Final Report

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Studying the role of selective autophagy in *Drosophila* health and neurodegeneration

Background

Drosophila being an excellent model of neurodegenerative diseases (Bhattacharjee et al. 2019) and both mine and the host lab's prior expertise being in autophagy (Bhattacharjee et al. 2018; Lörincz et al. 2017; Takáts et al. 2014), we set forward to understand what are the physiological consequences of loss of selective autophagy in context of a model organism? In mammals, selective autophagy (i.e. the autophagic degradation of selective cargo such as lipid droplets, mitochondria) is driven by diverse array of selective autophagy receptors (SAR) (Johansen and Lamark 2011). For example, in aggrephagy which recognizes and degrades protein aggregates, the identified SARs include p62/SQSTM1, NBR1, CALCOCO2/NDP52 etc. In Drosophila however, only a single such receptor for degradation of protein aggregates is known: the fly homologue of p62/SQSTM1, known as ref(2)P. These receptors on one hand recognize cargo by their ubiquitin-associated (UBA) domains, and connect them to the autophagosomal LC3 by the virtue of having so-called LC3-interaction region(s) (LIRs) (Birgisdottir, Lamark, and Johansen 2013). We posited that a specific knock-in in the single LIR of ref(2)P would disable its recognition by LC3, and by extension its autophagic degradation. Notably, protein aggregates that are degraded by aggrephagy and the ubiquitin-proteosome pathway are linked to proteinopathies, a subclass of neurodegenerative diseases (Bartlett et al. 2011).

Results

We were able to successfully create a two amino acid substitution (W454A, I457A) in the endogenous ref(2)P LIR W454-Q-L-I457 by CRISPR/Cas9. The flies named *ref(2)P*^{LIRm}, were found to be viable and fertile. These flies were then isogenized to the control, and the lifespan, locomotor abilities were tested. The mutants had a moderate lifespan defect (p=0.005), and no climbing defects (p>0.05). As expected, we found systemic accumulation of undegraded ref(2)P first from whole-fly western blots, and then by immunostaining specific tissues: the adult brain, the larval fat cells and the adult flight muscles. Interestingly, all p62 aggregates also contained polyubiquitin signal. Aggregate sizes, and their colocalization with ubiquitin increased with age. Since protein aggregates in brain have been implicated in proteinopathies, we next tested the tolerance of these mutants against paraquat (PQ) – a neurotoxin. Surprisingly, we found that *ref(2)P*^{LIRm} animals tolerated PQ more than their isogenic control counterparts. Since PQ is known to induce free radical formation, and p62 having a role in the Nrf2 antioxidant pathway, we wondered whether the mutant flies have a basally elevated

antioxidant response which provides immunity against reactive oxygen species. Notably, it has been described earlier that ref(2)P interacts with Keap1, which is a negative regulator of the fly Nrf2 homologue and transcription factor CncC (Jain et al., J Biol Chem 2015). We tested these mutants for their expression of several genes in the Nrf2 pathway, namely *CncC*, *GstE1*, *Gclc*, *cat*, *Keap1* and *ref(2)P* itself (Sykiotis and Bohmann 2008). Expression of *Keap1*, *GstE1*, *CncC* and *cat* were found upregulated in the *ref(2)P^{LIRm}* animals. We also verified with immunostaining the presence of endogenous Keap1 accumulates colocalizing with ref(2)P- and polyUb positive aggregates in the 30 day adult brain, and in the larval fat cells. Keap1-p62 aggregates in mammals is known to the removal of the inhibitory effect on the Keap1-Nrf2 complex, and thus facilitating the nuclear localization of Nrf2. Here, similarly we found that GFP-CncC localized in the nucleus significantly more in *ref(2)P^{LIRm}* than the control animals. Notably, silencing of CncC drastically reduced the PQ tolerance in *ref(2)P^{LIRm}* animals.

Next, we wondered whether the LIR mutant p62-polyUb co-aggregates can be disrupted, and whether soluble ref(2)P^{LIRm} can still recognize cargo during induced ubiquitin autophagy. To this end, we overexpressed GFP-tagged 4xUb, which was previously shown to disrupt p62 filaments in vitro. We discovered that GFP-4xUb expression drastically reduced the size of ref(2)P aggregates, as well as their colocalization with ubiquitin. To see if this newly soluble Ref(2)P can recognize cargo, we induced mitochondrial damage by CCCP, which decorates mitochondrial outer membrane with ubiquitin chains and thus serves as a signal for selective autophagy. While *ref(2)P^{LIRm}* cells did not colocalize with ATP5a (a mitochondrial marker), *ref(2)P^{LIRm}, tub>GFP-4xUb* cells showed distinct colocalization, indicating the soluble nature and cargo capture abilities of LIR mutant ref(2)P when ubiquitin is overexpressed. This work was published in Autophagy, 2022 (Bhattacharjee et al. 2022).

The focus on characterizing BNIP3 as a potential fly mitophagy receptor in this project was significantly hampered by a prior publication by Schmid et al. (Nat Aging 2022; 2(6):494-507) which reported similar findings. Thus, we decided to instead focus on the lysosome, the cells' recycling center. The aim was to understand how lysosomes, only acidic and proteolytically active during fusions (with an autophagosome, for example) are activated. My main goal, as outlined in the previous progress report, 2023, was to characterize the lysosomal responses to calcium release by the mucolipidosis type-IV associated lysosomal cation channel TRPML1. It was previously established that TRPML1 promotes autophagy by biogenesis of autophagosomes, and by fusion of autophagosome-lysosome which is regulated by migration of lysosomes towards (-) end directed transport (Rosato et al. 2019; Li et al. 2016). Interestingly, current research on TRPML hasn't

investigated its immediate early impact on autophagy. This is likely because TRPML agonists trigger very rapid calcium release (under 5 seconds), which might be too fast to be captured in standard autophagy assays. We developed a lysosomal Ca^{2+} sensor, GCaMP6m-TRPML1 to measure lysosomal Ca^{2+} release, and using this showed that lysosomes become acidic (pH 4.5-5.0) within 10 minutes of TRPML1 opening. This is also accompanied by autophagosome-lysosome fusion, although the existing lysosomes do not show any change in their overall distribution. This indicated that local fusion events are upregulated by lysosomal Ca^{2+} release. In support of this, we found that small, previously reported lysosome-adjacent carrier vesicles (Pols et al. 2013) containing the SNARE VAMP7 (which is a lysosomal SNARE protein required for fusion with autophagosomes and endosomes) were fusing with lysosomes and enriching VAMP7 on its membrane, thus providing a clear mechanism as to why we see increased local autophagosome-lysosome fusion. To understand whether fusion is necessary for acidification of the lysosome after TRPML1 activation, we knocked down VAMP7 as well as PI4KIIA, a lipid kinase that synthesizes the fusion-critical lipid PI(4)P on autophagosomes. Both shVAMP7 and shPI4KIIA lysosomes were calcium-depleted and thus could not respond to agonist stimulation of TRPML1, and at least shVAMP7 lysosomes had lower cathepsin activity.

The only known cellular agonist of TRPML1, the phosopholipid $PI(3,5)P_2$ is synthesized by the lipid kinase PIKfyve (Kim et al. 2014). PIKfyve-inhibited cells show abnormally large, fusion-deficient lysosomes resulting from a lack of lysosomal fission (Bissig et al. 2017; Choy et al. 2018). We wanted to know if agonist activation of TRPML1 would overcome the inhibitory effects of PIKfyve inhibition. Surprisingly, PIKfyve-inhibited cells showed normal GCaMP-TRPML1 response, indicating luminal Ca^{2+} stores remain intact. PIKfyve-inhibited lysosomes also showed very abnormal localization of lysosomal SNAREs VAMP7 and syntaxin 7, particularly in the lysosome lumen indicating a defect in post-fusion trafficking of lysosomal SNAREs. Agonist activation of TRPML1 in these cells restored normal SNARE localization on the lysosome membrane and restored autophagosome-lysosome fusion. A manuscript has been prepared for this work and will be submitted to a journal soon.

Conclusion

Together, I could achieve the first part of my proposed objective and show that protein aggregation in the brain can have surprising resiliency in a model organism, and corroborates other studies which indicate that soluble aggregates are severely more harmful than phase separated condensates like p62-polyUb aggregates (Ghag et al. 2018; Lamark and Johansen 2012).

I showed resiliency in changing my topic for the second half of this project, and instead focused on the lysosome to show how lysosomes are proteolytically activated during autophagy. Both part I (p62) and part II (TRPML1) have disease associations: p62 mutations are causative for Paget's Disease of bone, and TRPML1 mutation leads to rare neurodegenerative disorder known as Mucolipidosis type IV, characterized by undegraded lysosomal bodies (LaPlante et al. 2006; Bartlett et al. 2011). As a whole, the project aimed to advance our understanding of autophagic degradation mechanisms of neurodegenerative cargo, and hopes that these findings would be useful to that end.

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1. **Bhattacharjee, A**, Ürmösi, A, Jipa, A, Kovács, L, Deák, P, Szabó, Á, Juhász, G, 2022. "Loss of Ubiquitinated Protein Autophagy Is Compensated by Persistent Cnc/NFE2L2/Nrf2 Antioxidant Responses." *Autophagy* 18 (10):2385-2396. https://doi.org/10.1080/15548627.2022.2037852.
2. Laczkó-Dobos, H., **Bhattacharjee, A.**, Maddali, A. K., Kincses, A., Abuammar, H., Sebők-Nagy, K., Páli, T., Dér, A., Hegedűs, T., Csordás, G., & Juhász, G. (2024). PtdIns4p is required for the autophagosomal recruitment of STX17 (syntaxin 17) to promote lysosomal fusion. *Autophagy*. https://doi.org/10.1080/15548627.2024.2322493
3. Abuammar, H., **Bhattacharjee, A**., Simon-Vecsei, Z., Blastyák, A., Csordás, G., Páli, T., & Juhász, G. (2021). Ion channels and pumps in autophagy: A reciprocal relationship. *Cells* (Vol. 10, Issue 12). https://doi.org/10.3390/cells10123537

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2. EMBO Workshop on Integrating the Molecular, Mechanistic and Physiological Diversity of Autophagy, June 27 – July 01, 2022, Eger, Hungary. Short talk and poster.
3. 4th Nordic Autophagy Society Conference, November 10-12, 2021. Tromsø, Norway. Short talk.

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