DEPARTMENT OF CELL BIOLOGY

Layla Nassar

How I Wrote My Prospectus

After joining my lab, I met weekly with my advisor to solidify the ideas for my thesis project, ask questions, and discuss my preliminary data. He sent me important papers and review articles throughout this time so that I could better survey the current literature. This process helped to refine my thinking about my thesis work. Four months before my prospectus deadline, my advisor suggested that I put together my specific aims page to form a skeleton for the prospectus. I submitted my first draft of the specific aims page that week and worked on this page with him for over one month, going through several rounds of revision that defined the structure and scope of the project. I then wrote my introduction and each specific aim, sending drafts of each section for review by my advisor once the section was completed. After finishing my introduction and conclusion, I sent a full draft to my advisor and a few colleagues for final suggestions.

Advice for Prospectus Writers

Having another set of eyes on the prospectus throughout the writing process was critical for the progression of this document. Sharing early work can be stressful but was worth it for me; reviews of early drafts allowed me to make changes easily and think about my project in a clearer way. If a student is not able to work extensively on writing with their thesis advisor, I highly recommend scheduling consultations with the Graduate Writing Lab or finding a trusted colleague to provide feedback throughout the writing process. Make sure to start months in advance as this is not a document that will come together seamlessly last minute. An early start will also ensure a more comfortable and enjoyable writing process.

Molecular mechanisms that coordinate retrograde movement and degradative function of axonal lysosomes

Specific Aims

Lysosomes perform degradative functions that are critical for cell survival. Neurons are especially dependent on maintenance by lysosomes due to the axon's extreme length and the need to turnover damaged proteins and organelles that are far from the major site of protein synthesis in the cell body. Neurons therefore require efficient mechanisms to regulate the turnover of axonal macromolecules and avoid aberrant protein accumulation. I am interested in how axonal lysosome movement and maturation equips neurons to overcome the challenges imposed by their extreme size and polarity.

Lysosomes are moved within axons by motor proteins on microtubules running parallel along the axon, with microtubule minus ends pointing towards the cell body. Lysosome transport towards the neuronal cell body therefore occurs via molecular factors that tether the lysosome to dynein (the major minus end directed motor). **I hypothesize that axonal lysosomes rely on interdependent molecular pathways controlling both lysosomal movement and maturation via acidification to ensure effective degradative function.** Although several candidate mechanisms controlling lysosomal retrograde transport have been reported, the mechanisms that coordinate retrograde movement and lysosomal function remain poorly understood. I propose three parallel approaches for investigating these problems.

Specific Aim 1: Determine cellular and physiological consequences of JIP3 and JIP4 depletion

First, I will focus on the relationship of axonal lysosomes to Jnk-Interacting Protein 3 (JIP3) in conjunction with its ubiquitously expressed homolog Jnk-Interacting Protein 4 (JIP4). JIP3 has been implicated in human intellectual disability and neurodevelopmental disease, and molecular studies of JIP3 show that it regulates the abundance and maturation of axonal lysosomes. (Iwasawa et al. 2019, Platzer et al. 2019, Gowrishankar et al. 2017) Because of this, as well as the range of relevant JIP3 putative binding sites and its function as a molecular scaffold, JIP3 is a strong candidate for a factor that tethers lysosomes to dynein. *I hypothesize that the cumulative action of JIP3 and JIP4 will lead JIP3 and JIP4 KO neurons to have strong defects in the movement and maturation of axonal lysosomes.* To test lysosomal defects, I will directly measure changes in localization, speed and direction of axonal lysosomes' movement, acidification, and lysosomal protein levels between wildtype and JIP3+JIP4 DKO neurons. I will also test whether these defects can be rescued by reintroducing or overexpressing JIP3, JIP4, and both JIP3 and JIP4. Together, this will establish the ways in which JIP3 and JIP4 contribute to healthy function of neuronal lysosomes.

Specific Aim 2: Determine the molecular role of JIP3 in retrograde axonal transport

Second, I will study the function of JIP3 in retrograde transport of axonal lysosomes. *I hypothesize that JIP3 regulates axonal lysosomes through binding partners that connect the lysosomal maturation state to dynein.* I will identify protein-protein interactions of JIP3 through mass spectrometry in order to indicate whether lysosomal and retrograde transport proteins are molecular interactors of JIP3. I will then image endogenous JIP3 and putative molecular interactors using fluorescent tags to understand their relative levels and localization dynamics. Finally, I will characterize the roles of JIP3-interacting proteins by imaging lysosomal localization and movement in JIP3-interacting proteins' absences and in JIP3 KO neurons in order to indicate whether they work in conjunction with JIP3 to cause retrograde transport defects in lysosomes. These efforts will define the molecular role for JIP3 during lysosomal axonal transport.

Specific Aim 3: Elucidate the mechanisms that coordinate lysosomal acidification and transport

I will also explore the mechanisms that coordinate retrograde axonal transport of lysosomes with their acidification. Lysosomes moving towards the cell body become progressively acidified by as much as 100 times. (Hollenbeck 1993). Lysosomal acidification also activates degradative enzymes involved in turnover of lysosomal substrates, critical for the function of axonal lysosomes when returning to the soma. Although there is a clear correlation between lysosome movement and acidification, little is known about mechanisms that might coordinate these processes. *I hypothesize that regulators of lysosomal transport also regulate acidification via the lysosomal V-ATPase proton pump.* The V-ATPase reversibly associates and disassociates from the lysosomal membrane to mediate its acidification, and evidence suggests lysosomal V-ATPase stabilized by the Rab-interacting lysosomal protein (RILP), which is implicated in lysosomal retrograde transport via interactions with Rab7 and dynein. (De Luca et al 2014) I will therefore study the translocation dynamics of the V-ATPase in neurons by measuring changes in V-ATPase localization and axonal lysosome retrograde transport when the V-ATPase is inhibited, which will establish whether the V-ATPase is impacted by changes in lysosomal transport. I will also assess putative V-ATPase protein-protein interactions in order to probe the relationship between acidification and lysosomal trafficking. Together, this will elucidate the regulation and functional role of retrograde transport in lysosomal maturation.

Background

Neurons undergo an extraordinary level of cellular stress and require specialized mechanisms to maintain cellular integrity. This is especially true along the extreme length of the neuronal axon. The axon faces a particular challenge in moving and breaking down its aberrant proteins because cellular waste must be delivered to the cell body in order to be recycled into new proteins. Axonal clearance and recycling of nutrients are therefore accomplished through the movement and function of the axonal lysosome.

In axons, lysosomes travel along uniformly polarized microtubule networks, where the plus ends of microtubules point towards the distal axon and the minus ends of microtubules point towards the cell body. (Figure 1, Ferguson 2018) Lysosomes therefore rely on dynein, the major minus end directed motor, to move towards the cell body. However, in neurons, it is not clear which molecular mechanisms attach dynein to a lysosome and support lysosomal movement to the cell body. I am interested in elucidating the mechanisms that dynein-activating adaptors use to help define dynein cargo and regulate retrograde transport.

Several candidate proteins may play a role in the retrograde axonal transport of lysosomes. The most recognized example is the Rab-Interacting Lysosomal Protein (RILP), which has been shown to act as an adaptor between the lysosomal small GTPase Rab7 and dynein. (Cantalupo et al. 2001) However, it is unlikely that RILP is the only downstream effector of Rab7 responsible for retrograde transport; the Schiavo group reports that dominant-negative RILP mutants do not alter the dynamics of retrograde carriers in spinal cord motor neurons. (Villaroel-Campos et al. 2018) Another putative adaptor between retrograde transport and lysosomal machinery is the JNK-interacting protein (JIP3), which causes severe developmental delay in JIP3 haploinsufficient patients. (Platzer et al. 2019; Iwasawa et al. 2019) JIP3 is more highly expressed in neurons than in many other cell types, suggesting a specific role for JIP3 in mediating neuronal function. (Kelkar et al. 2005) In addition, JIP3 has also been implicated in accumulation of axonal lysosomes in *C. elegans*, zebrafish, and mice. (Gowrishankar et al. 2017, Edwards et al. 2013, Drerup et al. 2013) While these phenotypes are consistent with defects in retrograde transport, is not yet clear which mechanisms guide JIP3 function and lead to stalling of axonal lysosomes.

The lysosome's retrograde movement towards the neuronal cell body has been posited to happen in conjunction with the lysosome maturation process. (Figure 1, Ferguson 2018, Maday et al. 2012) In order to mature into a lysosome and recycle its contents, the lysosome must first acidify. Acidification activates the lysosomes' distinctive hydrolytic degradative enzymes, allowing the lysosome to digest proteins into macromolecules, including amino acids. However, the mechanisms and functional implications of this putative coupling are not clear. I propose to test whether retrograde axonal transport and acidification in lysosomes are coordinated and, if so, which molecular mechanisms functionally tie the two processes. These studies will examine the lysosome in both its movement and acidification in order to understand the molecular mechanisms that facilitate movement and drive acidification. I propose studying this from the perspective of a putative dynein-activating adaptor and the putative source of acidification within the lysosome.

Significance

JIP3 haploinsufficiency causes severe neurodevelopmental in both motor and intellectual disability as well as neuroanatomical defects in the corpus callosum and anterior commissure. (Platzer et al. 2019; Iwasawa et al. 2019) JIP3 haploinsufficiency exacerbates axonal swelling phenotypes in Alzheimer's disease mouse models, where axons that already swell and fill with lysosome-like organelles at amyloid plaque sites become severely worse. (Gowrishankar et al. 2017) Both of these contexts highlight a necessity of JIP3 for proper neuronal function, but the neuronal cell biology of JIP3 is not yet fully understood.

In addition, one outstanding question in neuronal cell biology is how neuronal sub-compartmentalization is specified. Lysosomes and late endosomes are heterogeneous by nature and highly dynamic. Transport and distribution analyses using multiple fluorescent markers of lysosomal maturity may reveal biased distributions in subsets of lysosomes and uncover their specialized functional roles. I propose completing these studies using iPSC-derived neurons (iNeurons), which provide the genetic malleability and quick growth necessary for the proposed experiments. (Fernandopulle et al. 2017, Boecker et al. 2019)

Specific Aim 1: Determine cellular and physiological consequences of JIP3 and JIP4 depletion

JIP3 haploinsufficient patients suffer neurodevelopmental defects including motor disability, intellectual delay, and striking neuroanatomical defects in the CNS. (Platzer et al. 2019; Iwasawa et al. 2019) In cultured neurons, lysosomes accumulate within the axon of JIP3 KO neurons, forming a traffic jam of lysosomes that accumulate in axonal swellings. (Figure 2a-b, Gowrishankar et al. 2017) However, the specific mechanism of action for JIP3 is uncertain, and it remains unknown how the actions of JIP3 are coordinated with other proteins in the same or parallel pathways. I will examine JIP3 along with its homolog JNK-Interacting Protein 4 (JIP4), a scaffold with a similar structure to JIP3. (Isabet et al. 2009) However, unlike JIP3, which is found primarily in neurons, JIP4 is ubiquitously expressed in human cells. Preliminary data suggest that defects in JIP3 and JIP4 are cumulative in neurons. (Figure 2c-f). Based on the preliminary finding that JIP3+JIP4 double KO (DKO) neurons have a more severe lysosomal accumulation phenotype than JIP3 KO neurons alone, it is possible that JIP3 and JIP4 are playing distinct roles in axonal transport. However, it is also possible that the overall combined levels of JIP3 and JIP4 are calibrated for proper axonal transport. To shed new light on the roles of JIP3 and JIP4 in axonal transport and maturation of lysosomes, I seek to define the specific JIP3+JIP4 DKO defects in neurons, re-express and overexpress JIP3 and JIP4 in JIP3+JIP4 DKO neurons, and measure defects in JIP3 haploinsufficient neurons.

1.1 Define JIP3+JIP4 DKO neuron phenotypes

Rationale: Because preliminary imaging suggests that JIP3+JIP4 DKO neurons' lysosomes aggregate more than lysosomes in JIP3 KO neurons, I hypothesize that JIP3 and JIP4 both regulate the coordination of lysosomal acidification and transport. This is supported by the Ferguson lab's previous studies of JIP3 in neurons (Gowrishankar et al. 2017) as well as unpublished observations from JIP4 KO HeLa cells. However, the specific cumulative defects of both mutations in neurons have not yet been described. To test my hypothesis, I will track multiple lysosomal markers in JIP3+JIP4 DKO neurons in culture during the lysosome transport and maturation process.

Experimental Design: To test defects in lysosomal maturation and movement, I will image both wildtype (WT) and JIP3+JIP4 DKO neurons and label each for markers of lysosomal acidification and movement. To visualize lysosomal acidification, I will use an Oregon Green 488 covalently attached to a Texas Red-labeled dextran in order to label lysosomal pH in a quantitative and ratiometric manner. Oregon Green is pH-sensitive when excited at 490nm, (Haggie and Verkman 2009) and dextran is simultaneously shuttled along the endocytic pathway and accumulates in lysosomes, though lysosomal enzymes do not degrade it. Together, these markers will provide a ratiometric comparison to measure lysosomal pH. I will also visualize lysosome movement by expressing tagged forms of specific lysosomal and late endosomal proteins such as LAMP1-GFP and mCherry-Rab7 through transient transfection. I will also measure the relative level of lysosomal acidification in comparison to lysosomal protein levels. To visualize these dynamic processes in neurons, I will perform live cell time-lapse imaging in order to define dynamic properties such as retrograde movement, anterograde movement, and non-motile populations of lysosomes based on the presence of these markers as well as a change in the colocalization of these markers between the WT and DKO conditions.

Expected Outcomes: I expect that lysosomes will move more slowly in JIP3+JIP4 DKO neurons and that JIP3+JIP4 DKO lysosomes will accumulate more readily than when compared to JIP3 KO neurons or WT neurons, indicated by the movement of Oregon Green-488 and the lysosomal proteins.

I also expect that specific subtypes of lysosome or lysosome-like organelles will emerge based on their level of acidification, the presence of known lysosomal proteins, and their movement properties such as velocity, likelihood to stall, and direction of movement. Together, these would indicate defects in lysosomal function in due to the combined action of JIP3 and JIP4.
Limitations and Alternative Approaches: Although this aim is conceptually straightforward, it is critical to model endogenous expression levels, as protein overexpression could cause artifacts in protein dynamics. This aim relies heavily on light microscopy to visualize lysosomal dynamics in the lysosome, and visualizing endogenous JIP3 and JIP4 can be technically challenging because it is found in relatively low abundance in the cell. To avoid this, I will use bright fluorophores such as a HaloTag or mClover3. If newly described endogenous tagging tools do not work to tag endogenous JIP3 and JIP4, I can also use a CHoP-In CRISPR knock-in tool in order to produce endogenous tags. (Manna et al. 2019) I will also quantify organelles based on established protocols in the lab; to standardize measurements, I will quantitate puncta using Cell Profiler software. (Carpenter et al. 2006) While overall lysosomal acidification defects would be interesting to confirm, as this may imply that lysosomes are less able to degrade their contents, lower overall lysosome acidification will need to be accounted for when quantifying numbers of lysosomes through imaging. I will therefore measure numbers of lysosomes through a combination of distinct puncta with lysosomal protein expression (i.e. LAMP1, Rab5, and Rab7 levels) as well as through markers indicating lysosomal acidification, such as through the lysosomal dye cresyl violet. (Ostrowski et al. 2016)

1.2 Test JIP3- versus JIP4-dependent regulation of lysosome transport
Rationale: JIP3 and JIP4 are structurally similar, but unpublished results indicate that JIP3 cannot rescue JIP4 KO cells. I am interested in whether JIP3 and JIP4 have discrete roles in the axon and whether they work independently of one another. I will test this by determining baseline levels of JIP3 and JIP4 using endogenous tags. I will transfect JIP3 or JIP4 alone into JIP3+JIP4 DKO neurons and assess lysosomal movement and function to determine the specific pathologies of each. I will further increase overexpression of JIP3 or JIP4 alone in JIP3+JIP4 DKO neurons to see if either protein can make up for the absence of the other.
Experimental Design: To define the baseline levels of JIP3 and JIP4, I will image fluorescently tagged endogenous JIP3 and JIP4 in WT neurons. I will use newly developed tools in iPSC-derived neuron in conjunction with a bright fluorophore such as a Halo-tag or mClover3 to better visualize endogenous JIP3 localization dynamics. (Boecker et al. 2019). Based on this level of fluorescence, I will transfect JIP3 or JIP4 into JIP3+JIP4 DKO neurons at near-endogenous levels; this will help me to understand dynamics of each protein when reintroduced. Transfecting both JIP3 and JIP4 back into JIP3+JIP4 DKO neurons will serve as an important control. I will also increase gene dosages of JIP3 and JIP4 to severely overexpress the individual proteins in JIP3+JIP4 DKO neurons. This will help me to understand if the introduction of either JIP3 or JIP4 alone can provide a full rescue of JIP3+JIP4 DKO pathology. To complete this, I will increase the amount of DNA added to the neurons in a dose-dependent manner. I will then test lysosomal responses when exposed to the varying concentrations of either the JIP3 or JIP4 plasmid. In these neurons, I will directly measure lysosomal movement and acidification using the parameters described in Aim 1.1. In cells with JIP3 or JIP4 overexpression, I will focus on the localization of lysosomes as well as the velocity and direction of lysosomal movement through axons.
Expected Outcomes: I expect that, based on their extremely similar structures, JIP3 and JIP4 may be able to substitute for each other in the neuron and that lysosomal transport depends on the overall level of combined JIP3 and JIP4. (Isabet et al. 2009) However, because JIP3 binds JIP4, it is also possible that JIP3 cannot make up for JIP4 and vice versa; this may indicate that the two work together in neurons to perform their molecular functions. I expect that reintroduction of JIP3 to JIP3+JIP4 DKO neurons will be insufficient to fully rescue lysosome acidification and movement defects in JIP3+JIP4 DKO neurons when added at a similar level to the original endogenous level found in WT neurons. However, by adding the genes in a dose-dependent manner, JIP3 or JIP4 may also be able to rescue lysosomal movement defects in JIP3+JIP4 DKO neurons. If this is the case,

severely overexpressed JIP3 or JIP4 may cause increased retrograde movement overall, indicated by increased numbers of lysosomes in the soma and decreased numbers of lysosomes in the axon. However, despite their 57% identical sequence similarity and conserved domain organization, JIP3 and JIP4 may not be able to rescue each other and therefore may not function redundantly. If this is the case, these efforts will elucidate whether there are specific, discrete functions of JIP3 and JIP4 in retrograde transport.

Limitations and Alternative Approaches: It is possible that the levels of JIP3 and JIP4 are not rate limiting in their respective pathways; this may cause abnormal localization of lysosomes throughout the neuron. WT neurons will therefore serve as important controls in these studies. It is also possible that over-expression sequesters binding partners of lower abundances into non-productive complexes. If transfected neurons are severely abnormal, I will decrease the amount of plasmid added to cells. This analysis depends on JIP3 and JIP4 causing significant changes in lysosomal transport through the axon. It is possible that overexpression by increasing gene dosage will not cause robust changes in protein expression in neurons. If this is the case, I will isolate specific transfected clones and search for a clonal population enriched with more JIP3 or JIP4. This will allow me to systematically determine the response of neurons to greater or less production of JIP3 and JIP4.

1.3 Measure lysosomal transport in JIP3 haploinsufficient neurons

Rationale: I also plan to measure the relationship between JIP3 and lysosomal localization in neurons with JIP3 haploinsufficiency, which has been implicated in neurodevelopmental defects. (Platzer et al. 2019; Iwasawa et al. 2019) JIP3 haploinsufficiency has also been shown to exacerbate defects in 5xFAD mice, a model for Alzheimer's disease. (Gowrishankar et al. 2017) Based on this, I hypothesize that reduced levels of JIP3 through haploinsufficiency will have both cellular and physiological consequences. To test cellular function of JIP3 haploinsufficiency, I will use JIP3 haploinsufficient iPSC-derived neurons to study neuronal defects due to JIP3. I will replicate these results by measuring changes in the brain anatomy of JIP3 haploinsufficient mice in areas previously implicated in developmental delays, such as the corpus callosum.

Experimental Design: To study JIP3 haploinsufficient lysosomal defects, I will compare level of acidification and lysosomal movement in JIP3 haploinsufficient iPSCs compared to JIP3 KO and WT iPSCs. I will study these defects based on markers described in Aim 1.1. I will also perform brain dissections on JIP3 haploinsufficient mice, fix brains, cut coronal sections through the relevant brain regions (especially within the axonal tracts), stain sections, and then examine these sections under the microscope. I will focus on brain regions where neuroanatomical defects have been found in JIP3 haploinsufficient patients, including in the corpus callosum tissue and the anterior commissure. I will also search for specific neuroanatomical defects seen in the patients such as cerebral atrophy. (Platzer et al. 2019; Iwasawa et al. 2019) I will compare these results to the brain anatomy of WT mice as a control.

Expected Outcomes: I expect that JIP3 haploinsufficient iPSCs will have a similar, though less severe, phenotype to JIP3 KO iPSCs. I expect that JIP3 haploinsufficient iPSCs will not have as many swellings and that individual swellings will be smaller. Lysosomes may move more in the retrograde direction as well as more quickly, as partial function of JIP3 would be restored. I also expect to see reduced corpus callosum size and cerebral atrophy in JIP3 haploinsufficient mouse brains, which indicate that the human pathology is mirrored in JIP3 haploinsufficient mice. This would render JIP3 haploinsufficient mice a valuable tool in studying the clinical pathology of JIP3 haploinsufficient patients.

Limitations and Alternative Approaches: It's possible that JIP3 haploinsufficient neurons do not have axonal swellings or experience axonal swellings at a low frequency. If this is the case, I will focus on measurements overall movement of lysosomes and comparative acidification levels between lysosomes.

Specific Aim 2: Determine the molecular role of JIP3 in retrograde transport

Both JIP3 and JIP4 contain binding sites for several putative molecular partners including microtubule motors, signaling proteins, and small GTPases. Although JIP3 and JIP4 are structurally similar, it is unclear why neurons require JIP3 for axonal transport of lysosomes, particularly because many cells do not express JIP3 at all. I seek to understand the molecular role JIP3 plays, along with its molecular interactors, in regulating retrograde axonal transport of lysosomes. Currently, the best recognized adaptor responsible for recruitment of lysosomes to retrograde transport machinery is Rab-Interacting Lysosomal Protein (RILP). (Cantolupo et al. 2001) RILP is a Rab7 effector protein that binds to dynein to control dynein-mediated transport of lysosomes on microtubules. However, while overexpression of RILP causes increased retrograde transport of lysosomes, there is little evidence that RILP is the only, or even primary, adaptor responsible for lysosomal retrograde transport. (Surana et al. 2019, Villaroel-Campos et al. 2018) JIP3 shares regions of sequence homology with RILP, suggesting that it may bind many of the same targets that RILP does. (Figure 3, Matsui et al. 2012) JIP3 KO neurons also show defects consistent with those found in impaired retrograde transport. (Gowrishankar et al. 2017) Together, this suggests that JIP3 may function in a similar way to RILP in order to control retrograde transport.

2.1 Find molecular interactors of JIP3

Rationale: While molecular interactors of JIP3 have been proposed based on the structure of JIP3, the relevance of their binding with JIP3 in a cellular system is not clear. I propose a systematic analysis of JIP3 binding partners using mass spectrometry. I hypothesize that molecular motors and lysosomal membrane proteins bind with JIP3 to tether lysosomes to dynein, promoting retrograde transport. To test this, I will perform a mass spectrometry screen based on binding partners of JIP3.

Experimental Design: I will immunoprecipitate JIP3 using an HA tag fused to the N-terminus (Vilela et al. 2019) from WT neurons and perform a mass spectrometry screen to analyze interacting partners of JIP3. I will then perform a co-immunoprecipitation of JIP3 with top candidates found via mass spectrometry to confirm their binding. While I will approach the screen in an unbiased manner, I will also pay some attention to likely binding partners involved with retrograde transport. This will include previously identified partners with RILP and partners that have been posited to be involved with the activation of JIP3 during dynein binding, such as small GTPases Rab36 and Arf6. (Matsui et al. 2012, Isabet et al. 2009) I will validate these results via co-immunoprecipitation confirming the binding of these individual proteins with JIP3.

Expected Outcomes: I expect that both retrograde transport machinery and lysosomal machinery interact with JIP3. I expect that various Rab GTPases previously implicated in JIP3 or RILP binding will appear, such as Rab7 or Rab36. I expect that dynein will bind within the JIP3 RILP Homology 1 (RH1) domain based on structural studies, that Arf6 will bind within the leucine zipper II (LZII) domain, and that Rab36 will bind within the RILP Homology 2 (RH2 domain). (Vilela et al. 2019, Matsui et al. 2012, Isabet et al. 2009)

Limitations and Alternative Approaches: When searching for novel interactor candidates, interactors may not be detected for technical reasons, such as salt-sensitive binding or detergent use. Because of this, if expected candidates of previously characterized interactions do not appear, I will try performing the screen again by fusing JIP3 with the lysosome targeting sequence of p18. (Sancak et al. 2011) If there are still no putative candidates, it is possible that JIP3 is not present at high enough levels in the cell to detect interactions, so I will overexpress JIP3 before performing the immunoprecipitation and repeat the mass spectrometry experiment. If none of these troubleshooting methods work, I will purify lysosomes from WT, JIP3KO, and JIP3+JIP4 DKO cells and compare their protein compositions by mass spectrometry. For this experiment, it is possible that top hits do not bind with JIP3 but are found in high abundance in lysosomes. To avoid this false positive, I will focus on proteins that have putative binding sites on JIP3. I could also test individual candidate JIP3 molecular partners based on structural studies of JIP3, JIP4, and RILP. Possible candidates would include the small GTPases Rab36 and Arf6. Though this approach is also valuable, I will perform the screen first to find novel and physiologically relevant targets.

2.2 Test molecular interactors' co-localization with JIP3 and interactor KO phenotype

Rationale: Molecular interactors of JIP3 identified through biochemical experiments must also be validated for cellular relevance in neurons. In particular, I will focus on JIP3 binding partners that could act as activators of retrograde transport, and, secondarily, molecular partners responsible for driving retrograde transport. I hypothesize that lysosomal GTPases, which activate RILP in retrograde transport, also activate JIP3, allowing JIP3 to tether the lysosome to dynein for retrograde transport. (Vilela et al. 2019, Matsui et al. 2012)

Experimental Design: I propose to measure endogenous levels of both JIP3 and its molecular interacting partners by imaging fluorescent knock-in reporters in neurons. I will also create knockouts or knockdowns of specific JIP3 molecular interactors to test whether axonal transport is impacted. I will also test for rescue of this interactor knockout or knockdown by transiently transfecting the interactor into the interactor KO. I will use the lysosomal protein markers and acidic organelle dyes described previously in order to ensure that the proteins are colocalizing with lysosomes.

Expected Outcomes: If the interactor were required for efficient lysosomal retrograde transport, I would expect to see interactor KO defects in lysosomes that appear similar to JIP3 KO defects. I would expect this to be true particularly for knockouts or knockdowns of Rab GTPases, which would likely not abolish all retrograde transport, unlike dynein, which likely would abolish nearly all retrograde transport as it is the major molecular motor responsible for retrograde axonal transport. However, I would expect GTPase KOs to abolish JIP3 recruitment to lysosomes.

Limitations and Alternative Approaches: It is possible that binding of the interactor and JIP3 can happen outside of a lysosomal context. To avoid this, I will begin by using a lysosomal protein that is strongly tethered to the lysosome, such as Rab7, and therefore less likely to associate with JIP3 outside of a lysosomal context. For interactors primarily involved in retrograde transport, I will study known mediators of retrograde movement such as dynein and dynactin. If the rescue phenotypes are not robust when the interactor is transfected into the interactor KO neuron, I could also induce dimerization of JIP3 and the interactor using blue light. (Benedetti et al. 2019,) This would ensure that the interactor is efficiently binds JIP3 and is directly responsible for increased retrograde transport.

2.3 Characterize putative binding sites of JIP3 and its molecular interactor

Rationale: Once the binding between JIP3 and its molecular interactor is determined, I will use structural analysis data to posit where the interactor binds JIP3. JIP3 contains two regions of RILP homology and other structural motifs, such as leucine zippers, have been identified as important for the binding of JIP3 molecular partners. (Figure 3, Vilela et al. 2019, Matsui et al. 2012, Isabet et al. 2009) Because small GTPases bind JIP3 at its Leucine Zipper II domain (LZII) and RILP Homology 2 (RH2) domains while dynein should bind within the RILP Homology 1 (RH1) domain*, I hypothesize that dominant negative mutant copies of JIP3 will cause functional consequences in retrograde transport.* To do this, I will identify specific, critical binding sites of JIP3, mutate JIP3 in these regions, add dominant negative mutant JIP3 to neurons, and assess neurons for functional consequences.

Experimental Design: I will create truncated versions of JIP3 to perform co-immunoprecipitations and indicate where new JIP3 interacting partners bind JIP3. I will design more precise, smaller truncations based on protein sequence alignment data, which will allow me to determine likely binding sites. I will then mutate putative binding sites to these JIP3 binding proteins, using dynein and previously identified GTPase interactions as controls (specifically Rab36 and Arf6, whose binding has been previously identified). Then I will measure the functional consequences when the mutated copies of JIP3 are transfected into JIP3 KO neurons. I will specifically measure defects in lysosomal movement and acidification based on the criteria outlined in Aim 1.1. Together, this will elucidate the specific relationship between JIP3 and its binding partner.

Expected Outcomes: I expect that dynein binding takes place within the RH1 domain of JIP3 based on previous structural studies (Vilela et al. 2019) and that binding sites within the LZII and RH2 bind small GTPases. (Matsui et al. 2012, Isabet et al. 2009) Other lysosomal components may bind within the WD40 domain, which has been mutated in JIP3 haploinsufficient patients. (Iwasawa et al. 2019,

Platzer et al. 2019) This would be the first specific indication of WD40 binding identified in JIP3. I expect that protein sequence alignment will determine the specific binding sites of these interactors and that the binding sites will overlap with mutations present in clinical studies of JIP3 haploinsufficiency. (Iwasawa et al. 2019, Platzer et al. 2019) I also expect that mutated copies of JIP3 will present similar defects to JIP3 KO neurons and that lysosomes will be unable to move.
Limitations and Alternative Approaches: While unlikely, it is possible that protein sequence alignment data does not provide promising candidates for putative JIP3-interactor binding sites. Another option would be to systematically mutate regions of JIP3 while performing immunoprecipitations to narrow down the domains of JIP3 responsible for binding with the interactor. It is also possible that defects in transfected mutant JIP3 into the JIP3 KO neurons are not robust. Another method would be to mutate the region in both JIP3 and JIP4 and transfect both mutant copies into JIP3+JIP4 DKO neurons.

Specific Aim 3: Elucidate the mechanisms that coordinate lysosomal acidification and transport
Acidification of the lysosomal lumen is required for function of many lysosomal enzymes, transporters, and channels. (Mindell 2012) While LAMP1-positive vesicles are found throughout the axon, fewer of these compartments are fully acidified, degradative lysosomes. (Maday et al. 2012) Acidified lysosomes and endosomes share a dynamic relationship within the axon, and, along with the low relative abundance of acidified lysosomes, this has made it difficult to visualize degradative lysosomal dynamics clearly. However, there appears to be a temporal coupling between acidification and retrograde transport of lysosomes in the axon, and axonal lysosomes moving in retrograde towards the soma become progressively acidified by as much as 100 times. (Cheng et al. 2015, Hollenbeck 1993; Overly 1995, Overly 1996) Together, this suggests a relationship between lysosomal acidification and movement, but there is not yet a well-established, direct mechanism explaining this phenomenon. Because lysosomal acidification is thought to be mediated through the function of the lysosomal V-ATPase, one possible mechanism linking acidification and transport is through the V-ATPase binding with a retrograde transport adaptor. In fact, RILP has been implicated in stabilizing the lysosomal V-ATPase during retrograde transport via interactions with Rab7 and dynein. (De Luca et al. 2014) Due to the structural similarities and functional implications between RILP and JIP3/4 described in the previous aim, a similar regulation may take place via JIP3 or JIP3/4 in the context of neurons. I seek to study transport properties of the V-ATPase during inhibition of lysosomal acidification and elucidate the relationship between mediators of acidification and mediators of transport.

3.1 Determine localization of V-ATPase subunits in WT, JIP3 KO, and JIP3+4 DKO axons
Rationale: The V-ATPase reversibly separates into two protein complexes: the transmembrane V0 subunit and the V1 subunit, which is cytosolic when unbound from V0. (Bodžeta et al. 2017, Forgac 2007) Because the reversible association and dissociation of the V-ATPase has been proposed as a regulation mechanism of pH, tracking the localization of V1 subunits could provide an opportunity to visualize whether lysosomes are able to acidify. To this end, I will test the localization of the ATP6V1G1 (V1G1) subunit along with markers of lysosomal maturation state. *I hypothesize that V-ATPase subunits accumulate within the distal axon*. This would provide a mechanism for increasing acidification specifically in lysosomes moving in retrograde direction.
Experimental Design: To measure the localization of the V-ATPase, I have designed an endogenous fluorescent tag of the V1G1 subunit using the CHoP-In CRISPR system and tested this tag in HeLa cells. (Manna et al. 2019) I plan to replicate this experiment in neurons in order to measure the localization of the V1 complex in the axon. I will co-express this fluorescent tag with fluorescently tagged copies of proteins found in lysosomes and endosomes including LAMP1-GFP, mCherry-Rab7, and mCherry-Rab5 to focus on specifically lysosomal V-ATPase. Because V-ATPases should also be enriched in synaptic vesicles, I will track synapse locations using fluorescent tags of VAMP2, a synaptic vesicle protein, and I will track dendrites through the dendritic marker MAP2B in order to specify my search to lysosomes in the axon. To measure level of acidification in V-ATPase positive puncta, I will use the ratiometric pH indicator Oregon Green 488-dextran. Once I establish the natural

dynamics of V1 in WT neurons, I will repeat this in JIP3KO and JIP3+JIP4 DKO neurons in order to understand localization dynamics of the V1 complex when axonal transport is hindered.
Expected Outcomes: I expect to visualize V1G1 enriched at the distal axon in WT neurons, which would suggest that the V-ATPase assembles in the distal axon to regulate acidification. In JIP3 KO and JIP3+JIP4 DKO neurons, V1G1 should also be clustered in axonal swellings where lysosomes accumulate. I also expect the V-ATPase to co-localize more strongly with lysosomal markers, such as LAMP1, than with early endosomal markers, such as Rab5, which would be indicative of increasing acidification as the lysosome matures. I expect that puncta with more V1G1 expression will also have higher acidification, suggesting a direct relationship between amount of V1 subunit and level of acidification. Inhibiting lysosomal transport through JIP3 and JIP3+JIP4 mutations may also decrease overall level of acidification in lysosomes, suggesting that mediators of transport also control acidification.
Limitations and Alternative Approaches: In addition to Oregon Green 488-dextran, a genetically encoded ratiometric reporter indicating pH could be used, such as LAMP1-mApple-pHlourin. It is also possible that acidification is related to transport but is regulated upstream of JIP3/JIP4 function. If V1G1 do not appear to correlate with strictly lysosomal markers, I will look at localization of V1G1 with Arf6 in addition to late endosomes and lysosomes. Luminal acidification has been shown to regulate Arf6 at endosomes, and Arf6 also binds JIP3. (Hurtado et al. 2006)

3.2 Find V-ATPase protein-protein interactions with retrograde machinery
Rationale: Because lysosomes moving towards the cell body become progressively acidified by as much as 100 times, it's possible that a specific molecular interaction could regulate both processes. (Hollenbeck 1993) The lysosomal V-ATPase is stabilized by the Rab-interacting lysosomal protein (RILP), which is implicated in lysosomal retrograde transport via interactions with Rab7 and dynein, so similar molecular scaffolds (such as JIP3 and JIP4), GTPases (such as Rab36 and Arf6), and other molecular factors may act on the V-ATPase in a similar manner. (Vilela *et al.* 2019, De Luca et al. 2014) *I hypothesize that retrograde transport machinery binds to the V-ATPase in order to functionally tie lysosomal movement to acidification.* To test this, I will further study the reported interaction between RILP and V1G1 and assay putative V-ATPase interactions in order to probe the relationship between acidification and lysosomal trafficking.
Experimental Design: I will first test V-ATPase binding interactions through an immunoprecipitation and subsequent mass spectrometry screen of V1G1 fused to a HA tag at the N-terminus in WT, JIP3 KO, and JIP3+4 DKO neurons. This will identify whether the V-ATPase dynamics change in response to defects in axonal transport and whether the V-ATPase associates with other known proteins involved in retrograde transport. Based on this information, I will also test V1G1 for interactions with proteins involved in retrograde transport through co-immunoprecipitation. These candidates will include putative adaptors (i.e. JIP3, JIP4, and RILP), small GTPases (i.e. Rab7, Rab36, and Arf6) and dynein. I will also include any putative candidates from the mass spectrometry experiments detailed in this aim and in Aim 2. Based on these results, I will perform lysosome-specific purifications between candidate KO neurons and WT neurons to identify levels of cytosolic V1 vs bound V-ATPase in these lysosomes. These neurons can then be assayed for lysosomal dynamics through measuring acidification levels with Oregon Green 488/dextran and lysosome localization through LAMP1-GFP, mCherry-Rab7, and mCherry-Rab5.
Expected Outcomes: I expect that interactions that have been previously characterized will once again appear immunoprecipitations, including RILP, Rab7, and Arf6. I also expect that similar proteins may bind V1G1 including JIP3 and JIP4, which share significant homology with RILP. (Vilela et al. 2019) The V1 subunit may dissociate more in KOs of these candidates than in WT neurons, indicating that acidification is hindered. In addition, I expect acidification and transport to both be hindered by mutations affecting transport such as JIP3 KO and JIP3+JIP4 DKO neurons, further indicating a link between retrograde transport and acidification.
Limitations and Alternative Approaches: While the previously described experiments will provide important clues to lysosomal dynamics, the mass spectrometry screen is exploratory and may not

initially pull out interesting candidates. Following a reverse genetic approach through previously reported interactors of V1G1 will serve as a positive control for immunoprecipitation experiments. Because the V-ATPase has other functions in the cell, it is also possible that it associates with many of the above proteins. To avoid this, when testing localization and lysosome dynamics in cells, I will prioritize proteins that are upregulated in the WT mass spectrometry when compared to JIP3 KO and JIP3+JIP4 DKO. It is likely that not all of the V1 subunit can be cytosolic based on inhibition of acidification alone. In order to establish a baseline level of V-ATPase dissociation, I will normalize cytosolic V1 data to cytosolic V1 when cells are exposed to concanamycin, a V0c inhibiting drug that decreases lysosomal acidification. Also, while these results could confer association between proteins, they will not indicate direct protein-protein binding. To confirm the direct association, I could conduct further experiments by making systematic mutations within the putative binding regions of each protein.

Summary

These studies will provide novel insights into regulation of the retrograde axonal transport of lysosomes. It will also address the consequences of irregular retrograde transport on lysosomal function, including in lysosomal maturation and localization. These experiments will elucidate the cellular importance of JIP3 and JIP4 in retrograde transport of lysosomes, the molecular mechanisms that JIP3 uses to act to facilitate lysosomal retrograde transport, and the relationship between lysosomal acidification and retrograde transport.

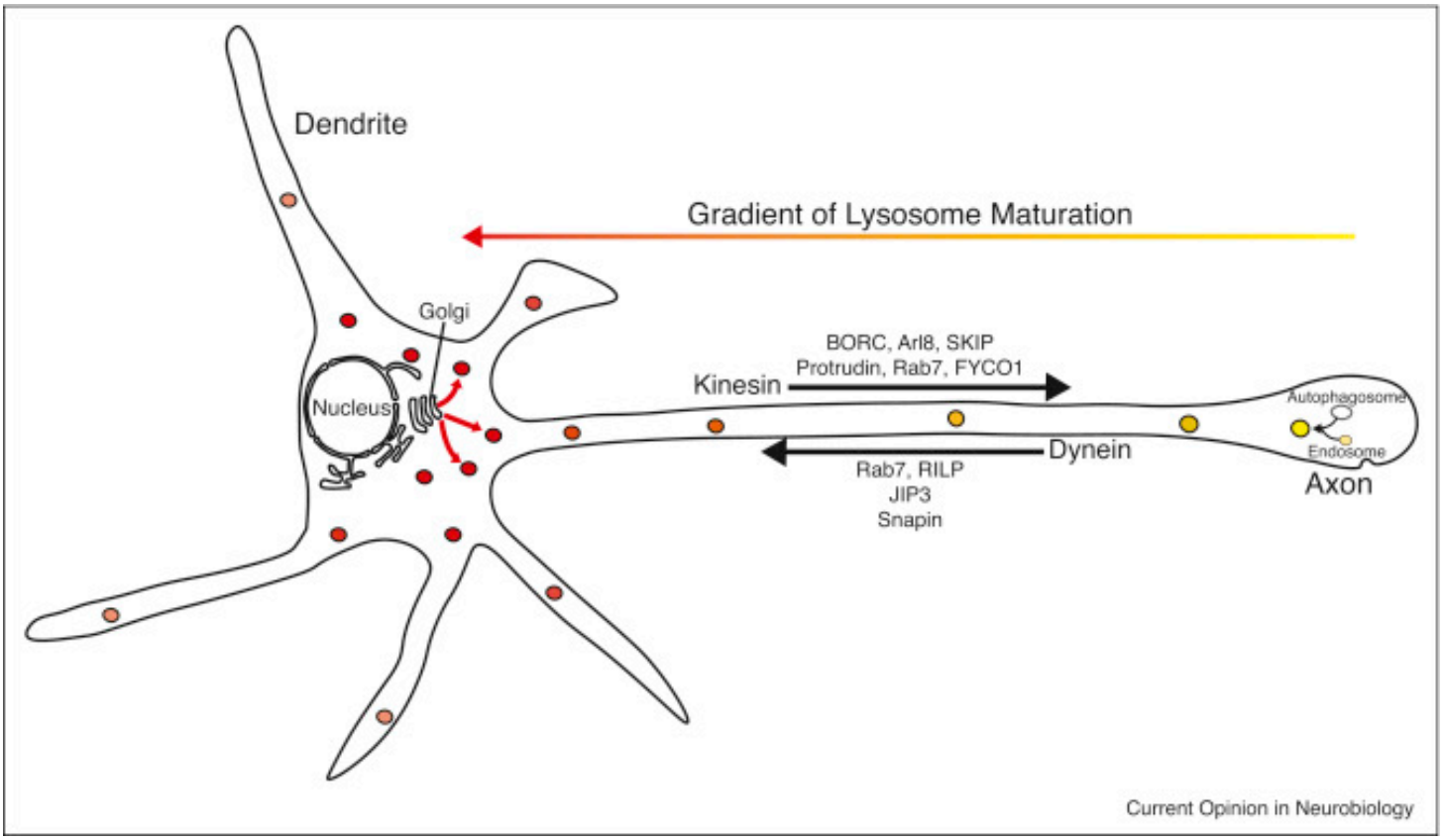

Figure 1: Schematic diagram of lysosomal maturation and axonal transport. As lysosomes move in retrograde towards the cell body, they progressively acidify. A number of proteins have been identified as putative adaptors between lysosomes and molecular motors guiding axonal transport. Published in Ferguson 2018.

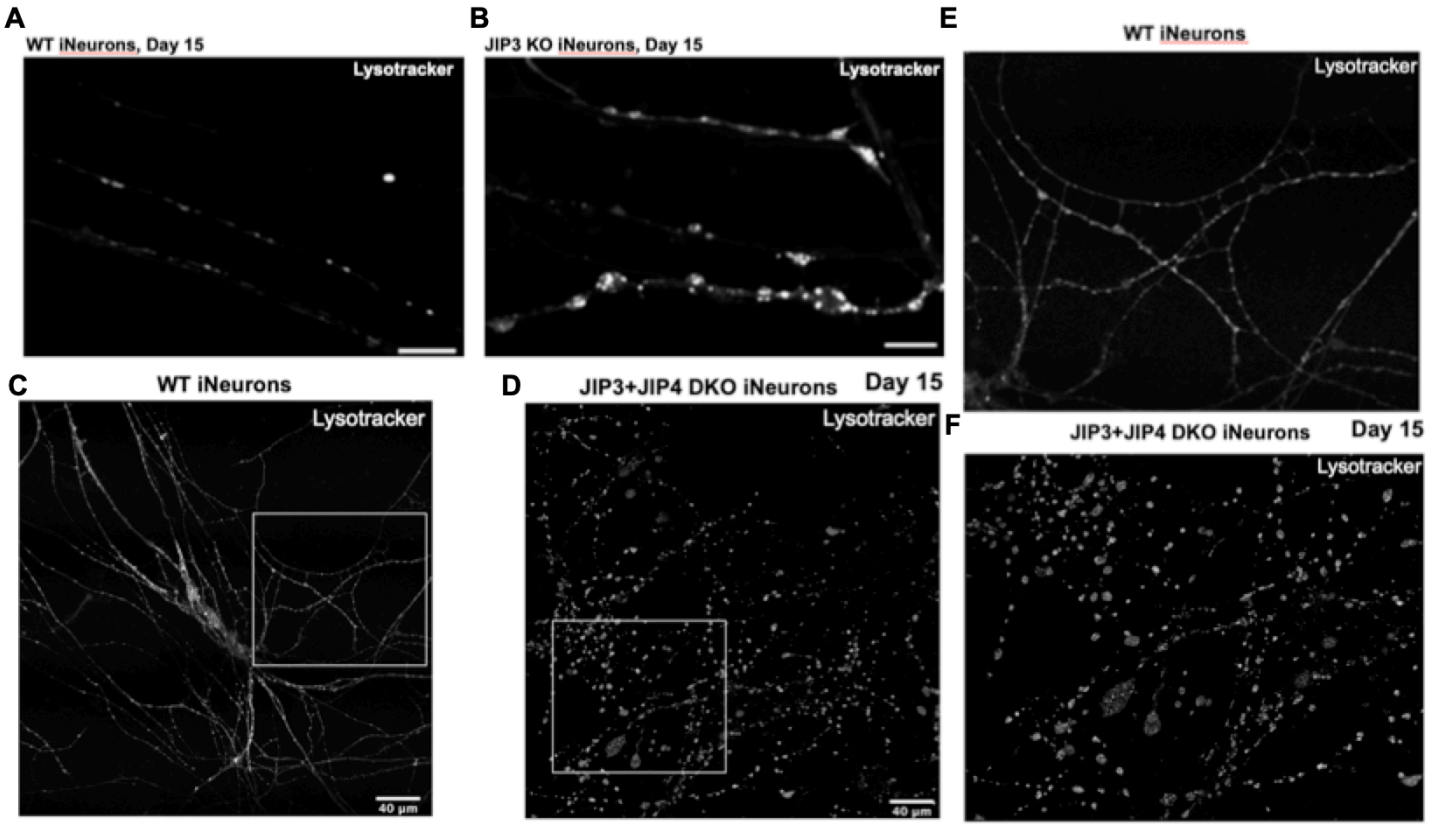

Figure 2: JIP3+JIP4 DKO and JIP3 KO experience axonal swellings 15 day old iNeurons stained for LysoTracker. a) image of WT axon and LysoTracker (scale bar = 10 uM); b) image of JIP3 KO axon and LysoTracker with swell (scale bar = 10 uM); c) image of a WT axons and dendrites with LysoTracker (scale bar = 40 uM); d) image of JIP3+JIP4 DKO axons and dendrites with LysoTracker (scale bar = 40 uM); e) zoomed in image of WT axon from panel c; f) zoomed in image of JIP3+JIP4 DKO axon from panel d. Data collected by Nisha Mohd Rafiq.

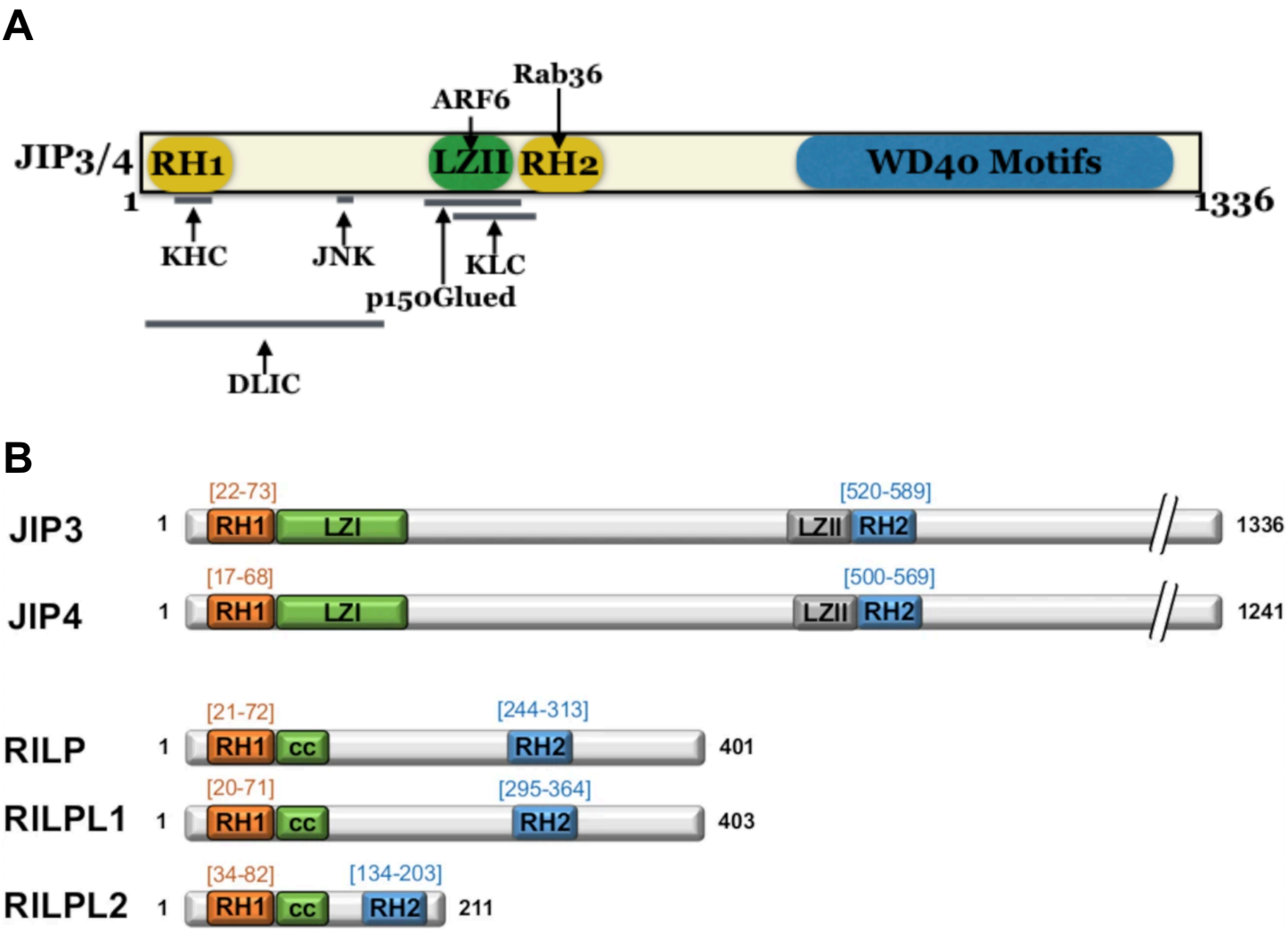

Figure 3: Illustration of JIP3/4 protein domain organization and binding sites for known interacting proteins (RH=RILP homology, LZ=leucine zipper). A) JIP3 and JIP4 contain binding sites for mediators of retrograde transport. (KHC = Kinesin Heavy Chain, JNK = c-Jun N-terminal Kinase, DLIC = Dynein Light Intermediate Chain, KLC = Kinesin Light Chain) Figure by Shawn M. Ferguson. b) RILP and JIP3/4 are structurally similar (cc = coiled coil domain) Published in Vilela et al. 2019.

References

Benedetti L, Barentine AES, Messa M, Wheeler H, Bewersdorf J, De Camilli P (2019) “Light activated protein interaction with high spatial subcellular confinement.” *PNAS* 115(10):2238-45

Boecker AC, Olenick MA, Gallagher ER, Ward ME, Holzbaur ELF (2019) “ToolBox: Live-Imaging of intracellular organelle transport in iPSC-derived neurons.” *Traffic* doi: 10.1111/TRA.12701

Bodžeta A, Kahms M, Klingauf J (2017) “The Presynaptic v-ATPase Reversibly Disassembles and Thereby Modulates Exocytosis but Is Not Part of the Fusion Machinery.” *Cell Rep* 20(6):1348-59

Cantalupo G, Alifano P, Roberti V, Bruni CB, Bucci C (2001) “Rab-interacting lysosomal protein (RILP): the Rab7 effector required for transport to lysosomes.” *The EMBO Journal* 20(4):683-693

Carpenter AE, Jones TR, Golland P, and Sabatini DM (2006) “CellProfiler: image analysis software for identifying and quantifying cell phenotypes.” *Genome Biology* 7(10):R100

Cheng XT, Cai Q, Sheng ZH. (2015) “Axonal autophagosomes recruit dynein for retrograde transport through fusion with late endosomes.” *J Cell Biol.* 209(3):377-86

De Luca M, Cogli L, Di Fiore PP, Bucci C (2014) “RILP regulates vacuolar ATPase through interaction with the V1G1 subunit.” *Journal of Cell Science* 127:2679-2708

Drerup CM, Nechiporuk AV. “JNK-interacting protein 3 mediates the retrograde transport of activated c- Jun N-terminal kinase and lysosomes.” *PLoS genetics* 2013;9(2):e1003303.

Edwards SL, Richmond JE, Miller KG. (2013) “An Organelle Gatekeeper Function for Caenorhabditis elegans UNC-16 (JIP3) at the Axon Initial Segment.” *Genetics* 194(1):143- 61

Fernandopulle MS, Prestil R, Gan L, Ward M (2017) “Transcription Factor-Mediated Differentiation of Human iPSCs into Neurons” *Current Protocols in Cell Biology* 79(1):e51

Ferguson SM (2018) “Axonal Transport and the Maturation of Lysosomes.” *Current Opinion Neurobiology* 51:45-51

Forgac M (2007) “Vacuolar V-ATPases: rotary pumps in physiology and pathophysiology.” *Nature Reviews Mol Cell Bio* 8(11):917-29

Gowishankar S, Wu Y, Ferguson SM (2017) “Impaired JIP3-dependent axonal lysosome transport promotes amyloid plaque pathology.” *Journal of Cell Biology* 216(10):3291

Haggie PM & Verkman AS (2009) “Unimpaired lysosomal acidification in respiratory epithelial cells in cystic fibrosis.” *Journal of Biological Chemistry* 284(12):7681-76

Hollenbeck PJ (1993) “Products of endocytosis and autophagy are retrieved from axons by regulated retrograde organelle transport.” *Journal of Cell Biology* 121:305-315

Hurtado-Lorenzo A, Skinner M, … Brown D, Marshansky V (2006) “V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway.” *Nature Cell Biology* 8(2):124-36

Isabet T, Houdusse A, Menetrey J (2009) “The structural basis of Arf effector specificity: the crystal

structure of ARF6 in a complex with JIP4." *EMBO J.* 28(18):2835-45

Iwasawa S, Yanagi K, Kikuchi A, Kobayashi Y, Haginoya K, Shoji W, Kure S (2019) "Recurrent de novo MAPK8IP3 variants cause neurological phenotypes." *Annuals of Neurology* 85(6):927-933

Kelkar N, Standen CL, Davis RJ. "Role of the JIP4 scaffold protein in the regulation of mitogen-activated protein kinase signaling pathways." *Mol Cell Biol.* 2005;25(7):2733-43.

Maday S, Wallace KE, Holzbaur EL (2012) "Autophagosomes initiate distally and mature during transport toward the cell soma in primary neurons." *J Cell Biol.* 196(4):407-17.

Manna PT, Davis LJ, Robinson MS (2019) "Fast and cloning-free CRISPR/Cas9-mediated genomic editing in mammalian cells." *Traffic* doi: 10.1111/tra.12696

Matsui T, Ohbayashi N, Fukada M (2012) "The Rab interacting lysosomal protein (RILP) homology domain functions as a novel effector domain for small GTPase Rab36: Rab36 regulates retrograde melanosome transport in melanocytes." *Journal of Biological Chemistry* 287(34):28619-31

Mindell JA (2012) "Lysosomal acidification mechanisms." *Annu Rev Physiol.* 74:69-86

Ostrowski PP, Fairn GD, Grinstein S, Johnson DE. (2016) "Cresyl violet: a superior fluorescent lysosomal marker." *Traffic* 17(12):1313-21.

Overly CC, Lee KD, Berthiaume E, Hollenbeck PJ (1995) "Quantitative measurement of intraorganelle pH in the endosomal-lysosomal pathway in neurons by using ratiometric imaging with pyranine." *PNAS* 95:3156-60

Overly CC & Hollenbeck PJ (1996) "Dynamic Organization of Endocytic Pathways in Axons of Cultured Sympathetic Neurons." *The Journal of Neuroscience* 16(19):6056-64

Platzer K, Sticht H, Edwards SL, Allen W, Angione K, Bonati MT, Brasington C, Cho MT, Demmer LA, Falik-Zaccai T, Gamble CN, Hellenbroich Y, Iascone M, Kok F, Stöbe P, Stumpel C, Wilson C, Lemke J, Di Donato N, Miller KG, Jamra R (2019) "De Novo Variants in MAPK8IP3 Cause Intellectual Disability with Variable Brain Anomalies." *The American Journal of Human Genetics* 104:1-10

Sancak Y, Bar-Peled L, Zoncu R, Markhand AL, Nada S, Sabatini D (2011) "Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids." *Cell* 141(2):290-303

Sleigh JN, Rossor A, Fellows A, Tosolini AP, Schiavo G (2019) "Axonal transport and neurological disease." *Nat Rev Neurol*. doi:10.1038/s41582-019-0257-2 6.

Surana S, Villaroel-Campos D, Lazo O, Moretto E, Tosolini AP, Sleigh JN, Schiavo G (2019) "The evolution of the axonal transport toolkit." *Traffic* doi:10.1111/tra.12710

Vilela F, Velours C, Chenon M, Llinas P, Menetrey J (2019) "Structural characterization of the RH1-LZI tandem of JIP3/4 highlights RH1 domains as a cytoskeletal motor-binding motif" *Nature Scientific Reports* 9(16036)

Villaroel-Campos D, Schiavo G, Lazo OM (2018) "The many disguises of the signaling endosome" *FEBS Letters* 592(21):3615-3632