Project Summary/Abstract

With no effective treatments and cures, ageing-related neurodegenerative diseases (NDs) continue to present a pressing threat to our society. Strong evidence links NDs to dysfunctional endolysosomal trafficking, a cellular homeostasis process that recycles and degrades protein cargos. One representative ND with defect in endolysosomal trafficking is Huntington's disease (HD), a dominantly transmitted disease caused by hereditary mutations that amplify the glutamine tract (polyQ) in Huntingtin (HTT). Converging evidence reveal that HTT exists in a 'core complex' with HAP40, and that both proteins are conserved in *Drosophila* (fruit flies), a powerful model for studying genes involved in human diseases including HD. While analyzing HTT and HAP40 proteins, computer modeling revealed a membrane-binding site in HAP40 that is exposed when bound to HTT, and validation experiments in flies demonstrated that HTT binds endosomes in a HAP40-depedent manner, thus implicating a structural basis for HTT's engagement on endosomes during trafficking. Further endocytic and biochemical assays in *Drosophila* and cultured mammalian cells demonstrated that HTT/HAP40 recruits Rab4, a regulator of protein recycling, to [redacted], a regulator of [redacted] endosomes/lysosomes. Using cultured mammalian cells, we found that trafficking of [redacted], a known cargo of Rab4, is disrupted upon HTT depletion. This established an intriguing functional link for HTT/HAP40 and Rab4 in an important homeostasis process commonly dysregulated in NDs. This led to **our *central hypothesis* that HTT is a conserved regulator of Rab4 and Rab4-mediated endolysosomal trafficking, a cellular process potentially disrupted by polyQ expansion in HD.** In this proposed study, we will *first* use structural-functional domain-mapping mutagenesis (Aim1a), together with unbiased phenotype-based RNAi screens in fruit flies (Aim1b) to address 1) how HTT recruits Rab4 to [redacted] late endosomes, and 2) the key intermediate players involved given that HTT is known to not bind directly to Rab4. The *second* objective will be to test the physiologic and functional significance of the mechanism using super-resolution imaging of Rab4-cargo trafficking ([redacted]) in cultured mammalian cells (HeLa, HEK293) (Aim2a), and HTT's genetic interaction with Rab4 (Aim2b) to test if HTT activates or inhibits Rab4. In parallel, using our established HD models that express full-length mutant HTT of varying polyQ lengths (75Q,128Q, 145Q), we will systematically test in vitro and in vivo their effect on Rab4 functions and cargo trafficking (Aim3a). Lastly, we will test whether Rab4 depletion or over-expression modulates the severity of HD using our fruit fly neurodegenerative assay (Aim3b). The proposed study will address important questions regarding how HTT, the HD-causing gene, regulates endosomal trafficking, a process commonly dysregulated in NDs. Findings from this study will shed light on new targets for HD specifically and NDs in general.

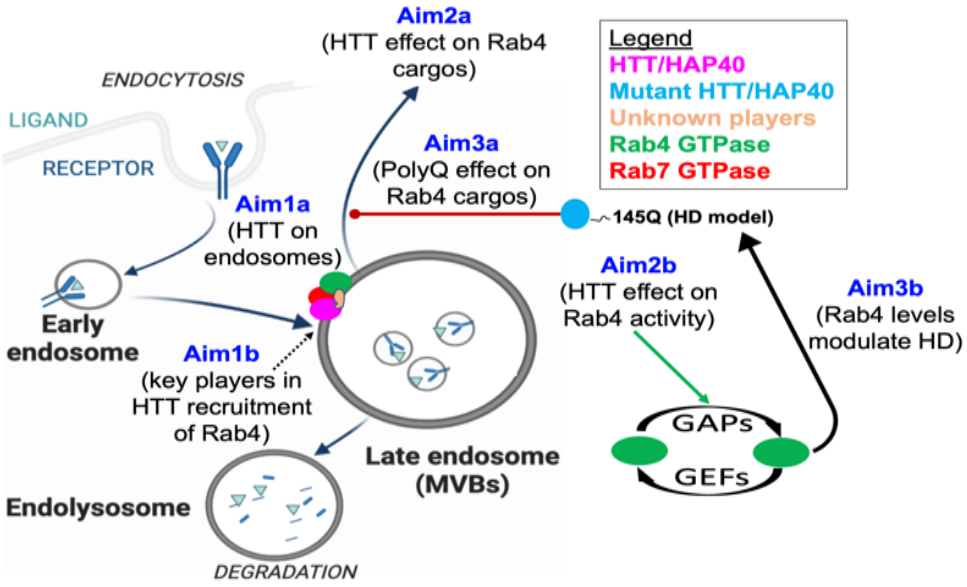


Figure 1. Graphical abstract of the proposed aims testing HTT/HAP40's role in Rab4 functions in normal and HD states.

Specific Aims

With little effective treatments and cures, ageing-related neurodegenerative diseases (NDs) present a pressing threat to our society. Increasing evidence links NDs to endolysosomal trafficking pathways, as their dysfunction often causes abnormal accumulation of misfolded proteins in the brain, a common hallmark of most NDs. One representative ND is Huntington's disease (HD), a dominantly transmitted fatal disease caused by amplifications of the poly-glutamine tract (polyQ) in Huntingtin (HTT). Importantly, HTT has been suggested to function in endolysosomal trafficking. However, the *in vivo* evidence of such functional involvement and the exact step(s) and mechanism of action by HTT as well as its targets in this biological process remains unclear. Our *long-term goal* is to develop effective treatments for HD and other NDs through better understanding of endolysosomal trafficking, a major cellular homeostasis mechanism.

Converging evidence from biochemical, structural, and genetic studies revealed that HTT exists in a complex with HTT-associated protein 40 (HAP40). Both proteins are highly conserved in *Drosophila*, a powerful genetic model for functional study of conserved genes. While analyzing HTT and HAP40 homologues in *Drosophila*, we obtained *in vivo* evidence supporting HTT's functional role in endosomal trafficking. In *Drosophila* egg chambers, a tissue that not only can be easily imaged and genetically manipulated, but is also highly active in endosomal trafficking, we found that HTT interacts directly with endosomal membranes and co-localizes with [redacted], a regulator of late endosomes. Further, in HTT knockout (KO) fly lines, we found that the number of endosomes were increased but their overall sizes decreased. Interestingly, HTT depletion did not affect [redacted] endosomes, but abolished their association with Rab4, a regulator of fast-recycling endosomes, implying a critical role of HTT in recruiting Rab4 to [redacted] endosomes. In agreement with this finding in fly, we found aberrant localization of cargo recycled by Rab4 in HTT-KO mammalian cells.

Together with existing reports that link Rab4 with HTT and HD, these findings led to my **central hypothesis that HTT is a conserved regulator of Rab4 and Rab4-mediated endolysosomal trafficking, a cellular process potentially disrupted by polyQ expansion in HD.** Using my experience with molecular and structural modeling, together with the powerful genetic tools in *Drosophila* and biochemical assays in mammalian cells, I propose the following aims to rigorously test this hypothesis and interrogate the underlying mechanism:

Aim 1: Elucidate the mechanism underlying HTT-regulation of Rab4 in endolysosomal trafficking.
We found that the structural architectures of HTT/HAP40 core complexes, in particular a solvent-exposed membrane-association loop, are well-conserved between humans and *Drosophila*. This suggests a structural basis for how HTT functions on endosomes to regulate Rab4. We will perform structural-functional analysis on HTT/HAP40 to test this hypothesis in vivo (Aim1a); Separately, given the report that that HTT does not directly bind Rab4, we *hypothesize* that HTT recruits Rab4 to [redacted] endosomes through 'unknown' intermediate players. Using the existing genome-wide RNAi lines in *Drosophila*, I have been conducting phenotype-based RNAi screens to identify such key "intermediate" players in HTT-mediated Rab4 regulation (Aim1b).

Aim 2: Determine the functional consequence of HTT recruitment of Rab4 to [redacted] endosomes.
Given the preliminary findings that HTT-KO causes dissociation of Rab4 from [redacted] endosomes and the defective trafficking of Rab4 cargos, I *hypothesize* that HTT recruits Rab4 to [redacted] endosomes for proper recycling and/or degradation of specific Rab4 cargos. Using both biochemical assays and super-resolution imaging in mammalian cells, I will test this hypothesis by examining the trafficking dynamics of these cargos in HTT-KO cells, to define the exact step(s) and potential cause(s) of the observed trafficking defects (Aim2a). In parallel, I will perform genetic interaction assays between HTT and Rab4 in *Drosophila* to determine how HTT affects Rab4's activation or function (Aim2b)

Aim 3: Test the cellular and physiologic consequences of HD-causing mutant HTT on Rab4.
A recent comprehensive proteomic study showed that HTT interacts with many regulators of endolysosomal trafficking in age- and polyQ-dependent fashions. Together with our preliminary data, I *hypothesize* that the regulation of Rab4 by HTT could be potentially disrupted by polyQ expansion in mutant HTT. Using HD models established in mammalian cells and *Drosophila* that express HTT with different polyQ lengths (Q23, Q72, Q145) I will test this hypothesis by examining whether subcellular localization and function of Rab4 are affected in HD (Aim3a), and whether Rab4 itself modulate neuronal toxicity and HD pathogenesis (Aim3b).

Overall, the proposed aims will elucidate the underlying function(s) of HTT, the causative gene in HD, in endolysosomal trafficking and how polyQ expansion alters HTT's normal function in this cellular homeostasis process. This work will have broader impacts on new molecular targets for HD specifically and NDs in general.

A. Significance

Huntington's disease (HD) and endolysosomal trafficking HD is an autosomal dominant, hereditary disease caused by CAG expansion in *HTT* gene that leads to an expanded polyglutamine tract (polyQ) in the corresponding encoded protein[1]. Although the cause of the disease is straightforward genetically, the precise etiology and pathogenesis remains unknown. Currently, there are no treatments for HD, and it remains paramount to identify promising therapeutic strategies.

A hallmark of many NDs is the development of abnormal protein aggregates in the brain because of defective endolysosomal trafficking[2-4]. HTT has been implicated in endolysosomal trafficking, however its exact mechanism and how it becomes altered in HD remains unexplored. Prior studies showed that HTT functions by interacting with dynein (retrograde to cell soma) and kinesin (anterograde to axon terminal) to mediate the translocation of endosomes during trafficking[5]. Moreover, rigorous experiments, including a recent comprehensive co-IP/mass-spec study, found that HTT plays a more complex role by interacting with other effectors in the endocytic process, interactions that are altered in a polyQ-length and/or age-dependent fashion (hallmarks of HD)[6,7]. Thus, a better understanding of HTT's role in the endosomal pathway will offer insight into the specific mechanism of HD, NDs in general, and the identification of potential molecular targets (Fig 1).

HTT regulates Rab4-endosome functions HTT is functionally conserved in *Drosophila*[8]. While characterizing the HTT homolog (dHtt) in this powerful genetic model organism, we obtained in vivo evidence that HTT functions in endosomal trafficking. For example, we show that HTT localizes to endosomal membranes and recruits Rab4, the fast-recycling regulator, to [redacted] endosomes (Figs 2, 4). Previous cell-based studies described Rab4 as functioning only on early endosomes during fast rec cling of cellular cargos and not overlapping [redacted][9]. However, our in vivo data demonstrate an HTT-mediated mechanism by which Rab4 is present and potentially active on late endosomes to modulate Rab4-cargo trafficking. *These findings raise questions on how HTT associates with endosomes to regulate Rab4 recruitment*.

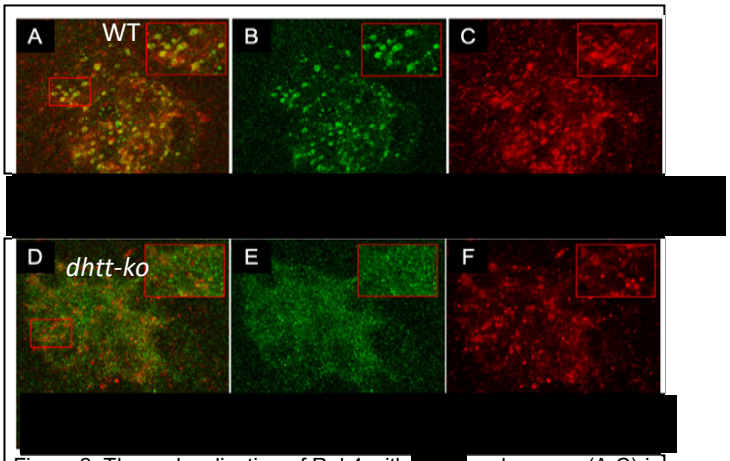


Figure 2. The co-localization of Rab4 with [redacted] endosomes (A-C) is lost in dhtt-ko (D-F) fly egg chamber border cells.

HTT exists in a core complex with HAP40 HTT is a large 350-kDa protein that consist primarily of highly ordered, anti-parallel, double-helix motifs known as HEAT repeats[10]. These structural repeats have been suggested to serve as docking sites for proteins, thus allowing HTT to regulate diverse cellular events including endolysosomal trafficking, selective autophagy, and transcriptional activities[11-13]. From recent cryo-EM studies[13,14], and genetic studies from our lab[15], HTT was found to exist as part of a large globular 'core complex' containing a 40-kDa protein, HAP40 (Fig 3). HAP40 is a critical regulator of HTT's conformational stability[15-17].

HTT and HAP40 proteins are mutually dependent. Through rigorous in vivo and in vitro studies, we found that HAP40 modulates mutant HTT toxicity in a *Drosophila* model of HD[15], and that HTT and HAP40 protein levels, not mRNA levels, are mutually dependent: HTT undergoes lysosomal degradation in the case of HAP40 depletion, and HAP40 undergoes proteasomal degradation in the case of HTT depletion. Further, it was found that apo-HTT (unbound) is aggregate-prone and readily degraded. Thus, in the disease context, interrogation of the functions of the full-length core complex (HTT/HAP40) with polyQ expansion is critically important.

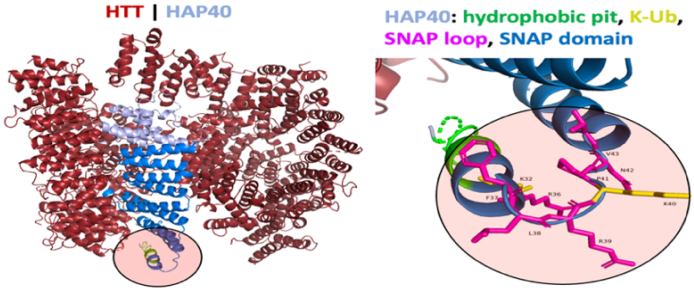


Figure 3. Core complex (HTT/HAP40) showing a conserved SNAP membrane binding loop (pink) on HAP40 that is exposed when bound to HTT (red).

HAP40 has a potential membrane-binding domain The cryo-EM model showed that HTT is composed of two solenoid domains connected by one bridge domain that together ensheath around HAP40[13]. However, the highly flexible intrinsically

disordered regions (IDRs) that make up ~25% of the core complex were not resolved in the original structure which convolutes further structural-functional characterization. In my pilot study using AlphaFold2 to predict the unresolved IDRs, I found that HAP40 has a SNAP membrane-association loop that is conserved in humans and flies, is solvent-exposed in a complex with HTT (Fig 3) and is permissive to binding negatively charged lipids such as phosphatidyl-serine (PS) on endosomes. To investigate the endosomal functions of HTT and HAP40 in a physiological setting, we established a robust endocytic assay using *Drosophila* egg chambers which contain two regions, migrating border cells and the oocyte that 1) harbor conserved endocytic machineries, 2) highly active in endocytosis, 3) can be easily imaged, and 4) amenable for high-throughput genetic manipulation[18,19]. I found that **HTT interacts with endosomal membranes in a HAP40-dependent fashion,** suggesting a mechanistic clue for HAP40's SNAP domain (Fig 4). Further, we discovered that similar as HTT-knockout (HTT-KO), HAP40-depletion also reduced overall endosome size but had no effect on their maturation shown by normal granule formation and yolk contrast (Fig 5), supporting a role in membrane fusion by HTT/HAP40 core complex. Given that SNAPs are critical for membrane fusion and our finding of a conserved SNAP domain in HAP40, **they led to our hypothesis that HAP40 may use this conserved structural domain to mediate HTT's interaction with endosomes to regulate Rab4.**

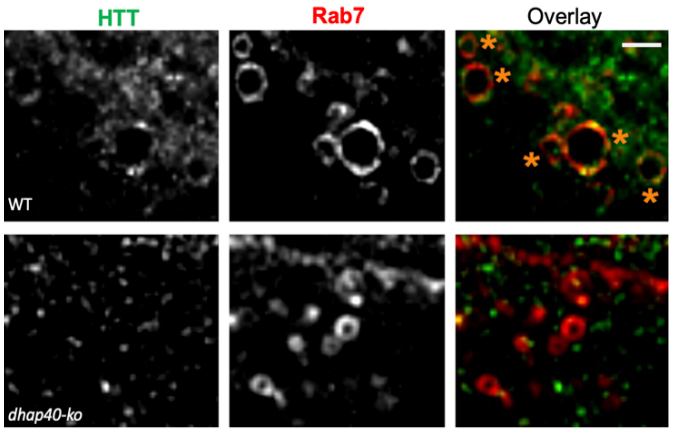


Figure 4. HTT interaction with [redacted] endosomes is dependent on the presence of HAP40 in vivo (fruit fly egg chamber oocytes).

Overall, this study aims to decipher the regulatory mechanism of HTT/HAP40 on Rab4 and evaluate its role under pathogenic conditions. Our study will systematically address these questions *first* by using rigorous molecular modeling and in vivo domain-mapping mutagenesis to obtain structural-functional insight, and systematic in vivo screens using the powerful fruit fly system to identify key intermediate players in the process given that HTT does not directly bind Rab4 (Aim1). In parallel, we will test the functional significance of HTT-mediated recruitment of Rab4 to [redacted] endosomes using in vitro biochemical assays, prioritizing the fate of Rab4 cargos (Aim2). *Lastly*, using our established HD model in flies and mammalian cells, we will test exactly how polyQ expansion alters Rab4 and contributes to HD pathogenesis (Aim 3). This will help to identify new molecular targets for HD specifically and ND in general.

B. Innovation

Our study is innovative because it utilizes computer-based protein modeling combined with a high-throughput *in vivo* genetic platform and biochemical and imaging tools in mammalian cells to address important questions regarding HD pathogenesis. Employing computer-based molecular modeling with unbiased RNAi screens in fruit flies, we will uncover the mechanism for how HTT recruits Rab4 (Aim1). To interrogate the functional significance of HTT-mediated recruitment of Rab4 to [redacted] (Aim2), we will employ advanced super-resolution imaging and computational techniques, together with many unique and innovative fly strains that our lab has created, such as *dhtt*- and *dhap40-null* flies, transgenic fly lines that allow targeted expression of normal and mutant human HTT and HAP40 orthologs (UAS-based system), as well as engineered endogenous tagging lines for dHtt and Rab genes (Table 1). The availability of these tools allows us to readily test how Rab4 functions become altered in the presence and absence of HTT, and whether polyQ disrupts Rab4 activity or if Rab4 forms modulate HD pathogenesis (Aim3). By integrating multi-disciplinary approaches, this project will provide a full-scope view of the molecular and structural bases underlying a novel HTT-mediated mechanism in endosomal trafficking.

Fly lines	Type	Purpose
RabXN-YFP	Genomic tagging	Live-imaging and co-localization studies
UAS-RabXN-YFP UAS-RabXN-RFP	Driven by promoter-specific Gal4	Rescue, co-localization, over-expression studies
dHtt-GFP dHtt-mCherry	Genomic tagging	Live-imaging and co-localization studies
dHtt-DsRed	Genomic insertion	Loss-of-function allele
dHtt-GS	Genomic tagging	Rescue and. purification experiments (co-IP)

Table 1. List of Rab and dHtt fly lines harboring reporter tags. X = RabGTPases 4, 5, 7; N=Wild type (WT), constitutive active (CA), and dominant negative (DN)

C. Approach

AIM 1: Elucidate the mechanism underlying HTT-regulation of Rab4 in endolysosomal trafficking.

Rationale: Determining how HTT becomes stabilized on endosomal membranes, its functional role of recruiting Rab4 to [redacted] endosomes, the effectors involved, and the physiologic outcomes using Rab4 cargos as a readout, will shed light on an important biological question for how the HTT protein, involved in HD, regulates the fate of cellular cargos. *Hypothesis: HTT binds endosomal membranes via HAP40 to recruit key intermediates that bring Rab4 to [redacted] endosomes.* **Overview of Experimental Design:** To test how HTT interacts with endosomes via HAP40 to regulate Rab4 functions, I will dissect its membrane binding mechanism through a series of structural-modeling-guided mutagenesis studies on HAP40, followed by their functional evaluation using our established fly egg chamber endocytic assays (Fig 5). Lastly, I will next identify the key players in the process using our high-throughput fly egg chamber screening platform to drive RNAi expression in a tissue-specific manner using endosomal size, Rab4 co-localization with [redacted], and Rab4-cargos as readouts.

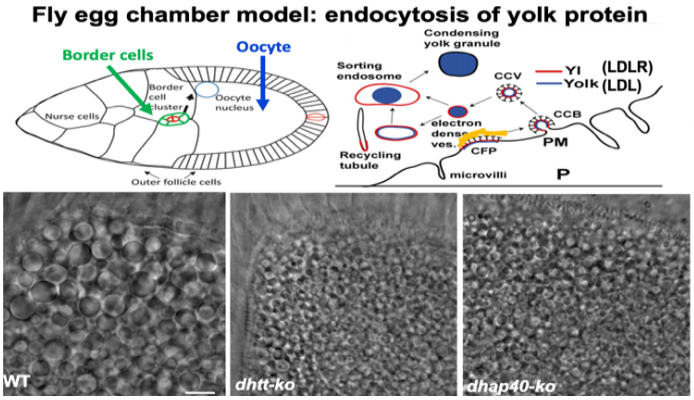


Figure 5. Fly egg chambers that will be used for endocytic assays (top) and notable phenotypes in dhtt- or dhap40-ko flies (bottom).

Aim 1A: Structural-functional analysis of predicted SNAP membrane-binding domain in HAP40. A key finding from our modeling was the identification of an exposed IDR that aligns with a SNAP-membrane binding loop (residues 22-40), which exists as part of a much larger SNAP domain on HAP40's N-terminus (residues 1-185). We hypothesize that this motif is functionally important because it is 1) conserved across species, and 2) potentially regulated via two lysine residues that are known to be ubiquitinated (Fig 3). In contrast, the C-terminal half of HAP40 (residues 186-371) is buried and stabilizes the interactions with HTT. Thus, rigorous structural-functional analysis of HAP40 protein will give insight into the specific mechanism by which HTT binds to [redacted] endosomes to recruit Rab4. We will perform structural-functional studies using domain-mapping mutagenesis in fruit flies, and assaying for defects in endolysosomal trafficking using our egg chamber assays (Fig 5). Experimental Approach: To validate the functional importance of the SNAP-domain in HAP40, we will generate "deletions" by replacing protein domains described above with large or small reporter proteins (GFP or Myc) on HAP40 including: 1) "SNAP loop mutant; 2) "N-terminal mutant"; and 3) "C-terminal mutant" (Fig 6). Analysis and Rigor: Mutational constructs will be validated by sequencing and reporter staining. Mutated HAP40 will be introduced into *dHap40-null Drosophila* through P-element insertion, and their activities will be interrogated in N=3 independent experiments using our established fly endocytic assays. Endosomal phenotypes will be tested using confocal microscopy and analyzed on ImageJ/Fiji, with WT and *dhap40-ko* flies as negative and positive controls. Interpretations: If the SNAP binding loop is critical for the core complex to associate with endosomes, then I expect the dHap40 SNAP loop mutant to phenocopy the *dhap40-ko* phenotype. However, it alone may fail to provide a robust effect on endosomal binding if other regions of the SNAP domain (red, Fig 6) contribute to membrane binding. The N-terminal mutant will sequester potentially missed membrane binding sites on HAP40, whereas the C-terminal mutant will primarily disrupt the stability of the core complex, thus being a loss-of-function control for our study.

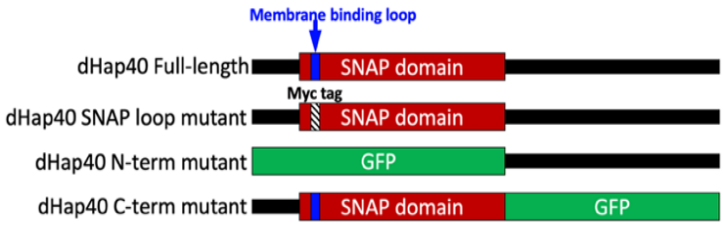


Figure 6. Proposed dHap40 mutant constructs for domain-mapping.

Aim 1B: Identify key players involved in HTT recruitment of Rab4 to [redacted] endosomes. One important question is how HTT specifically controls Rab4 recruitment. Given the report that HTT does not directly interact with Rab4, we hypothesize that other unknown players act as key intermediates between HTT and Rab4. Identifying such regulators that are important for HTT's recruitment of Rab4 will allow us to have a whole picture of the exact cellular process in HTT/HAP40-mediated recruitment of Rab4 and related endolysosomal trafficking events. *Drosophila* is ideal model to identify such novel players given that flies are amenable for unbiased,

phenotype-based high-throughput screening and importantly, genome-wide RNA interference (RNAi) lines against almost all the genes in the fly genome are available[20]. Accordingly, I will conduct a phenotype-based in vivo RNAi screen in the fly to search for such key players. Experimental Approach: We have optimized a robust screening platform using tissue-specific Gal4 drivers to direct the expression of gene-specific dsRNA from UAS-based transgenic RNAi lines in flies that co-express GFP-tagged Rab4. This allows convenient observation of defects in Rab4 co-localization [redacted] upon knockdown of key intermediates between HTT and Rab4. I will use two Gal4 drivers for complementary screening: 1) oocyte-specific Gal4 (oocyte-Gal4), and 2) a potent broadly expressed tubulin-based Gal4 (Tub-Gal4). As a validation of our approach, preliminary data show that when Tub-Gal4 drives UAS-dHtt-RNAi, compared to negative controls, it can recapitulate the HTT-KO phenotype: Rab4 loses its association with [redacted]. Although Tub-Gal4 gives a robust phenotype, it often has issues of animal lethality due to its strong and ubiquitous expression, precluding analysis of those essential genes. To bypass this, we will also use the oocyte-specific oocyte-Gal4 driver to deplete target genes in the oocyte, a tissue that is highly active in endolysosomal trafficking but dispensable for animal viability.

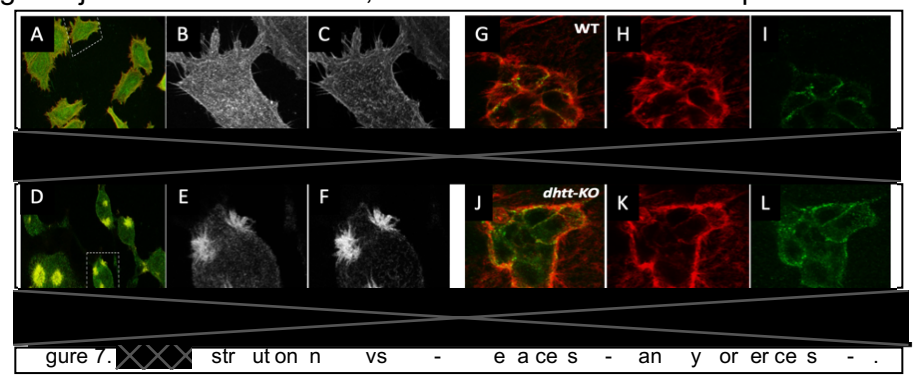
To perform the assay, Gal4 females (Tub or oocyte) will be crossed with UAS-RNAi males, and their F1 progenies carrying both Gal4 and UAS-RNAi will be dissected and analyzed for Rab4 and endosomal phenotypes. I will prioritize the screen first on the known HTT- and Rab4- interactors that are conserved across species. Importantly, many HTT (>1000) and Rab4 (>50) interactors, either directly or indirectly interact with HTT, HAP40 and/or Rab4, have been isolated through various biochemical and proteomic studies, and most of them are conserved[6,7,21]. Given the limitations of the sensitivity and specificity of co-IP/mass-spec, many of the reported hits are likely low confidence. To address this, I will first use stringent criteria to select interactors with the 'highest confidence' that have been reported in multiple independent studies. If we do not identify our hit within the first few rounds of screening, I will expand our search using less stringent criteria which is both feasible and cost-friendly with our system. Analysis and Rigor: With our platform, I have already begun and will be able to continue to conveniently screen 100-200 cost-effective RNAi lines per month. Confocal images will be taken on a Leica and/or a Nikon AXR microscope to check Rab4's localization and other endosomal phenotypes as described above. Five representative samples (N=5) from each F1 genotype will be imaged to ensure data robustness. Samples with clear phenotypes will be repeated to confirm reproducibility. Sterility will be recorded to test whether the eggs laid by F1 females (F2 generation) hatch. Given that *dhtt-* and *dhap40-ko* flies are fertile and viable, we expect RNAi suppression of partners to be similar. [redacted] staining will serve as an internal control for the study, since [redacted] endosomes are not affected in *dhtt-* and *dhap40*-ko (aside from becoming smaller). Interpretations: We expect RNAi knockdown of key regulators on HTT recruitment of Rab4 to mirror the phenotype of *dhtt-ko* and *dhap40-ko* flies, causing the disassociation of Rab4 from [redacted] endosomes and other similar defects such as smaller endosomes and normal [redacted] levels.

Potential Pitfalls/Alternative Approaches: Although our preliminary computer simulations help to inform our experimental clues for Aim1, the predictions alone may be inaccurate. An alternative is to focus only on regions predicted by AlphaFold2 with the highest IDDT scores, a validated measure for how computer predictions compare to known experimental data[27]. Nonetheless, domain-mapping in Aim1a will still be feasible and valuable to identify the structural sites critical for the core complex functions in endolysosomal trafficking. For the screening in Aim1b, it is possible that the key partners proposed in the two priority gene groups will not give us ideal 'hits'. An alternative will be to expand our search to include genes involved in lipid and membrane fusion, such as 1) SNAP/SNARE genes, given the small endosome phenotypes in *dhtt-ko* and *dhap40-ko* flies that implicate potential membrane fusion defects, and 2) other components of the endocytic process, such as lipid regulators and V-ATPase pump components that are known to be important for endosomal trafficking and maturation[28,29].

AIM 2: Determine the functional consequence of HTT recruitment of Rab4 to [redacted] endosomes.
Rationale: Elucidating the functional significance of HTT recruitment of Rab4 to [redacted] endosomes, and the physiologic outcomes using Rab4 cargos as a readout, will shed light on an important biological question for how the HTT protein, involved in HD, regulates the fate of cellular cargos. *Hypothesis: HTT recruits Rab4 to [redacted] endosomes to facilitate proper recycling and degradation of Rab4-cargos.* **Overview of Experimental Design:** To test the raising question on the functional significance of HTT recruitment of Rab4 to [redacted] endosomes, we work with Dr. Travis Moore to use cultured mammalian cells (HeLa, HEK293T in a WT and HTT-KO background) to perform super-resolution imaging (STED and SIM) of Rab4 cargos such as [redacted].

Using these powerful microscopies, we will be able to detect whether HTT depletion causes defects in Rab4 cargo translocation (STED) or trafficking over time (SIM). We will further test whether HTT gene dosage affects the functions and activation of Rab4 GTPase in vivo by testing various Rab4 forms (CA, DN, KO) in a WT, heterozygous and HTT-KO backgrounds. Given that our group has already established these tools, we are in a strong position to address how HTT genetically or functionally interacts with Rab4.

Aim 2A: Test how HTT modulates Rab4 cargo trafficking in normal states in vitro. The fact that HTT is a critical regulator of Rab4 suggests that HTT levels should alter subcellular trafficking of Rab4's cargo. In support of this hypothesis, our preliminary studies in both fl e chambers and cultured mammalian cells (HeLa) show that HTT depletion causes defects in trafficking of [redacted], a known cargo of Rab4[22-24], leading to abnormal actin accumulations (Fig 7). Rab4 activity and its membrane association are intimately linked to its binding status with GTP or GDP, with Rab4 in GTP-bound form being active and recruited to endosome, while GDP-bound Rab4 is inactive and released from the endosomal membrane via GDP-dissociation inhibitor (GDI) proteins[25,26]. One potential mechanism of Rab4 recruitment by HTT/HAP40 is to control Rab4 activation, such as through stimulating Rab4 GEFs, or by preventing its inactivation by inhibiting Rab4 GAPs. Additionally, as endosomal trafficking is a highly dynamic process involving continuous steps of cargo recognition and endocytosis to its subsequent sorting for recycling or degradation fates, it is unclear at which step(s) HTT affects [redacted] and whether its regulation is indeed through Rab4. Using [redacted] as the readout of HTT-mediated cargo trafficking, I will carry out super-resolution microscopy (STED and SIM) to examine how HTT affects the trafficking dynamics of Rab4 and [redacted], and whether HTT's effect on [redacted] trafficking can be modulated by distinct Rab4 forms. Experimental Approach: In cultured normal, and stable HTT-KO and HAP40-KO cells that our lab has established[15], I will carry out biochemical assays to examine the functional status of Rab4 by transiently transfecting constructs harboring [redacted] and Rab4-mTagRFP to trace their subcellular localization over time. In parallel, by co-expressing dominant negative (DN, permanently GDP-bound) or constitutive active (CA, permanently GTP-bound) Rab4 in normal or HTT-KO cells, I will examine whether HTT's effect on [redacted] trafficking is affected by perturbed Rab4 activity. Analysis and Rigor: Image acquisition will be performed at UT Microscopy core and analyzed using ImageJ/Fiji. For in vitro studies, conditions will be run in triplicate and repeated N=3 independent times. Interpretations: We expect that in the absence of HTT or HAP40, [redacted] will show an increased recycling to cell surface through Rab4 and a concurrent reduced degradation through [redacted] and lysosomes, which will be consistent with the dissociation of Rab4 from [redacted] endosomes.



gure 7. [redacted] str ut on n vs - e a ce s - an y or er ce s - .

Aim 2B: Test if HTT depletion causes loss- or gain-of-function of Rab4 in vivo. Experimental Approach: Complementing the above cell-based studies, I will co-express [redacted] together with over-expressed form of Rab4 (WT, CA, DN) in border cells of *Drosophila* egg chambers and use regular laser-scanning confocal microscopy to determine their spatial relationship over time in normal, *dhtt-ko* and *dhap40-ko* backgrounds. In parallel to HTT/Rab4-genetic interaction tests, we will determine whether there are functional defects in i [redacted] trafficking and signaling by examining the subcellular distribution of phosphorylated [redacted], a downstream effector of activated [redacted]. Given that border cell migration involve active [redacted] dynamics, we will perform functional migration assays characterizing the position of border cells at stage 8 (migration not initiated), 9 (border cells de-laminate and start their migration) and 10 (border cells reach the oocyte, the final target) in flies overexpressing WT, CA or DN Rab4 in the presence or absence of endogenous HTT. Data Analysis and Rigor: Confocal images of at least n=20 egg chambers will be collected from N=18 adult flies dissected for each genotype. Interpretations: We expect that overexpression of Rab4-WT or Rab4-CA will cause migration defects due to an increased lysosomal degradation of [redacted], and loss of HTT or HAP40 will rescue these defects by compromising the Rab4-mediated degradation of [redacted] and promoting its recycling back to cell surface.

Potential Pitfalls/Alternative Approaches: It is possible that the Rab4-cargos are shared by Rab11, a protein involved in slow recycling[30]. We can introduce a dominant-negative (DN) form of Rab11 or Rab11RNAi, tools

that are available[31], in flies and mammalian cells to identify Rab4-specific cargos. Further, as an alternative to [redacted], we can also test other known Rab4 cargos, e.g., glutamate transporters EAATs[32] in HTT-KO settings.

AIM 3: Test the cellular and physiologic consequences of HD-causing mutant HTT on Rab4.
Rationale: It is believed that in addition to a gained toxicity from expanded polyQ tract, alteration of HTT's normal physiological functions is also an important contributing factor in HD pathogenesis[33-35]. Given the strong effect of HTT and HAP40 on Rab4 regulation in vivo, it is important to determine how polyQ expansion in HTT affects Rab4 regulation and whether Rab4 modulates HD pathogenesis. Results from the study should give critical insight into the underlying pathogenic mechanism and uncover novel therapeutical targets for HD. *Hypothesis: HTT regulates Rab4-cargo trafficking dynamics in a polyQ-length-dependent fashion.* **Overview of Experimental Design:** To test if polyQ expansion alters HTT/HAP40's regulation on Rab4, we will follow a similar paradigm as in Aim1 and 2, including to examine in vivo and in vitro the effect of polyQ expansion in HTT on recruitment of Rab4 and its cargos to [redacted] endosomes. Given that *Drosophila* HTT (dHtt) does not harbor a polyQ tract, we will focus on mammalian cell lines and transgenic fly lines that express human HTT with different polyQ lengths (23, 75, 145), and compare their effect on Rab4 protein function or activation (Aim3a). Lastly, using an established fly model of HD, I will test if Rab4 depletion modulates HD pathogenesis (Aim3b).

Aim 3A: Test how mutant HTT alters Rab4 normal functions. Longer polyQ in HTT might enhance (gain-of-function) or reduce (loss-of-function) its regulation on Rab4 or confer a novel cellular function (neo-function) on Rab4, such as by mis-targeting Rab4 and its cargos. Experimental Approach: In *Drosophila*, our lab has established a set of transgenic fly lines for normal (Q23) and mutant (75Q, 128Q, 145Q) full-length human HTT, which allow targeted expression of these proteins in different fly tissues including fly neurons[15]. We will test whether mutant HTTs affect the sizes of endosomes, Rab4's subcellular distribution, and as well as the trafficking dynamics of Rab4 cargos (*e.g.,* [redacted], glutamate transporters (EAATs), GABA receptors (GABARs) or neuroglians (Nrg)) in neuromuscular junction (NMJ) and dendritic arborization (DA) neurons. Data Analysis and Rigor: For in vivo studies, I will image N=5 animals per genotype. I will analyze NMJ by the number and morphology of pre-synaptic boutons and the localization of Rab4 cargos, whereas for DA neurons, I will measure dendritic complexity and pruning (via Sholl Analysis). Interpretations: If flies expressing polyQ-HTT show phenotypes similar as HTT-KO such as mistargeted Rab4 or its cargo [redacted], it would suggest polyQ expansion compromises HTT's normal functions. Conversely, opposite effects on Rab4 and its cargo [redacted], such as increased Rab4 association with [redacted] or decreased [redacted] levels would indicate a GOF effect by polyQ. Any novel phenotypes could indicate a neo-function effect by polyQ on HTT.

Aim 3B: Test whether distinct Rab4 forms modulate HD pathogenesis in vivo. Recently we showed that in a *Drosophila* model of HD, dHap40 depletion partially suppressed the neurodegenerative severity induced by mutant HTT [15]. Using the same well-validated HD model, I will test how Rab4 affects neurodegeneration associated with mutant HTT. Experimental Approach**:** To test if Rab4 depletion or over-expression modulates mutant HTT toxicity, I will use multiple neuronal Gal4 drivers (Nsyb-, Elav- and GMR-Gal4) to direct the expression of normal (Q23) and mutant HTT (75Q, 128Q, 145Q) in Rab4-KO background, or co-expression together with WT, CA or DN-Rab4, and interrogate if any of the genetic manipulation modulates HD pathogenesis. Data Analysis and Rigor: Imaging will be performed on neuronal cell bodies within the adult eye stained with phalloidin, and the number of intact photoreceptor units (i.e., rhabdomeres and ommatidia) will be quantified to evaluate the severity of neurodegeneration. Interpretations: If Rab4 is important for mutant HTT toxicity, then we expect modulation of Rab4 would affect the severity of neurodegeneration in our HD models.

Potential Pitfalls/Alternative Approaches: One pitfall of Aim3 is the relevance of *Drosophila*-based findings on neurodegeneration to mammalian cells. To circumvent this, we can use our established HD models in cultured neuronal cells from human (SHS5Y cells) and rat (i.e., PC12 cells) that stably express normal or mutant full-length HTT. Using cell-death assays, we can assess whether altering the expression and activity of Rab4 will affect neuronal toxicity induced by polyQ-HTT. In addition, we can use similar methods as described in Aim2 to assess the trafficking of Rab4 cargos in these neuronal cells. This would expand our study on mutant HTT in mammalian neuronal cells that are highly amendable for super-resolution imaging methodologies, biochemistry and gene expression manipulations, potentially providing an alternative avenue to dissect the relationship between Rab4 and mutant HTT.

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