Title: **A yeast-two-hybrid screening identifies novel Atg8a interactors in *Drosophila***

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Abstract

Macroautophagy/autophagy-related protein Atg8/LC3 is important for autophagosome biogenesis and required for selective degradation of various substrates. In our recent study, we performed a yeast-two-hybrid screening to identify proteins that interact with Atg8a, the *Drosophila* homologue of Atg8/LC3. The screening identified several Atg8a-interacting proteins. These proteins include: i) proteins which have already been experimentally verified to bind Atg8a, such as Atg1, Dor, Ref(2)P and Kenny, ii) proteins that their mammalian homologues are known to interact with Atg8-family members, like Ank2, Atg4, and Nedd4 and iii) several novel Atg8a-interacting proteins, such as STK38/Trc and Tak1. We showed that Tak1, as well as its co-activator, Tab2, both interact with Atg8a and are substrates for selective autophagic clearance. We also identified that Sh3px1 interacts with Tab2 and is necessary for the effective regulation of the IMD pathway. Our findings suggest a mechanism for the regulatory interactions between Tak1/Tab2/Sh3px1 and Atg8a, which contribute to the fine-tuning of the IMD pathway.

Keywords: autophagy, Imd, Tak1, Tab2, Sh3px1, inflammation, LIR-motif

Macroautophagy is an evolutionary conserved process, whereby cells generate nutrients by degrading own intracellular constituents. A group of specific interactions between cargo and autophagy-related proteins are key in promoting the targeted uptake of certain autophagy substrates over others. The most well-characterized among such interactions is when substrates that contain specific sequences known as LC3-interacting regions (LIRs) bind to the LIR-docking site (LDS) of the autophagy-related (Atg) protein Atg8 (LC3/GABARAP families in mammals). Considerable interest and effort are invested into characterizing the Atg8 interactome, and by extension, studying the regulatory effect of autophagy on cellular processes such Atg8-interacting proteins participate in.

We conducted a high-throughput yeast-2-hybrid (Y2H) screen using *Drosophila* 3rd instar larva library to identify the Atg8a-interactome (1) . We identified 34 Atg8a-interacting proteins in total. These include proteins that have been experimentally verified to bind Atg8-family members (8 proteins), as well as novel interactors for which a direct association with Atg8 has not been previously reported (26 proteins). Prominent examples of known *Drosophila* Atg8a-interactors included the autophagy proteins Atg1, Atg4 and the adaptor protein refractory to sigma P [Ref(2)P]. In addition, the Y2H results showed that the fruit fly homologues of ANK2, DOR/TP53INP2 and NEDD4 (Ank2, Dor and Nedd4 respectively) among others, were also candidate Atg8a-binding proteins. ANK2, DOR/TP53INP2 and NEDD4 have been characterized in mammals to associate with LC3/GABARAP family members via LIR-LDS-dependent interactions. With regards to the group of novel Atg8a-interacting members, we first selected tricornered (Trc), as its mammalian homologue Ser/Thr kinase 38 (STK38) is known to associate with the autophagy-related protein BECLIN-1 (Atg6 in *Drosophila*) and both STK38 and Trc have been shown to impair autophagosome formation upon depletion. By employing glutathione S-transferase (GST) Pulldown assays as well confocal imaging of *Drosophila* tissue we observed that Trc associated with Atg8a, both *in vitro* and *in vivo*, but in a LIR-independent manner.

We next focused our attention to TGF-beta activating-kinase 1 (Tak1). Tak1 is the apical kinase in the immune-deficiency (IMD) pathway of the innate immune response and forms a complex with its co-activator, Tak1-binding protein 2 (Tab2), to convey its downstream signalling effects. Using a proteomics-based approach we also identified that Tab2 associates with the sorting nexin SH3-and-PX domain-containing 1 (Sh3px1) and corroborated their interaction further in GST pulldown assays. Sh3px1 is known to promote autophagy by participating in autophagosome formation and has already been shown to interact with Atg8a.

The interactions between Tak1, Tab2, Sh3px1 and Atg8a prompted us to investigate their relationship further. We showed that both Tak1 and Tab2 interact with Atg8a *in-vitro* using GST pulldown assays. The interaction between Tab2 and Atg8a does not seem to be LIR-LDS dependent, whereas for Tak1, the binding to Atg8a was found to be conveyed by the LIR motif bearing the sequence EGWVVI between amino-acid positions 667-672.

We found in addition that both Tab2 and Tak1 are also substrates for autophagic clearance.

To study the physiological effect the inactivation of the Tak1 LIR motif may have on immune response, we used *tak1* LIR mutant flies created by CRISPR to examine the mRNA levels of signature IMD-regulated anti-microbial peptide (AMP) genes, whose transcription follows the activation status of IMD pathway. In qPCR assays we observed that young as well as older adult Tak1 LIR mutant flies presented with persistently elevated levels of the AMP genes studied, compared to controls. This finding underscored that the LIR motif of Tak1 is necessary for the efficient regulation of the IMD pathway.

We concluded our work by integrating our findings into the proposal of a working model regarding how the interactions between Tak1, Tab2, Sh3px1 and Atg8a could help shape IMD signaling (Figure 1). Based on our observations, we posit that selective autophagy is upregulated following IMD activation and removes Tak1/Tab2 signaling complexes through the interaction of both Tak1 and Tab2 with Atg8a. In this context, Sh3px1 is an essential mediator that associates with Tab2 and Atg8a and further tethers the complex on autophagosomes. Our findings suggest that both the LIR motif of Tak1, as well as Sh3px1 are indispensable for the efficient removal of the Tak1 complex from the IMD cascade, as loss of either results in IMD overactivation. In our graphic representation of the model, we depict each member of the tripartite Tak1-Tab2-Sh3px1 complex to interact with a different Atg8a molecule. However, of the three, only Tak1 is so far verified to associate with Atg8a in a LIR-LDS-dependent manner and likely occupies the cognate site on Atg8a. We observed that Tab2 does not rely on the LDS site of Atg8a to bind the autophagy adaptor and neither a LIR-dependent interaction for Sh3px1-Atg8a is known to date. As such, it is possible that some or all members could bind to different sites on the same Atg8a moiety. Furthermore, Sh3px1 is reported to transiently associate with Atg8a during autophagosome formation and is not itself an obligatory substrate for autophagy. This, together with its association with Tab2 as we showed, and our observations that Tab2 does not accumulate as strongly compared to Tak1 upon autophagy inhibition, invite an interesting possibility that perhaps dissociation of the Tak1/Tab2 complex could also be taking place. Our findings that all three proteins are capable

in-vitro of binding directly to Atg8a, may perhaps allude to a failsafe mechanism, that increases the likelihood of Tak1 being targeted for degradation and removed from active complexes.
In summary, the combined interactions between Tak1, Tab2, Sh3px1 with Atg8a can promote the removal of the Tak1/Tab2 complex by autophagy and aid in termination of IMD signaling. Our study highlights the physiological importance of selective autophagy in the innate immune response of metazoans and demonstrates the plasticity of its participating regulators.

Reference

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Figure 1. A working model for regulation of the IMD pathway by selective autophagy via the Tak1/Tab2/Sh3px1-Atg8a interactions. Based on findings from the current study, we posit with this 2D schematic representation, that both components of the Tak1/Tab2 complex interact with Atg8a. Tak1 binds Atg8a via its functional LIR motif, whereas Tab2 does not necessarily rely on a LIR motif to interact with Atg8a. Sh3px1 binds Tab2 and Atg8a and likely further stabilizes the Tak1/Tab2 complex on the autophagosome membrane. Both Tak1's LIR motif and Sh3px1, seem to be equally required for the efficient sequestration of the complex by autophagosomes, as in either's absence the IMD pathway is overactivated based on our current observations.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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