Huntingtin S421 phosphorylation increases kinesin and dynein engagement on early endosomes and lysosomes

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Summary Statement
Huntingtin phosphorylation at S421 increases the outward motility of early endosomes and lysosomes by enhancing the microtubule binding and force generation of kinesin and dynein.

ABSTRACT

Huntingtin (htt) acts as a scaffolding protein that recruits motor proteins to vesicular cargoes, enabling it to regulate kinesin- and dynein-dependent transport. To maintain the native stoichiometry of huntingtin with its interacting partners, we used CRISPR/Cas9 to induce a phosphomimetic mutation of the endogenous htt at S421 (S421Dhtt). Using single-particle tracking, optical tweezers, and immunofluorescence, we examined the effects of this mutation on the motility of early endosomes and lysosomes. In S421Dhtt cells, early endosomes and lysosomes exhibited more outward motility towards the cell periphery compared to wild-type (WT) cells. In contrast, overexpression of htt had variable effects on the processivity, run length, and directional bias of both early endosomes and lysosomes. Kinesins and dyneins exerted greater forces on early endosomes and lysosomes in cells expressing S421Dhtt. Additionally, endosomes had higher binding rates, increased resistance to detachment under load, and enhanced recruitment of kinesins and dyneins in S421Dhtt cells. These data indicate that phosphorylation of the endogenous huntingtin causes early endosomes and lysosomes to move towards the cell periphery by activating both kinesins and dyneins.

List of Symbols and Abbreviations

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<thead>
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<tr>
<td>α</td>
<td>Processivity parameter alpha</td>
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<tr>
<td>BDNF</td>
<td>Brain derived neurotrophic factor</td>
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<td>DIC</td>
<td>Dynein intermediate chain</td>
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<td>Early</td>
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<td>EGF-qdot</td>
<td>Epidermal growth factor coated quantum dot</td>
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<td>HD</td>
<td>Huntington’s Disease</td>
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<td>KHC</td>
<td>Kinesin heavy chain</td>
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<td>ks</td>
<td>Kolmogorov-Smirnov statistical test</td>
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<td>latA</td>
<td>Latrunculin A</td>
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<td>NZ</td>
<td>Nocodazole</td>
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<td>$R_g$</td>
<td>Radius of gyration</td>
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<td>S421Ahtt</td>
<td>Phosphoresistive huntingtin with the mutation S421A</td>
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INTRODUCTION

Huntingtin (htt) is an essential, multifunctional, and ubiquitous protein. It consists of several disordered regions and 34 α-helical HEAT repeats (Fig. 1A) (Guo et al., 2018).

Htt is essential for embryonic development (Duyao et al., 1995; Nasir et al., 1995; Schmitt et al., 1995; Zeitlin et al., 1995), and is ubiquitously expressed in tissues including the brain, ovaries, and testis into adulthood (Li et al., 1993; Sharp et al., 1995). It has diverse roles within the cell, including regulation of gene expression (Dunah et al., 2002; Futter et al., 2009; Holbert et al., 2001; Li et al., 2002; Marcora et al., 2003; Zuccato et al., 2003), cell morphology (Lo Sardo et al., 2012; Thion et al., 2015), cell survival (Leavitt et al., 2006), and intracellular transport (Cason et al., 2021; Gauthier et al., 2004; Liot et al., 2013; Twelvetrees et al., 2010; Wong and Holzbaur, 2014). Htt enhances transport of brain derived neurotrophic factor (BDNF) vesicles (Gauthier et al., 2004; Zala et al., 2008), autophagosomes in the mid-axon (Cason et al., 2021; Wong and Holzbaur, 2014), TrkB signalling vesicles (Liot et al., 2013), and synaptic vesicles through GABA\(_R\) signalling (Twelvetrees et al., 2010). The remarkable ubiquity and variety of htt’s roles underscore the importance of understanding its function in health and disease.

Teams of kinesins and dyneins transport signalling and degradative cargoes. There are multiple kinesins that drive outward transport towards microtubule plus ends at the cell periphery. Different ratios of kinesin-1, kinesin-2, and kinesin-3 associate with distinct cargo populations, allowing different motility characteristics for each population of vesicles (Bentley et al., 2015). Each type of kinesin has a unique binding rate and velocity, which leads combinations of motors to create a motility barcode for cargoes. Unlike kinesins, there is only one form of cytoplasmic dynein that drives organelle transport. Dynein forms a complex with dynactin and activating adaptors such as BicD, Hook1, and others to direct inward transport towards microtubule minus ends at the cell center (Cason et al., 2021; McKenney et al., 2014; Schlager et al., 2014). Scaffolding and adaptor proteins work together to regulate the recruitment and activity of both kinesin and dynein motors on each cargo based on its identity.
Huntingtin recruits teams of kinesin, dynein, and myosin VI motors to cargoes, allowing it to control the cargo’s direction of movement along microtubules and switching between actin and microtubules (Caviston et al., 2011). Htt interacts with molecular motors indirectly through adaptors; kinesin-1 and the dynein adaptor dynactin interact with htt through HAP1 (Engelender, 1997; Li et al., 1998; McGuire et al., 2006; Twelvetrees et al., 2010), and myosin VI interacts through optineurin (Sahlender et al., 2005). In addition, dynein interacts directly with htt through its intermediate chain, suggesting a prominent role for htt in regulating inward transport (Caviston et al., 2007). Htt’s interaction with myosin VI suggests htt regulates transitions to the actin cytoskeleton for short range transport or tethering. Upon htt deletion or mutation, intracellular transport of many vesicular cargoes is severely inhibited (Caviston and Holzbaur, 2009; Hilditch-Maguire, 2000).

Polyglutamine expansion mutations in htt lead to Huntington’s disease (HD), disrupting multiple cellular functions and leading to neurodegeneration in medium spiny neurons of the striatum (Gil and Rego, 2008). HD mutations cause htt to form novel interactions. Notably, through increased self-association mutant htt forms aggregates throughout the cytoplasm and nucleus (Davies et al., 1997). These interactions cause aberrant initiation of autophagy, mTOR signalling, mitochondrial fission, and palmitoylation (reviewed in Wanker et al., 2019). Additional defects occur in neuronal transcription, endoplasmic reticulum homeostasis, mitochondrial function, calcium signalling, synaptic activity, and intracellular transport (reviewed in Saudou and Humbert, 2016), demonstrating the importance of htt’s native interaction network in maintaining homeostasis (Colin et al., 2008; Lemarié et al., 2021; Zala et al., 2008).

Huntingtin is regulated by multiple post-translational modifications, including palmitoylation, phosphorylation, sumoylation, ubiquitination, and acetylation. The functions and mechanisms of many of these modifications remain unknown. Its palmitoylation site at C214, regulated by HIP14 in the Golgi, facilitates interactions with vesicular membranes (Yanai et al., 2006). Palmitoylation is reduced in cells expressing the polyglutamine-expanded htt protein that leads to HD, while restoring palmitoylation
reduces aggregation and cytotoxicity in vitro (Lemarié et al., 2021). Sumoylation sites may contribute to nuclear import and transcriptional regulation (Steffan et al., 2004). Ubiquitination and acetylation regulate huntingtin-mediated autophagy (Wong and Holzbaur, 2014). Htt has 40 known phosphosites, many of which have unknown biological functions (reviewed by Saudou and Humbert, 2016). The phosphorylation state of serine 421 affects axonal transport direction and velocity of BDNF vesicles and autophagosomes (Colin et al., 2008; Wong and Holzbaur, 2014), while mitochondrial transport is unaffected by this phosphosite (Xu et al., 2020). Each of htt’s post-translational modifications contribute to its role in transport of both signaling and degradative cargoes. Further, post-translational modifications are reduced in HD, disrupting the balance of htt’s interactions with its binding partners (Colin et al., 2008; Lemarié et al., 2021; Zala et al., 2008).

Post-translational modifications of scaffolding proteins can influence transport direction. The phosphomimetic mutation (S421Dhtt) mimics a constitutively phosphorylated amino acid and biased BDNF transport towards the cell periphery, while phosphoresistive mutations (S421Ahtt) led to increased transport towards the cell center (Colin et al., 2008). The S421Dhtt mutation is neuroprotective in cells with the mutation that leads to HD (Zala et al., 2008), which motivates our attempt to understand the native role of this phosphosite in cells with normal (WT) htt. It has also been generalized for various scaffolding proteins including RILP, Miro/TRAK1/TRAK2, JIP1, JIP3/4 that post-translational modifications can modify transport direction (Fu and Holzbaur, 2014). These studies suggest that huntingtin phosphorylation directs transport towards the cell periphery.

To elucidate the effects of S421 phosphorylation at native expression levels and determine the mechanisms of regulation for both early endosomes and lysosomes, we applied CRISPR-based gene editing and single-molecule biophysics. Previous studies analyzed the motility of cargoes overexpressing S421Dhtt or S421Ahtt constructs and performed Western blotting to determine S421’s role in transport regulation (Colin et al., 2008; Zala et al., 2008). Western blot and immunofluorescence in overexpressing COS7
cells suggests that S421 phosphorylation increases the recruitment of kinesin to cargoes (Colin et al., 2008; Zala et al., 2008). However, only a subset of the motors bound to a cargo might be active (Encalada et al., 2011). In addition, the critical stoichiometry of interactions between scaffolding proteins and motors is disrupted in overexpression systems. We generated an endogenous mutation to replicate htt’s native interactions and expression levels and observed its effect on organelle transport. We measured the forces generated by the motors driving early endosome and lysosome transport with optical tweezers to determine whether phosphorylation of huntingtin modifies motor activity. Single particle tracking allowed us to obtain nanometer-resolution tracking of cargo motility. Combining single-molecule biophysical approaches with gene editing provided mechanistic insight into htt’s regulation of intracellular transport by S421 phosphorylation.

To understand how S421 phosphorylation of htt regulates intracellular transport, we examined early endosome and lysosome transport in CRISPR-generated S421Dhtt HEK293T cells. Motility assays demonstrated an increase in outward (microtubule plus-ended) motility for both early endosomes and lysosomes in cells expressing S421Dhtt. Additionally, lysosome run length increased upon S421 phosphorylation. Examining the forces generated by microtubule motors on early endosomes and lysosomes, we observed both kinesin and dynein motors had higher binding rates and increased resistance to unbinding under load with S421Dhtt for both cargoes. Cells with S421Dhtt showed higher forces in both outward and inward directions on both early endosomes and lysosomes. A higher fraction of kinesins and dyneins associated to microtubules upon S421 phosphorylation. The phosphorylation-induced change in directional bias and force generation for lysosomes was more subtle than those for early endosomes. We hypothesize that the native motility characteristics of the cargoes dictate the effect of S421 phosphorylation on htt: inward-directed early endosomes become bidirectional while bidirectional lysosomes increase their displacement. Our data indicates phosphorylation of htt at S421 increases motor recruitment and activity of early endosomes and lysosomes. Additionally, overexpression of WT and S421D htt suggests overexpressed htt disrupts the transport complex of htt, motors, and adaptors required for transport, affecting cargo run length, processivity, and direction.
RESULTS

125 Endogenous S421D mutation does not affect cytoskeletal organization or huntingtin localization

Introducing a phosphomimetic S421D mutation in the endogenous huntingtin did not alter huntingtin expression levels and localization or the organization of the cytoskeleton and golgi. In generating the S421D HEK293T cell line, we aimed to preserve the native expression levels and stoichiometry of interactions between htt and its many interactors. We used a lentiviral CRISPR cassette to generate this S421D point mutant cell line (Fig. 1B) and identified a single positive clone with the desired mutation using next-generation sequencing (Fig. 1C). S421D htt did not result in appreciable changes to the actin nor microtubule morphology (Fig. 1D). To determine whether S421D htt caused major defects in dynein function, we probed the organization of the Golgi apparatus (Burkhardt et al., 1997; Caviston et al., 2007; Corthésy-Theulaz et al., 1992). We observed no changes in the percentage of cells with organized versus dispersed Golgi, indicating dynein function was not severely disrupted (Fig. 1E). Importantly, the S421D mutation did not affect huntingtin’s localization nor expression level (Fig. 1F, G). Overexpression of mCherry-WT htt or mCherry-S421D htt leads to elevated expression of both constructs at similar levels (Fig. 1G). In iPSC-derived neurons expressing polyglutamine-expanded huntingtin (180Q), S421D htt led to increased mitochondrial surface area while the number of mitochondria remained similar (Xu et al., 2020). Immunofluorescence of the mitochondria in HEK293T cells showed a decrease in the mitochondrial number and area upon S421D mutation (Fig. 1H-J), suggesting that S421D htt mitochondrial regulation may be cell-type dependent. Together, immunofluorescence and huntingtin expression level characterization demonstrated that the S421D mutation did not substantially alter the cytoskeleton, Golgi apparatus, nor localization of huntingtin while mitochondrial morphology was affected.

154 Huntingtin phosphorylation directs early endosomes towards the cell periphery

155
Introducing the S421D phosphomimetic mutation in the endogenous huntingtin gene induced an outward directional bias in early endosomes but did not alter run length nor processivity. We hypothesized that the processivity, run length, and fraction of outwardly-directed early endosomes would increase upon S421 phosphorylation based on previous work with BDNF vesicles in neurons (Colin et al., 2008). We quantified the processivity of organelle trajectories using the slope of the log-log plot of mean-squared displacement ($\alpha$). For cargoes transported by microtubule-based motor proteins, we expect that $\alpha > 1$ is indicative of directed transport, while $\alpha = 1$ indicates diffusive motion. The radius of gyration ($R_g$) is an indicator of run length that determines the average position of a trajectory and measures a radius containing half of the points in the trajectory from its average position. We transfected cells with Rab5-eGFP to label early endosomes for particle tracking (Fig. 2A, C). The endogenous S421D mutation increased the outward motility of early endosomes towards the cell periphery without changing their processivity or run length (Fig. 2E, F, I). The outward directional bias was previously described when truncated WT htt and S421D htt were overexpressed in primary neurons (Colin et al., 2008; Zala et al., 2008). To dissect the effects of htt phosphorylation and expression levels, we transiently transfected full length mCherry-WT htt (WT+WT htt) or mCherry-S421D htt (WT+S421D htt). These transfections lead to a ~4-10-fold increase in htt expression (Fig. 1G). Overexpression of S421D htt increased the processivity, run length, and unexpectedly induced inward motility of early endosomes (Fig. 2E, F, I). Interestingly, overexpression of WT htt increased the outward motility of early endosomes (Fig. 2I). These unexpected results in overexpression conditions may be due to the change in stoichiometry of htt’s interactions with its native binding partners. The gene edited S421D HEK293T cells demonstrate an outward bias of early endosomes, while htt overexpression alters motility. This indicates that S421 phosphorylation is either recruiting additional kinesins on early endosomes or increasing the activity of the kinesins present on the early endosome.

S421D increases run length and outward bias of lysosomes
S421 phosphorylation increased lysosome run lengths and directed them toward the cell periphery. We hypothesized S421Dhtt’s effects to be universal for all htt-associated cargoes, and therefore expected increased outward bias, processivity, and run length in lysosomes. This hypothesis is supported by previous work showing that htt promotes outward motility in the mid-axon of neurons for autophagosomes, which eventually mature into lysosomes (Cason et al., 2021; Wong and Holzbaur, 2014). In both WT and S421D experiments, the raw trajectories demonstrated the expected bidirectional motility (Fig. 2B, D). S421Dhtt induced an increase in run length and outward motility of lysosomes, without affecting their processivity (Fig. 2G, H, J). When we overexpressed htt, we observed a minor increase in processivity for WT htt and a substantial increase in run length regardless of the phosphorylation state (Fig. 2G, H, J). Unexpectedly, WT htt overexpression led to an inward directional bias (Fig. 2J), while overexpression of S421D htt reversed the effect by increasing outward motility. Taken together, our results indicate that htt overexpression modifies processivity, run length, and directional bias while phosphorylation shifts lysosome motility towards the cell periphery and increases their run length. This suggests S421 phosphorylation at native expression levels is increasing the number of active kinesin motors, as was observed for early endosomes.

Tethering on actin contributes to the directional bias of early endosomes

Our results suggest that huntingtin’s phosphorylation state modulates transient actin tethering and contributes to the transport direction of early endosomes. Huntingtin interacts with optineurin, which recruits myosin VI and could regulate cargo association to actin (Sahlender et al., 2005). We used latrunculin A (latA) to depolymerize actin filaments and reduce cargo association with actin. We expected an increase in run length and processivity upon latA treatment since cargo tethering and confinement by actin are reduced, allowing long range, processive motility by microtubule motors to dominate. We determined the optimal concentration for motility assays via imaging cells labelled with SiR-actin. We determined 250 nM latA was optimal as it resulted in significant depolymerization of actin filaments without loss of native cellular morphology (Fig. S1A). As expected, treatment with latA increased run length and processivity of Rab5 early
endosomes (Fig. S1B, C). Additionally, the phosphorylation-induced directional bias became less prominent with latA, suggesting that short-range actin-based motility contributes to the observed directional bias of early endosomes (Fig. S1D). WT early endosomes have increased outward motility upon latA treatment, indicating that allowing kinesin and dynein to dominate transport biases it outwards (Fig. S1D). The S421D mutation did not further shift the directional bias in actin-depleted cells, suggesting that both loss of actin tethering and htt phosphorylation act through similar mechanisms.

Lysosomes have a higher dependence on microtubule-based transport

Depolymerization of microtubules demonstrated no significant changes in processivity or run length for early endosomes, while lysosomes showed significant decreases in both run length and processivity. We used 10 μM nocodazole (NZ) to depolymerize microtubules and observe how strongly the motility parameters were affected. We expected that upon NZ treatment, both processivity and run length would decrease because microtubule-based motors drive the long range processive transport of both early endosomes and lysosomes. We observed this expected decrease in run length and processivity for lysosomes, suggesting that they rely heavily on microtubule-based transport to drive their motility (Fig. S1F, I, J). The large change in processivity and run length for lysosomes demonstrates the magnitude of changes in these parameters upon large scale disruption of the cytoskeleton. Early endosomes demonstrated a surprising lack of effect of nocodazole treatment on their processivity and run length, perhaps due to their inherently low processivity and run lengths compared to lysosomes (Fig. S1E, G, H). The lysosomes in NZ-treated cells reached similar values for processivity and run length to the early endosomes in the control condition, suggesting that the values for early endosome motility were close to their minimum. Combining this result with our conclusions from the latA treatment demonstrates that early endosome transport may be more dependent on switching between actin-based and microtubule-based transport (Fig. S1). These results indicate early endosome transport has a lower dependence on microtubules compared to lysosomes, perhaps due to its inherently short run lengths and lower processivity.
S421 huntingtin phosphorylation activates kinesins and dyneins on early endosomes

To determine how changes in motor activity contribute to the motility we observed, we measured the force generation of motors on early endosomes with optical tweezers. We incubated cells with fibronectin-coated 500 nm polystyrene beads for 10-50 mins (Blocker et al., 1997) and used optical tweezers to measure the forces that motors exerted in living cells (Fig. 3A, B). At this stage of maturation, the early phagosomes (termed early endosomes) are positive for Rab5 and other early endosome markers and represent a subpopulation of the vesicles labeled by Rab5-eGFP. Early endosome force traces demonstrated the expected bidirectional motility biased inwards (Fig. 3B). We predicted kinesins would specifically be activated, or dyneins inactivated to generate the directional bias we observed for early endosomes (Fig. 2I). Unexpectedly, the stall force distributions for early endosomes shifted towards higher forces in both outward and inward directions with S421Dhtt (Fig. 3C), suggesting both kinesins and dyneins were activated. In cells, each kinesin motor exerts forces of 2-7 pN, and each dynein exerts approximately 2-3 pN (Blehm et al., 2013). Looking closely at the distributions, we observe frequent kinesin forces around 3 pN and 6 pN for cells with WT htt (Fig. 3C). Both of those populations are observed in S421Dhtt cells, in addition to high force events near 9 pN and 12 pN. Observing the inward directed forces, WT cells show frequent dynein forces near 1.5 pN and 5 pN, while forces shift to ~ 3.5 pN, 9 pN, and 13 pN in S421Dhtt cells. Together, these forces likely correspond to ~1-2 active kinesins and 1-3 active dyneins in WT cells, and 1-4 active kinesins and 2-6 active dyneins in S421D cells, which is slightly lower than previous estimates for late phagosomes (Chaudhary et al., 2018; Hendricks et al., 2010). We then sought to determine how the force-dependent unbinding rates of each motor were affected by S421Dhtt, predicting that the kinesin motors would have slower unbinding and therefore induce the kinesin-based directional bias (Fig. 2I). The unbinding rates of kinesin and dynein teams on early phagosomes in WT cells is similar (Fig. 3D, Table S1). We observed slower unbinding under load of both kinesin and dynein teams with S421Dhtt, consistent with more kinesin and dynein motors engaged with the...
microtubule (Fig. 3D, Table S1). Next, we measured the binding rate of early endosomes moving inwards and outwards and found the binding rate for early endosomes was increased for both directions in S421Dhtt cells (Fig. 3G, H). The binding rate was more strongly enhanced for outward forces, suggesting an increased number of active kinesins enhances binding compared to WT htt (Fig. 3G). Observing the cumulative distribution functions, we note that outward and inward motor binding rates in S421Dhtt cells were very similar (Fig. 3H). This suggests that the kinesins are being activated to the same level as the dyneins, reducing the native inward bias for these cargoes. In summary, the force data indicate that huntingtin phosphorylation at site S421 increases the net force generation, affinity, and binding strength of both kinesin and dynein motors in early endosomes.

**S421Dhtt increases lysosome microtubule binding and resistance to unbinding under load**

Like for early endosomes, we hypothesized that kinesins would have increased activity on lysosomes as they also demonstrated an increased run length and outward directional bias (Fig. 2H, J). We measured the force characteristics of phagolysosomes by modifying the optical tweezers assay described for early endosomes with a longer incubation time of 1-2 hrs. The phagolysosomes measured in the optical tweezer experiments, termed lysosomes hereafter, represent a subpopulation of lysotracker-positive lysosomes, compared to our earlier analysis tracking all lysotracker-positive vesicles (Fig. 2). Sample force traces demonstrated typical bidirectional motility of lysosomes (Fig. 3B). We observed an increase in forces in both directions for lysosomes as for the early endosomes, although to a lesser degree (Fig. 3E). The balance of kinesin to dynein forces remained similar in both control and S421Dhtt conditions, as expected for these bidirectional cargoes. Predicting the number of motors as described for early endosomes, the force distributions suggest that 1-2 additional kinesins and 1-2 additional dyneins are engaged in S421Dhtt cells. The unbinding rates indicate that kinesin teams are more resistant to unbinding than dyneins for lysosomes (Fig. 3F). In S421Dhtt cells, we observed a slightly lower resistance to unbinding for lysosomes moving outward, while...
the unbinding resistance was higher than WT for inward-moving cargoes (Fig. 3F, Table S1). Finally, we examined the binding rates for lysosome motors to determine how S421Dhtt affected microtubule affinity. S421Dhtt increased the binding rate for both inward- and outward-directed cargoes compared to WT (Fig. 3I, J). The increased forces and enhanced binding we observed in S421Dhtt cells suggests htt phosphorylation recruits and/or activates both kinesins and dyneins.

**S421Dhtt increases kinesin and dynein association to microtubules**

As we did not observe increased kinesin activity compared to dynein upon S421 phosphorylation, we asked if more kinesins were engaged with microtubules to generate the observed directional bias. We used immunofluorescence imaging to approximate the differences in the number of motors associated with microtubules in each cell type. We fixed the cells with methanol to remove cytosolic components and determine the relative number of motors that were bound to microtubules in each condition at the time of fixation (Fig. 4A, B). We then created intensity heatmaps of all images from the microtubules and kinesin-1 heavy chain (KHC) or dynein intermediate chain (DIC) and plotted them on the same axes (Fig. 4C-F). We detected higher intensities in the motors channel for both KHC and DIC in the S421Dhtt cells (Fig. 4C-F). The higher intensity indicates a higher concentration of motors on extracted microtubules in the S421Dhtt cells. Upon calculating the correlation coefficients between the microtubule and motor channels for each individual image, we observed that the dynein and microtubule intensities were more highly correlated in S421Dhtt cells than WT cells. This assay demonstrates that S421Dhtt increases the fraction of kinesin and dynein motors associated to microtubules. This supports our data from the optical tweezers analysis, which showed a higher binding rate and lower unbinding rate for motors in the S421Dhtt cells in both early endosomes and lysosomes. Further, the signal for microtubule-bound kinesin and dynein motors is higher in S421Dhtt cells, suggesting htt S421 phosphorylation increases the engagement of both kinesins and dyneins with microtubules.

**DISCUSSION**
Our results show that huntingtin phosphorylation at S421 increases the number of active kinesin and dynein motors on endosomes. Surprisingly, huntingtin activates microtubule motors on endosomes at varying stages of maturation, from early endosomes to lysosomes. Huntingtin also regulates the motility of neuronal signalling vesicles including BDNF (Colin et al., 2008; Zala et al., 2008). In contrast, most cargo adaptors are recruited to specific cargoes at specific stages of maturation (Cason et al., 2021; Mogre et al., 2021). The ubiquitous function of huntingtin on a wide array of cargoes illustrates its role as a central regulator of vesicular transport.

While kinesin and dynein teams exert more force and have enhanced microtubule binding on both early endosomes and lysosomes in S421D huntingtin cells (Fig. 3,4), its effect on the motility of different endocytic cargoes varies. For early endosomes, huntingtin phosphorylation results in a pronounced shift in motility from primarily inward towards the cell center to bidirectional motility (Fig. 2I). The processivity and displacement of early endosomes are only weakly affected (Fig. 2E, F). In comparison, lysosomes display a small but significant shift in motility towards the cell periphery in phosphomimetic htt cells compared to control cells (Fig. 2J) while their run length is strongly enhanced (Fig. 2H). Taken together, these results suggest that huntingtin phosphorylation regulates transport by enhancing the activity of both kinesin and dynein (Fig. 5A, B). However, its effects on the motility of different cargoes depend on their basal motility characteristics. In the basal condition, early endosome motility is strongly biased towards the cell center. Activating both kinesin and dynein results in a loss of the inward bias. Lysosome motility is approximately equally distributed towards the cell center and periphery, such that activating kinesin and dynein does not alter directionality, but rather increases the total run length.

Disrupting actin filaments resulted in an increased outward motility similar to the effect of S421Dhtt (Fig. S1A-D), suggesting a potential role for myosin VI-mediated tethering to actin filaments (Sahlender et al., 2005) in generating the directional bias (Al-Haddad et al., 2001). Yet, optical trapping and immunofluorescence show that S421Dhtt results in
enhanced forces and recruitment to microtubules for both kinesin and dynein (Figs. 3, 4), suggesting S421D phosphorylation instead acts through activating microtubule motors. Thus, we hypothesize that outward directional bias occurs in situations where microtubule motors dominate transport. That could be achieved either by reducing tethering on actin or by engaging more kinesins and dyneins.

Our findings are in broad agreement with previous studies showing that overexpressing S421D htt resulted in increased outward motility of BDNF and TI-VAMP vesicles compared to cells expressing phosphoresistive S421A htt constructs (Colin et al., 2008; Zala et al., 2008). Further, Colin et al. observed increased kinesin-1 recruitment to microtubules and enhanced interactions between the p150glued subunit of dynactin and KHC upon S421D htt expression (Colin et al., 2008). Interestingly, we did not see KHC-specific recruitment to microtubules with endogenous S421D htt compared to wild-type. Instead, we observed an increase in recruitment of both motors, with slightly higher dynein recruitment. These differences between previous results and our observations may reflect effects of htt overexpression. Alternatively, they may be due to differences in htt function in the embryonic kidney cells used here compared to previous studies in neurons, which are highly polarized and have higher endogenous htt expression.

Overexpression of htt alters the processivity, run length, and directional bias of early endosomes and lysosomes. The effects of overexpression are highly variable and often do not reflect the trends observed when inducing mutations in the endogenous htt (Fig. 2), suggesting that maintaining the stoichiometry of htt to its many interacting proteins is critical. We expect that excess htt in the cytoplasm is interacting with and sequestering the motors and adaptors required to generate the effect that we observe in transport upon overexpression (Fig. 5C). This result supports the known consequences of overexpression experiments, which cause adverse effects by changing the stoichiometry of interactions with other proteins in the cell. For example, overexpressing the p50 dynamin subunit of dynactin causes a dominant negative effect in which early endosomes and lysosomes become peripherally localized due to dynein sequestration (Burkhardt et al., 1997). Regardless of the phosphorylation state, overexpression of htt
affected the directional bias of early endosomes and lysosomes. Processivity and run
lengths were increased for lysosomes in cells overexpressing either WT or S421D htt,
however, early endosomes only demonstrated this effect overexpressing S421D htt. This
work, supported by many previous studies discussing expression levels (e.g. (Burkhardt
et al., 1997; Dambournet et al., 2018)) highlights the importance of examining protein
function at endogenous expression levels to ensure biological relevance of the effects.
This is particularly important for scaffolding proteins like huntingtin, which mediates
interactions among many adaptors, effectors, motors, and membranes.

Transport is tightly regulated on multiple levels within the cell to ensure specific cargoes
arrive at their intracellular destination. Scaffolding proteins such as htt are a primary
mechanism of regulation that mediate interactions between cargoes and motor proteins,
and are the only known level of regulation that is cargo specific (Cason et al., 2021; Colin
et al., 2008; Fu and Holzbaur, 2014). Many scaffolds specifically interact with a limited
set of cargoes and are regulated by post-translational modifications (Cason et al., 2021;
Fu and Holzbaur, 2013; Fu and Holzbaur, 2014; Reed et al., 2006; Sun et al., 2017).
Here, we illustrate the role of htt in regulating early endosome and lysosome transport.
Htt S421 phosphorylation activates kinesin and dynein on both early endosomes and
lysosomes. However, enhanced kinesin and dynein activity differentially affects early
endosomes and lysosomes, influencing their direction, motor activity, and affinity for
microtubules. Our results underscore the central role of huntingtin in directing endosome
transport and suggest that huntingtin mutations likely result in cargo-specific disruptions
to both signalling and degradative pathways.

MATERIALS AND METHODS

Cell Culture
We passaged HEK293T cells (donated by Dr. Kamen’s lab) at 80% or higher confluency
in a biosafety cabinet starting by rinsing with 2.5 mL 37 °C 1X phosphate buffered saline
(PBS, Wisent, St-Jean-Baptiste, QC, Canada), followed by adding 400 μL of 4 °C 0.25%
trypsin (Wisent) and incubating for 5 mins at 37 °C. Next, we used 5 mL of 37 °C complete
medium (Dulbecco’s Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), 2
mM Glutamax, 60 μg/mL penicillin 100 μg/mL streptomycin, all components from Gibco, Waltham, MA, United States) to neutralize the trypsin activity. Finally, we placed a 1/5-1/20 dilution of the resuspended cells in 37 °C fresh complete medium and transferred to the 37 °C, 5% CO₂ incubator. We tested all stocks of cells for mycoplasma.

**CRISPR Design and Molecular cloning**

We transfected HEK293T cells with 1.25 μg of lentiCRISPR v2 cassette and 1.25 μg of the single-stranded oligodeoxynucleotide (ssODN) for 24-36 hrs. lentiCRISPR v2 was a gift from Feng Zhang (Addgene plasmid # 52961; [http://n2t.net/addgene:52961](http://n2t.net/addgene:52961); RRID:Addgene_52961, Watertown, MA, United States) (Sanjana et al., 2014). Subsequently, we selected expressing cells using 2 μg puromycin for 48-72 hrs. Finally, we isolated single cells using fluorescence activated single cell sorting and grew them for 2-3 weeks in complete media containing 50% FBS. We verified clones containing the S421D sequence with next-generation sequencing.

gRNA sequence:
GTTCCACAATACTCCCACCTACGG

ssODN sequence:
ACGCCTCCACCGAGCTTCTGCAAAACCTGACCGCAGTCGGGGGCATTGGGCCAG
CTCACCAGCTGCTAAGGAGGAGTCTGGTGGCCGATCTCGTAGTGGGGATATTGTGG
AACCTATAGGCAAGTTATTAGCAAGGCTACTCTTTAACTTCTGACGTAATAC
TAGTTACACTCTATTGATTAGGCGGCTGCCCTGT

Sequencing primers:
Forward: CACCGTTCCACAATCTCCACCTACGA
Reverse: AACTAGTGAGGATATTGTGGAAC

**Plasmids and Transfection**

We transfected cells with 0.8 μg of eGFP-Rab5 or mCherry-S421D- htt or mCherry-WT- htt huntingtin constructs 24-48 hrs before imaging. mCherry-S421D- htt and mCherry-WT- htt constructs were generously donated from the Saudou lab, generated from the full length pARIS- htt (Pardo et al., 2010) using methods from their previous work (Humbert
et al., 2002). We replaced cell culture medium in the 35 mm round glass bottom dishes
(Mattek, Ashland, MA, United States) containing the cells with 0.2 mL serum-free DMEM. In separate 1.5 mL tubes, we added 2 μL/dish Lipofectamine 2000 (ThermoFisher Scientific, Waltham, MA, United States) to 50 μL/dish serum-free DMEM and added 0.8 μg/dish of DNA to 50 μL/dish of serum-free DMEM. We then mixed the DNA and lipofectamine, incubated for 20 mins at room temperature, and added 100 μL/dish to the cells. Four hours later, we replaced the serum-free medium with complete media.

Western Blotting
We lysed cells by manually twisting while lifting and descending a Dounce homogenizer 20-30 times in 400 μL PBS containing 0.01 M dithiothreitol (DTT, R0681, ThermoFisher Scientific) and 1X protease inhibitor cocktail (100X stock from PIC002, Bioshop, Burlington, ON, Canada) on ice. Subsequently, we centrifuged the cell solution at 1500 g for 10 mins at 4 °C, then centrifuged the supernatant at 16000 g for 10 mins at 4 °C. We added 20 μL of lysate for each sample to the SDS-PAGE gel, diluted in 2x Laemmli buffer (161-0737, Biorad, Hercules, CA, USA) with 2.5% 2-mercaptoethanol (M6250, Sigma, Burlington, MA, United States) after heating to 100 °C for at least 10 mins. To mark molecular weights, we loaded 5 μL of protein ladder (PI26634, ThermoFisher Scientific).

We used a 25 mM Tris (TRS001.1, Bioshop), 190 mM glycine (800-045-LG, Wisent), and 0.1% SDS (SDS999.100, Bioshop) running buffer, and ran the gel at 80 mA for 60-90 mins. To transfer, we used 25 mM Tris, 190 mM glycine, and 30% methanol (34860-4L-R, Sigma) transfer buffer for wet transfer at 100 V for 90 mins with a PVDF membrane. We blocked the membrane for 1 hour with blocking buffer: 5% bovine serum albumin (BSA, Bioshop) in tris-buffered saline with Tween 20 (TBST, 20 mM Tris, 150 mM NaCl (SOD004.1, Bioshop), 0.1% Tween 20 (P2287, Sigma). We diluted primary antibodies in the blocking buffer and incubated at 4 °C overnight. Next, we washed the membrane with blocking buffer 3 times for 5 mins and incubated with the secondary antibody diluted in blocking buffer for 90 mins with constant agitation. We washed the membrane 3 times with TBST before incubating with a 1:1 ratio of horseradish peroxidase substrate and enzyme mixture (P90720, EMD Millipore, Burlington, MA, USA). We imaged using a Biorad Chemidoc camera set to detect chemiluminescence. We subsequently stained the...
membrane with Coomassie G-250 (CBB555.25, Bioshop), using a previously established protocol from Goldman et al. titled Coomassie blue R-250 staining (Goldman et al., 2016). We used the following primary antibodies: 1/1000 dilution of T9026 α-tubulin, Sigma lot: 000089497, and 1/500 dilution of MAB2166 ms-huntingtin, Abcam, Cambridge, England lot: 3703568. The secondary antibody was rabbit anti-mouse ab6728, Abcam.

Motility Assay
In a biological safety cabinet, we mixed 200 μL of 37 °C complete medium with LysoTracker™ Deep Red (L12492, ThermoFisher Scientific) to a concentration of 70 nM and added it to HEK293T cells in MatTek 35 mm glass bottom dishes. Cells were incubated for 10 mins at 37 °C, and subsequently replaced the medium with 1 mL of 37 °C Lebovitz (11415064, Gibco) with 10 % FBS for imaging. We imaged cells in a 37°C chamber of a Nikon Eclipse Ti-E microscope in near-total internal reflection (TIRF) fluorescence settings using an EMCCD camera with an exposure time of 120 ms for 3 mins. We repeated the process for several cells within each dish and all cells were imaged within 1 hour of adding the Lysotracker. For Rab5 transfected cells, we replaced the medium with 0.5-1 mL of of 37 °C Lebovitz with 10 % FBS and imaged them as the lysosomes. For experiments using 250 nM latrunculin A (S428021, Sigma) or 10 μM nocodazole (M1404, Sigma), we added the drug to the Lebovitz medium a minimum of 15 mins prior to imaging.

Immunofluorescence: Cytoskeleton and Golgi Apparatus
This protocol was adapted from Jimenez et al. (Jimenez et al., 2020). We passaged HEK293T cells onto rectangular coverslips (12544-A ThermoFisher Scientific) in 100 mm petri dishes (83.3902, Sarstedt, Saint-Leonard, QC, Canada) ~24 hrs prior to fixation. We performed incubations for extraction, fixing and NaBH₄ (452882, Sigma) solutions on flat pieces of parafilm, with droplets containing 100 μL of the described solution. Using fine-tipped tweezers, we manipulated the coverslips and placed them cell-side down on the droplets. Firstly, we incubated the cell-adhered coverslips with 37 °C extraction solution (0.25% Triton-X-100 (X100, Sigma), 0.1% glutaraldehyde (G5882, Sigma)) in PEM (80mM PIPES (P6757, Sigma), 5mM EGTA (EGT101, Bioshop), 2mM MgCl₂ (M2866,
Sigma at pH=6.8) for 15-45 s. We next fixed the cells with 37 °C 0.25% Triton-X-100 and 0.5% glutaraldehyde in PEM and incubated for 10 mins. We subsequently transferred the coverslips to 0.1% NaBH₄ in PBS (Wisent) and incubated for 7 mins, then rinsed quickly with PBS twice before blocking. Next, we incubated the fixed cells with blocking buffer (2% BSA (ALB001, Bioshop) in PBS, (Wisent)) inside a glass petri dish for 30 mins with gentle rocking on an orbital shaker. Subsequently, we added a 1:400 solution of primary antibody in blocking buffer (anti-GM130 ab52649 Abcam, anti-tubulin Sigma T6199) in 100 μL droplets on a parafilm-coated humid chamber. We incubated coverslips with the primary antibody droplets overnight at 4 °C. We then performed 3x 10-minute washes with ~10 mL blocking buffer in glass petri dishes. After washing, we added a 1:400 dilution of fluorescent secondary antibody, goat anti-mouse 594 (A11032, ThermoFisher) and goat anti-rabbit (18772, Sigma), to the humid chamber with new parafilm and incubated it for 1 hr at room temperature protected from light. We then washed as described after primary antibody incubation. To label actin, we added phalloidin (P1951, Sigma) labeled with Alexa-647 (0.094 μM in PBS) and incubated for 20 mins on 100 μL droplets in the humid chamber. We transferred coverslips to PBS, then set up for imaging by sealing them to a glass slide with double sided tape and vacuum grease to a glass slide and adding PBS to the channel. Finally, we imaged the cells in ~1.5-2 μm thick z-stacks using near-TIRF microscopy, with 1 mW laser power at 640 nm, 561 nm, and 488 nm excitation, 600 ms exposure time, EM gain of 200 using an EMCCD camera of a Nikon Eclipse Ti-E microscope.

**Immunofluorescence: Motor Colocalization**

This protocol differs from cytoskeletal imaging in the fixation step; we incubated cells at -20 °C with 1 mM EGTA (EGT101, Bioshop) in -20 °C methanol, for 8 mins. Subsequent steps are identical to the cytoskeletal immunofluorescence protocol, except that the blocking buffer contained 0.2% saponin (4521, Sigma). We first imaged cells for the motor (kinesin or dynein) and saved their positions on the microscope. Following imaging, we washed the sample and probed for tubulin while maintaining the slide in the same position on the microscope, using another colour and shorter incubation times to allow higher throughput. The protocol was as follows: 1 hr incubation with primary antibody, wash 3x...
slide volume with blocking buffer, 45 mins incubation with secondary antibody, wash 3x
slide volume with PBS, this sequential antibody staining was done due to the redundancy
in antibody species available. The primary antibodies used for experiments were: 1/400
T9026 ms alpha-tubulin, Sigma lot: 000089497, 1/400 ms-kinesin heavy chain MAB1614
lot: 3593684, 1/400 MAB1618 ms-dynein, cytoplasmic lot: 3089121. Secondary
antibodies used for experiments were goat anti-mouse 488 (A21236, ThermoFisher) for
motors followed by Goat anti-mouse 647 (A21236, Sigma) for microtubules.

**Immunofluorescence: Huntingtin Localization**

We fixed the cells with prewarmed 4% paraformaldehyde at 37 °C for 10 mins, and
subsequently followed the protocol as outlined for cytoskeletal imaging. The huntingtin
antibody used was MAB2166 ms-huntingtin, Abcam lot: 3703568. The blocking buffer
contained 0.2% saponin (4521, Sigma).

**Immunofluorescence: Mitochondria**

We fixed cells using prewarmed 4% paraformaldehyde at 37 °C for 20 mins, followed by
3 washes with PBS. Using 50 mM NH₄Cl in PBS, we quenched fixation for 10 mins, and
washed again 3 times with PBS. We permeabilized cells using 0.1% TritonX-100 in PBS
for 10 mins with gentle rocking, followed by blocking with 10% BSA (ALB001, Bioshop)
for 30-45 mins with gentle rocking. We diluted the primary antibody 1/500 (TOMM40
18409-1-AP, Proteintech, Rosemont, IL, United States) with 5% BSA in PBS and
incubated overnight at 4 °C. We washed coverslips 3 times with gentle rocking using the
5% BSA in PBS. We diluted the secondary antibody 1/400 in the same solution and
incubated for 1 hr at room temperature. We repeated the washing described after the
primary antibody incubation, then mounted the slide using PBS as the imaging medium.
We imaged samples using an Olympus IX83 with a 100x objective using a disk scanning
unit to obtain high resolution z stacking in a 25 μm range using 0.5 μm slices.

**Intracellular Optical Tweezers**

We prepared a fibronectin (356008 Corning, Bedford, MA, United States) stock at a
concentration of 1.4 mg/mL in water and stored it at -20 °C until use. We coated
fluorescent yellow/green 500 nm beads (F8813, ThermoFisher Scientific) by centrifuging 20 μL of stock bead solution at 20000 rcf for 5 mins, then resuspended the beads in 25 μL of the fibronectin solution. We incubated the beads overnight at 4 °C, then washed with 40 μL PBS 3 times, centrifuging again at 20000 rcf for 5 mins each. We diluted this bead solution 1/20 in DMEM complete media, sonicated for 5 mins, and incubated with the cells for 10 mins at 37 °C. For imaging, we replaced the medium with Lebovitz supplemented with 10% FBS. Early endosome data was acquired from 15 mins–50 mins after bead internalization, while lysosomes were from 1 - 2 hours post-internalization. We identified cargoes moving processively within the cell, positioned the location of the center of the optical tweezer laser above the motile cargo, and opened the shutter to capture the bead. We acquired 60 s force traces holding the optical tweezer laser (12 W, 1064 nm) fixed in the original capture position, while monitoring the position signal and live image to ensure that the bead remained within one bead radius of the center of the tweezer. Immediately after this measurement, we calibrated the optical tweezers by applying a 275 s multifrequency sinusoid to calculate the position sensitivity (β), trap stiffness (ktrap), and viscoelastic properties (G’, G”’, Fig. S2E, F) of the cytoplasm as previously described (Chaubet et al., 2020).

**Immunofluorescence Analysis: Actin, Microtubules, Golgi, Huntingtin**

For each z-stack, we chose the sharpest image for each channel for qualitative analysis and observation as overlays.

**Mitochondrial Immunofluorescence Analysis**

We determined the range of z-values with samples in focus, and only used those slices for image analysis. We processed the images in Fiji with the white top hat 3D morphological filter, using a radius of 5 in x and y, and 2 in z. We then set an automatic threshold and converted the thresholded image to a mask. Next, we ran the analyze particles function to determine the shape, area, and number of mitochondria in each plane, repeated for all in focus z planes, and added all the values for each z plane together. We plotted the results from all cells using MATLAB.
Golgi Dispersal Analysis

Using ImageJ, we observed each of the Golgi images using the same linear LUT (minimum = 145 a.u. and maximum = 1885 a.u.) and then categorized the Golgi into organized, dispersed, or undetermined. Comparing the results from multiple experiments using this binary categorization and excluding the undetermined data, we determined the percentage of organized and dispersed Golgi observed in each cell type.

Motility Assay Analysis - Tracking

We used the Fiji plugin TrackMate (Tinevez et al., 2017) to generate tracks from the images for subsequent analysis. The settings used in TrackMate highly influence the results and are therefore shown in the tables below (Table 1 and 2). We chose the parameters by comparing the maximum intensity projection of all frames with the generated tracks and trying multiple values that would be considered logical for these cargos.

Table 1. TrackMate analysis settings used in the analysis of lysosomal cargoes using LysoTracker™.

<table>
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<th>Value</th>
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<tr>
<td>Intensity threshold</td>
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<tr>
<td>Linking maximum distance</td>
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<tr>
<td>Gap-closing maximum distance</td>
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<td>Gap-closing maximum frame gap</td>
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Table 2. TrackMate analysis settings used in the analysis of early endosomal cargoes using Rab5-eGFP transfected cells.

<table>
<thead>
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<tr>
<td>Linking maximum distance</td>
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</tr>
<tr>
<td>Gap-closing maximum distance</td>
<td>0.5 μm</td>
</tr>
<tr>
<td>Gap-closing maximum frame gap</td>
<td>2</td>
</tr>
</tbody>
</table>

Motility Assay Analysis - Quantitation
To analyze the raw TrackMate data, we wrote custom MATLAB codes, now available on GitHub: [https://github.com/hendricks-lab](https://github.com/hendricks-lab).

We calculated time averaged mean squared displacement according to the following formula:

$$ MSD = \frac{1}{\tau-t} \sum_{t=0}^{\tau-t} (x_{t+1} - x_t)^2 $$

We calculated $\alpha$ values by fitting a line to the log-log plot of the mean squared displacement for time delays from 120 ms-5 s. We obtained radius of gyration values using the following formula:

$$ R_g^2 = \frac{1}{N} \sum_{k=1}^{N} (r_k - r_{mean})^2 $$

**Optical Tweezers: Force Trace Analysis**

We calculated the trap stiffness and position sensitivity from the calibration data as previously described (Chaubet et al., 2020). Using the calibration data, we converted 60 s position traces to force traces (Fig. S2D) and calculated the viscoelastic moduli in each condition (Fig. S2B, C). We observed brightfield images of the position of the trap and the cell’s nucleus to define the directionality of the cargo’s movement. Any force event that had a maximum off-axis force, a force in the direction perpendicular to the defined microtubule axis, above 75% of the median on-axis forces, forces along the direction of the microtubule, were excluded. Stall forces were binned into 1 pN size bins and counted as stalls if the duration that they were above 0.5 pN duration was longer than 0.1 s. We calculated binding rates from the force traces by identifying the times that the cargoes unbound from the microtubules after a stall event and determining the time between unbinding and rebinding to the microtubule. We calculated force-dependent unbinding rates from the stall force event before a cargo became detached and categorized them by the direction the cargo was travelling immediately prior to detachment, as previously described (Berger et al., 2019). We performed an exponential fit to the data and presented
it in Table S1 following the equation below with \( U \) as the unbinding rate, \( U_0 \) as the force-independent unbinding rate, \( F \) as the force, and \( F_d \) as the detachment force.

\[
U = U_0 e^{F/F_d}
\]

**Statistical Analysis**

We analysed all pairwise histogram comparisons and cumulative distribution functions using the Kolmogorov-Smirnov (ks) test. We used Student’s two-tailed t-test for all pairwise analyses of violin plots. For optical tweezers stall force distribution analyses, in addition to the ks test we performed bootstrapping. We took 1000 bootstrap means of samples equivalent to the number of datapoints for the condition being tested. We then calculated 95% confidence intervals from the bootstrap mean distribution using the quantile function in MATLAB. To calculate p-values for bootstrap samples (not reported in Fig. 3), we subtracted the control from the S421D condition for each of the 1000 bootstrap means for the conditions to be compared and plotted the distribution (Fig. S2E). The p-value was determined by the fraction of data that overlapped with the 0 value, if this fraction was lower than 0.05, the difference between the values was considered statistically significant.

**ACKNOWLEDGEMENTS**

We thank Frederic Saudou (U. Grenoble) for generously sharing huntingtin constructs, Amine Kamen (McGill U.) for guidance on CRISPR/Cas9, and the members of the Hendricks lab for thoughtful comments on the manuscript.

**COMPETING INTERESTS**

The authors report no competing interests.

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preferential binding between rabs, kinesins, and specific endosomal


In vivo optical trapping indicates kinesin’s stall force is reduced by dynein during

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bi-directional movement of phagosomes along microtubules. J. Cell Biol. 137, 113–
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Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts

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Sequential dynein effectors regulate axonal autophagosome motility in a

Caviston, J. P. and Holzbaur, E. L. F. (2009). Huntingtin as an essential integrator of


coordinates the dynein-mediated dynamic positioning of endosomes and


FIGURE LEGENDS
Fig. 1. S421D HEK293T cell line does not affect cytoskeletal organization, huntingtin localization, or dynein function. (A) Huntingtin protein sequence schematic demonstrating the structured domains (HEAT repeats and other α-helical regions, orange), unstructured domains (blue), S421 phosphosite, polyglutamine repeat region (PolyQ), and proline rich domain (PrD). (B) CRISPR phosphomimetic mutant design guide RNA (gRNA), and ssODN homologous recombination template. (C) Chromatogram of the positive clone containing the phosphomimetic mutation S421D. (D) Immunostaining of actin (top) and microtubules (bottom) indicating no difference between WT (left) and S421D (right) HEK293T cells. Note immunofluorescence images are inverted such that the protein of interest is dark, and the background is white, and all scale bars in the figure are 20 μm. (E) In both WT and S421D HEK293T cells, the Golgi apparatus appeared compact (organized) in ~70% of cells and dispersed in ~30% of cells. WT data for actin, microtubules, and Golgi apparatus staining was from 14 experiments, 84 cells, and S421D data from 11 experiments, 69 cells. (F) Immunofluorescence of huntingtin protein localization in WT (left) compared to S421D (right) HEK293T cells. Immunofluorescence of huntingtin localization for WT cells from 3 experiments, 43 cells and for S421D from 2 experiments, 22 cells. (G) WT and S421D huntingtin endogenous expression levels (top left) detected by Western blot with the raw intensity data from three independent samples (bottom left), compared to levels when overexpressing full length mCherry-WT or mCherry S421D huntingtin (top right) and the fold change relative to native expression levels for three independent samples (bottom right). Samples collected on the same date are connected by a line. (H) Mitochondrial morphology of WT (left) and S421D (right) HEK293T cells. (I) Mitochondrial area of WT (grey) compared to S421D (blue) cells, p=6.59x10⁻⁸ by Kolmogorov-Smirnov (ks) test. (J) Number of mitochondria per cell in WT (grey) compared to S421D (blue), p=6.14x10⁻⁷ by ks test. Mitochondrial analysis for WT from 7 experiments, 88 cells, and S421D from 7 experiments, 94 cells.
**Fig. 2.** Huntingtin S421D mutation increases outward motility of early endosomes and lysosomes while overexpression affects all parameters. (A, B) Maximum intensity projection of early endosomes, labelled with Rab5-eGFP, and lysosomes, labelled with Lysotracker, for endogenous WT and S421D huntingtin expressing cells. All scale bars are 20 μm. (C, D) Sample trajectories for early endosomes (C) and lysosomes (D) in both WT and S421D huntingtin endogenously expressing cells, with their inward (in) or outward (out) directionality indicated by the arrows in the center. Different shades of colours were assigned randomly to facilitate visualization of overlapping segments. (E, G) Early endosome (E) and lysosome (G) processivity per cell, measured by α, the slope of the log-log plot of their mean-squared displacement. Means are represented with a filled line, while medians are dashed lines. (F, H) Radius of gyration (Rg) per cell of early endosomes (F) and lysosomes (H). Rg is a measure of the radius that contains half of the datapoints in the trajectory centered around its average position. (I, J) Early endosome (I) and
lysosome (J) directional bias per cell of processive trajectories toward plus ends of microtubules (outwards), the remaining fraction of processive trajectories move inwards. Overexpression conditions represented by +WT htt or +S421D htt. Directional bias is determined by fraction of time particles are moving outwards compared to inwards and averaged for each cell. The number of experiments, cells, and trajectories for each condition are the following; WT early endosomes: 65 cells, 41994 trajectories, 6 experiments; S421D early endosomes: 28 cells, 25476 trajectories, 3 experiments; WT+WT htt early endosomes: 28 cells, 17424 trajectories, 5 experiments, WT+S421D htt early endosomes: 39 cells, 22854 trajectories, 6 experiments; WT lysosomes: 55 cells, 25247 trajectories, 5 experiments; S421D lysosomes: 38 cells, 12670 trajectories, 4 experiments; WT+WT htt lysosomes: 29 cells, 7558 trajectories, 5 experiments; WT+S421D htt lysosomes: 28 cells, 7031 trajectories, 6 experiments. Statistical significance was determined via Student’s two-tailed t-test and p-values are indicated on each plot where applicable.
Fig. 3. Force trace analysis demonstrates S421D huntingtin induces higher binding rates, stronger binding, and higher forces in both directions for early endosomes and lysosomes. (A) Schematic of the optical tweezers assay including the endocytosed bead (green) inside the optical trap (red) along with a brightfield image of a cell during the experiment (top right). The scale bar is 20 μm and the arrowhead indicates the position of the optical trap during the experiment. (B) Sample force traces for early endosomes (early) and lysosomes in WT and S421Dhtt cells. The horizontal line indicates the zero axis, while positive values indicate outward forces and negative values indicate inward forces. (C, E) The fraction of early endosome or lysosome stall events at a given force with a minimum stall duration of 0.1 s, separated into 1 pN bins with the mean and 95% confidence intervals of bootstrap sampled means and p-values for each condition are shown above (for outward) or below (for inward) the distribution. The colours in (C-J) follow the colour legend from (B). (D, F) Force-dependent unbinding rates in both outward and inward directions (filled circles) for early endosomes or lysosomes fit with a single exponential (solid line). (G-J) Binding rates...
for each stall event in early endosomes and lysosomes of WT and S421D in both directions in (G, I) including the cumulative distribution function (CDF) for each condition in (H, J). Statistical significance by ks test is indicated by the p-value in the figure panel where applicable. All results from optical tweezers data for WT early endosomes from 10 cells, 5 experiments; S421D early endosomes: 16 cells, 8 experiments; WT lysosomes: 12 cells, 5 experiments, S421D lysosomes: 11 cells, 4 experiments. Statistical significance was determined by ks test for binding rates and force distributions and p-values are indicated where applicable.

**Fig. 4.** S421D huntingtin increases kinesin and dynein association to extracted microtubules. (A, B) Inverted immunofluorescence images demonstrating colocalization (bottom) kinesin heavy chain (kinesin-1, (A), top) or dynein intermediate chain (dynein, (B), top) on extracted microtubules (middle) in WT and S421D huntingtin cells. In the merge channel, motors (kinesin-1 or dynein) are indicated in magenta and microtubules in black. All scale bars are 20 μm. (C-F) Colocalization plots were created from plotting intensity values of each pixel of the microtubule and motor (kinesin or dynein) channels of all images.
Differences between kinesin-1 WT and S421D correlation coefficients resulted in $p=0.4748$, while dynein WT compared to S421D correlation gave $p=0.0533$ by a two-tailed Student’s t-test. Data for WT kinesin-1 from 17 cells, 3 experiments, S421D kinesin-1: 15 cells, 2 experiment, WT dynein: 32 cells, 4 experiments, and S421D dynein: 32 cells, 4 experiments.

Fig. 5. Model of huntingtin S421 phosphorylation and expression level mediating regulation of early endosome and lysosome transport. (A) Huntingtin S421 phosphorylation (S421-phos) increases activity of kinesin and dynein motor proteins on early endosomes, such that the predominantly inward motility shifts to being more outward. (B) Lysosomes move bidirectionally. When kinesin and dynein activity are enhanced by huntingtin phosphorylation, the movement remains bidirectional, but the displacement of trajectories increases. (C) Overexpression sequesters motors and adaptors or disrupts vesicle-bound huntingtin-adaptor-motor complexes regardless of cargo identity or huntingtin phosphorylation state.
**SUPPLEMENTARY MATERIALS**

**Table S1. Calculated parameters from exponential fits for unbinding rates of early endosomes and lysosomes.**

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<th>Condition</th>
<th>$U_0$</th>
<th>$F_d$ (pN)</th>
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</thead>
<tbody>
<tr>
<td>Early endosome WT outward</td>
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<td>0.42</td>
</tr>
<tr>
<td>Early endosome S421D outward</td>
<td>0.31</td>
<td>3.2</td>
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<tr>
<td>Early endosome WT inward</td>
<td>$7.2 \times 10^{-7}$</td>
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</tr>
<tr>
<td>Early endosome S421D inward</td>
<td>0.31</td>
<td>6.3</td>
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<tr>
<td>Lysosome WT outward</td>
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<tr>
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<tr>
<td>Lysosome WT inward</td>
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<td>14.6</td>
</tr>
<tr>
<td>Lysosome S421D inward</td>
<td>0.77</td>
<td>10.8</td>
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</table>
**Fig. S1. Cytoskeletal disruption affects cargo motility.** (A) Actin cytoskeleton labelled with SiR actin in S421D HEK293T cells treated with 100 nM-1 μM concentrations of latrunculin A (latA). Scale bars for all images are 20 μm, all images inverted. (B) Per cell α values of Rab5-eGFP labelled early endosomes. Means are represented with a filled line, while medians are dashed lines. (C) Radius of gyration (Rg) per cell is presented for early endosomes (C). (D) Directional bias per cell shown by fraction of time processive.
runs moving outward for Rab5-eGFP early endosomes. Note: control WT and S421D data for latrunculin A experiments is the same data reported in Fig. 2. (E, F) Maximum projections of the same cell with and without nocodazole (NZ) for early endosome and lysosome analysis. (G-J), Radius of gyration and alpha values for early endosomes and lysosomes before and after nocodazole treatment. (B-D) WT control data from 7 independent experiments (41994 trajectories, 64 cells), S421D data from 3 independent experiments (25476 trajectories, 28 cells), WT latA data from 3 independent experiments (17461 trajectories, 22 cells); S421D latA data from 5 independent experiments (19879 trajectories, 30 cells). (G, H) WT control data from 4 experiments, 22 cells, 6109 trajectories and WT+nocodazole data is from 4 experiments, 23 cells, and 7094 trajectories. (I, J) Contains control data from 4 experiments, 15 cells, 17073 trajectories, and nocodazole treated data from 4 experiments, 23 cells, and 6639 trajectories. Statistical significance of p<0.05 by Student’s two sample t-test indicated in the figure where applicable.
A  WT early  S421D early  WT lysosome  S421D lysosome

B  WT G'  WT G''  S421D G'  S421D G''

C  WT G'  WT G''  S421D G'  S421D G''

D  10pN

E  WT out-in  S421D out-in  WT out-in  S421D out-in

S421D-WT out  S421D-WT in  S421D-WT out  S421D-WT in
Fig. S2. Force measurement analysis approach, additional force traces, and viscoelastic properties of WT and S421D huntingtin cells. (A) Brightfield image of a WT and S421D cell containing a trapped phagocytosed 500 nm bead (red arrow) during measurement of early endosome (early) or lysosome forces. (B, C) Viscous (G’’) and elastic moduli (G’) for WT (black) and S421D (teal) early endosomes and WT (blue) and S421D (green) lysosomes. (D) Additional sample force traces for each condition in optical tweezers assays, the horizontal line indicates the zero axis. Positive value forces are directed outward and negative forces are directed inward. (E) Distributions of bootstrap mean differences evaluated for additional statistical analysis of force distributions for the differences shown on the x axis of each plot in both early (left) and late (right) endosomes.

Fig. S3. Western blot transparency for huntingtin blot in Fig. 1. (A) Full western blot (above 100 kDa) of the blot presented in Fig. 1G using the anti-huntingtin antibody. Ladder is indicated by L with molecular weights to the right of the blot. (B) Coomassie staining of the entire membrane post-antibody staining.