscopy suggests that Syx17 is present in the outer membrane of autophagosomes, supporting our model that completely formed autophagosomes undergo a maturation process and gain competence for fusion by recruiting Syx17.

Based on previously published localization data, we assume that ubisnap and Vamp7 are likely also involved in endocytosis (and perhaps other trafficking routes) in addition to autophagy. Indeed, mutation of Vamp7 or systemic knockdown of ubisnap results in pupal lethality, unlike mutation of Syx17. Syx17 was originally described as an ER SNARE, and we were also able to detect the Drosophila protein in the ER. This localization usually indicates a role in protein secretion, which we cannot rule out at this point. We hypothesize that Syx17 may act redundantly if it is indeed involved in secretory traffic, since previously characterized mutations in genes required for protein secretion cause lethality during the embryonic or early larval stages in Drosophila. In contrast, both of our Syx17 mutants (carrying open reading frame-disrupting transposon insertions) are viable, and survive for about 48–60 h after emergence from the pupal case. Previously published autophagy null mutants also die around or soon after eclosion (Atg1, Atg13), or live for more than a month (Atg7, Atg8a). These latter mutants also exhibit pathological aberrations, such as accumulation of cytosolic ubiquitinated protein aggregates in neurons, progressive neuronal death and loss of climbing ability. Similarly, 2-d old Syx17 mutant adults are hardly able to climb. No cytosolic aggregates are obvious in Syx17 mutant neurons, while about a fifth of their cytoplasm in the perikaryon is sequestered inside autophagosomes. Although a fraction of these cells are positive for apoptosis markers, blocking cell death genetically in mutants does not rescue their lethality or negative geotaxis defect. Based on these data, we suggest that neuronal dysfunction may be simply due to the loss of a large proportion of functional cytoplasm. Thus, these viable Syx17 mutants likely represent the first genetic model in which the fusion of autophagosomes with late endosomes and lysosomes is specifically blocked, potentially without severely affecting other trafficking pathways.

Previous Atg knockout studies in flies and mice already established partly similar pathological phenotypes. Based on our work, one may conclude that not only the formation of autophagosomes but also their effective clearance is essential. Significant current research efforts are focusing on the role that autophagy may play in various human diseases. While knockout studies (now including Syx17 besides several *Atg* genes) are extremely important for defining the role of these gene products, we think that the consequences of completely losing such critical genes may be too severe to be responsible for a major disease. To our knowledge, complete loss of a core *Atg* gene has not been documented in such human studies yet, but their polymorphisms or loss of genes encoding more peripheral players of autophagy may be associated with certain diseases. We hypothesize that partial loss of Syx17 function, for example, through altered post-translational modification such as phosphorylation, or defects in the currently unknown mechanism of its loading onto autophagosomes may contribute to human disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.