HHS Public Access

Author manuscript

Cell Rep. Author manuscript; available in PMC 2017 April 28.

Published in final edited form as:

Cell Rep. 2017 March 07; 18(10): 2452–2463. doi:10.1016/j.celrep.2017.02.034.

Anterograde transport of Rab4-associated vesicles regulates synapse organization in *Drosophila*

Swagata Dey[1,*], **Gary Banker**[2], and **Krishanu Ray**[1,#,*]

[1]Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai 400005, India

[2]Jungers Center for Neurosciences Research, Oregon Health and Science University, Portland, OR 97239, USA

Summary

Local endosomal recycling at synapses is essential to maintain neurotransmission. Rab4GTPase, found on sorting endosomes, is proposed to balance the flow of vesicles between endocytic, recycling and degradative pathways in the presynaptic compartment. Here, we report that Rab4-associated vesicles move bidirectionally in *Drosophila* axons but with an anterograde bias, resulting in their moderate enrichment at the synaptic region of the larval ventral ganglion. Results from FRB-FKBP conjugation assays in rat embryonic fibroblasts together with genetic analyses in *Drosophila* indicate that an association with Kinesin-2 (mediated by the tail domain of Kinesin-2α/KIF3A/KLP64D subunit) moves Rab4-associated vesicles towards the synapse. Reduction in the anterograde traffic of Rab4 causes an expansion of the volume of the synapse-bearing region in the ventral ganglion and increases the motility of *Drosophila* larvae. These results suggest that Rab4-dependent vesicular traffic towards the synapse plays a vital role in maintaining synaptic balance in this neuronal network.

eTOC summary

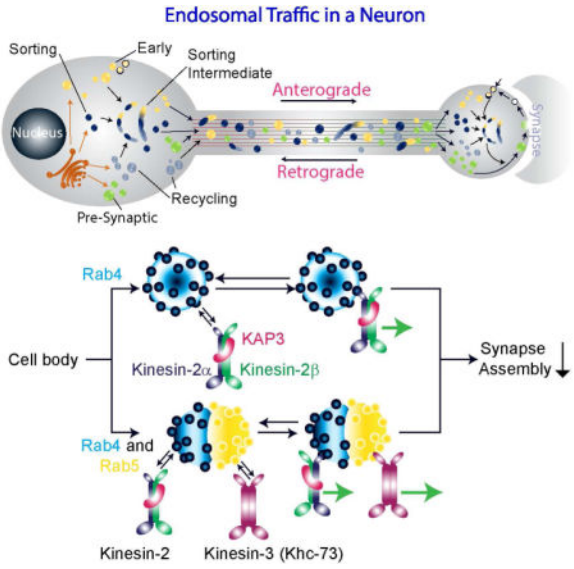
Dey et.al. show that in *Drosophila* larvae, binding to the Kinesin-2α tail propels Rab4-associated vesicles towards the synapse. Reduced Rab4 transport expands the synapse-bearing region of the ventral ganglion and enhances larval motility. Hence, Kinesin-2 mediated Rab4 trafficking appears to regulate synapse homeostasis in a neuronal network.

[*]**Correspondence:** Phone: +91-22-22782730, Fax: +91-22-22804610, krishanu@tifr.res.in, krishanu64@gmail.com, swagatad86@hotmail.com.

[#]**Lead contact**

Author contributions

SD performed all the experiments, collected and analyzed data. SD and KR planned the experiments, organized the data and wrote the manuscript. SD, KR, and GB planned experiments done in mammalian cells.



Graphical abstract

The logistics of endosomal transport and trafficking in neurons. The nascent Rab4 vesicles, a part of the sorting intermediate endosomes, are shown to bind to Kinesin-2α (KIF3A/KLP64D) tail. Rab4-associated vesicles are transported predominantly by heterotrimeric Kinesin-2 in *Drosophila* axons. Association of a fraction of these vesicles with a Kinesin-3 family motor, Khc-73, is suggested to facilitate their pre-synaptic delivery. This transport is indicated to play a crucial role in suppressing the synapse assembly, maintaining homeostasis in the *Drosophila* larval brain.

Keywords

Rab4; Kinesin-2α; Axonal Transport; *Drosophila*; Ventral Ganglion; Synapse

Introduction

The apical compartment of a neuronal cell extends into specific processes forming axons and synapses that are maintained by a distributed endosomal system (Lasiecka et al., 2009). For example, the vesicle recycling at the synapse, orchestrated by various RabGTPases and associated proteins (Rizzoli, 2014), renews the readily releasable pool of synaptic vesicles sustaining the neurotransmission (Hoopmann et al., 2010). Rab4 is thought to play an important role in balancing the vesicle traffic between the recycling and degradation pathways involved in various cellular processes such as metabolism, cell secretion and antigen processing (Lazzarino et al., 1998; McCaffrey et al., 2001; Shirakawa et al., 2000). Recruitment of Rab4 on sorting intermediate endosomes allows the transfer of vesicular cargoes from early endosomes to recycling endosomes in axons and at synapses (de Renzis et al., 2002; Hoogenraad et al., 2010; Jovic et al., 2010). The activity of Rab4 has also been implicated in the progression of growth cone in *Xenopus*, and the maintenance of dendritic spines in rat hippocampal neurons (Brown et al., 2007; Falk et al., 2014). Furthermore, endosomal abnormalities found in the cholinergic basal forebrain of patients with Alzheimer

disease correlate with elevated levels of Rab4 (Cataldo et al., 2000). All these reports suggest that Rab4 dependent vesicle sorting is essential for the development and maintenance of the nervous system.

Vesicular cargoes, including the RabGTPases, originate at the trans-Golgi network, and microtubule-dependent motors transport them to different destinations within a cell (Hunt and Stephens, 2011). Specific association to motors ensures differential and dynamic subcellular localizations of various RabGTPases according to tissue-specific activities and metabolic demands (Gurkan et al., 2005). For instance, the anterograde trafficking of early, sorting and late endosomes is dependent on Kinesin-3 motors, KIF13, KIF16B, and KIF1A/IBβ respectively (Bentley et al., 2015; Niwa et al., 2008; Ueno et al., 2011). Similarly, in *Drosophila*, the Rab5 positive early endosomes associate with Khc-73 which is the orthologue of mammalian KIF13 showing a conserved machinery of Kinesin-Rab interaction (Huckaba et al., 2011). The same vesicles also associate with Dynein for their retrograde movement (Schuster et al., 2011; Soppina et al., 2009). It is further indicated that multiple RabGTPases can be grouped together and transported to a destination within a cell where they can engage in different functions. Although the long-range transport of certain presynaptic RabGTPases has been studied in cultured neurons (Niwa et al., 2008), it is still unclear how the bidirectional movement of Rab-associated vesicles could localize them in distant compartments such as the synapse.

Here, we show that Rab4-associated vesicles are transported with an anterograde bias, induced by a specific interaction with the tail domain of Kinesin-2α subunit, which results in its moderate enrichment at the synapses. The rate of pre-synaptic inflow of Rab4 is positively correlated with its activation. Interestingly, we found that a decreased flow of the Rab4-associated vesicles expanded the region occupied by synapses in the neuropil region of the larval ventral ganglion and enhanced larval motility. Altogether, these results indicate that the flow of Rab4-associated vesicles could maintain synaptic homeostasis in a neuronal network.

Results

Synaptic Rab4 is maintained through a bidirectional traffic in axons with a moderate anterograde bias

Rab4, a key endosomal sorting molecule, is involved in post-Golgi membrane sorting throughout the neuron. We chose *Drosophila* central nervous system as a model for studying the molecular logistics of the Rab4 distribution in neuron and its effect on the nervous system. Both the endogenous and recombinant Rab4 were evenly distributed in the neurons of larval ventral ganglion with a moderate enrichment in the neuropil region (Fig.S1A–C). Overexpression of Rab4-mRFP in cholinergic neurons caused a marginal increase in the relative enrichment of the protein in the neuropil region. The ectopically expressed Rab4-mRFP colocalized with the endogenously expressed YFP-Rab4 (protein-trap), on particulate structures in the cell body and axons of the Lateral Chordotonal (*lch5*) neurons (Fig. S1D). These neurons are located in the lateral body wall of every abdominal segment (Campos-Ortega and Hartenstein, 1985). The endogenous Rab4 was also found in the synaptic terminals of neuromuscular junctions marked by Bruchpilot (Fig. S1E), as well as in the

neuropil region of the ventral ganglion (Fig.S1A–B). Together, these observations indicated that Rab4-mRFP could function like the endogenous protein which is also marginally enriched at the synapse. Due to the widespread labeling of punctae, it was technically challenging to observe the axonal movement of YFP-Rab4-associated vesicles in the protein-trap background. Therefore, we expressed Rab4-mRFP in cholinergic neurons and investigated its enrichment in the neuropil region of the ventral ganglion. We chose the proximal regions of the *lch5* neurons to investigate the long-range transport of Rab4-mRFP in single axons.

Both the Rab4-mRFP and Syt-GFP, selectively expressed in cholinergic neurons using the *chaGal4* driver, were enriched in the neuropil region (Fig.1A) and marked vesicles in both the cell body and axons (Fig.1B). Very few Rab4-mRFP (approx. 6%) vesicles were marked with the Syt-GFP (inset, Fig.1B) and *vice versa*. The fluorescence recovery after photobleaching (FRAP) assay for Rab4-mRFP and Syt-GFP in the synaptic boutons at the neuropil region also indicated that the turnover rates of Rab4-mRFP (18.3 ± 0.2%) and Syt-GFP (12.8 ± 0.6%,) were quite distinct from each other (Fig.1C; Movie S1). The inflow rate of Rab4-mRFP ($t_{1/2}$ = 0.75 ± 0.05 s) was significantly faster than that of the Syt-GFP ($t_{1/2}$ = 2.19 ± 0.26 s). The kymographs of Rab4-mRFP and Syt-GFP-bearing vesicles in *lch5* axons indicated no overlap between the vesicles marked by Rab4-mRFP and those marked by Syt-GFP (Fig. 1D; Movie S2). These observations suggested that Rab4 and Syt are transported independently in axons.

Segment-wise analysis of the traces provided an estimate of the traffic density, speed, and displacement of the individual runs (Fig.1E). Although the anterograde and retrograde traffic densities of vesicles marked by Rab4-mRFP were comparable, they were significantly higher than those of the Syt-GFP vesicles (Fig. 1F). The Rab4-associated vesicles moved with a relatively higher anterograde bias and had comparatively greater average speed and displacement than those of the Syt-GFP vesicles (Fig.1G, H). The retrograde movement parameters of these two types of vesicles were comparable. We confirmed that the overexpression of Rab4-mRFP in cholinergic neurons did not influence the transport characteristics in both the anterograde and retrograde directions (Fig.S1F). These data suggested that two distinct types of anterograde motors may transport vesicles marked by Rab4-mRFP and Syt-GFP, respectively, in the axons. Synaptotagmin containing vesicles are transported by the Kinesin-3 and Kinesin-1 family of motors in the anterograde direction (Gindhart et al., 2003; Hirokawa et al., 2009; Okada et al., 1995). Rab4 and a Kinesin-2 motor subunit (KIF3B) were copurified as a complex upon insulin stimulation and thought to participate in the translocation of GLUT4-containing exocytotic vesicles to the plasma membrane (Imamura et al., 2003).

Rab4 associated endosomes interact with members of the Kinesin-2 and Kinesin-3 families of motors in cultured fibroblasts

We adopted a candidate based approach to independently identify the motors that could bind to Rab4-associated vesicles in the tissue cultured cells by using an inducible FRB-bait and Bicaudal D2 (BicD2)-FKBP interaction assay (Bentley et al., 2015). We expressed Rab4 tagged with Emerald (a GFP variant) together with FRB tagged stalk-tail fragments of

various mammalian (Rattus norvegicus, Rn; Homo sapiens, Hs) kinesins in rat embryonic fibroblasts (Fig.2A). The BicD2-FKBP was tagged with tdTomato at the N-terminus to monitor the degree of colocalization. Upon addition of the linker-drug, AP21967 (a rapamycin analog), the FRB- and FKBP-tagged proteins undergo conjugation. If the FRB-Kinesin (bait) binds the Rab4-associated vesicles (target), conjugation with tdTomato-BicD2-FKBP leads to the recruitment of dynein, which moves the vesicles toward the cell center (Fig 2B). If the FRB-kinesin tail does not bind the Rab4 vesicles, their distribution remains unchanged (Fig.2B). This assay, conducted within a cellular environment, is likely to be physiologically more relevant than the results of coimmunoprecipitation experiments. Moreover, the readouts in this assay, based on coaccumulation of the Rab4 vesicles and tdTomato-BicD2-FKBP, are robust and unambiguous.

Among the kinesin tails expressed, we found a positive interaction between the Emerald-Rab4-associated vesicles and truncated heterotrimeric mammalian Kinesin-2 subunits (RnKIF3A$^{419\text{-}702}$ and HsKAP3B) as well as the members of the Kinesin-3 family of motors (RnKIF13A$^{386\text{-}1149}$ and RnKIF13B$^{442\text{-}1826}$, Fig.2C and S2C). This observation suggested a role for both the heterotrimeric Kinesin-2 and a specific member of the Kinesin-3 family motors in the trafficking of Rab4-associated vesicles. In all cases, except that of KAP3B, the readout was strong (Fig.S2), and all the cells showed coaccumulation of Rab4 vesicles with tdTomato-FKBP-BicD2 (Fig.2C). In the case of KAP3B, only 25% of the transfected cells showed accumulation of tdTomato-FKBP-BicD2 after treatment with the linker (Fig S2C). These cells also had a loose perinuclear aggregation of Rab4 associated vesicles (Fig 2C). KIF13A and KIF13B have been shown to interact with early endosomes that are Rab5-positive using this assay (Bentley et al., 2015). We also found that FRB-Rab4 could localize a fraction of GFP-Rab5 vesicles to the cell center in the presence of the linker-drug (Fig.S2B), which led us to speculate that KIF13A and KIF13B may interact with the subset of Rab4 vesicles that also contain Rab5. Of the two motor subunits of the heterotrimeric Kinesin-2, only the KIF3A stalk-tail fragment showed a strong interaction with the Rab4 vesicles, whereas no interaction was found with the KIF3B stalk-tail.

As the FRB-tagged bait proteins were expressed using a strong promoter, they are likely to be present at much higher levels than their corresponding endogenous binding partners. Thus, the majority of the expressed FRB-KIF3A or FRB-KIF3B is likely to remain as monomers. This is consistent with the result obtained following expression of FRB-KAP3B, which produced only a partial association with Rab4. Thus, our results indicate that the interaction between Rab4 and Kinesin-2 is mediated by the stalk-tail domain of Kinesin-2α.

The Kinesin-2α subunit selectively interacts with Rab4 vesicles

We next explored the specificity of Kinesin-2 interactions with different endosomal pools labeled by distinct RabGTPases (Rab5, Rab7, and Rab11) using the same assay (Fig.3A). Rab5 is found on early endosomal compartments, Rab7 on late endosomes, and Rab11 associates with the recycling endosomes. As expected, the assay revealed a strong interaction between KIF3A$^{419\text{-}702}$ and Rab4-positive endosomes, but not with endosomes labeled with Rab5, Rab7 or Rab11 (Fig.3B). No positive interaction was observed between KIF3B$^{391\text{-}747}$ and any of the Rab-labeled vesicles (Fig.3B).

Rab4 and Rab5 are present together on sorting intermediate endosomes (SIE) along with common adaptors like Rabaptin-5 or Rabenosyn-5 in A431, CHO and BHK cell lines (de Renzis et al., 2002; Vitale et al., 1998). We found that the KIF3A stalk-tail did not interact with Rab5-positive compartments, suggesting that the association of Kinesin-2α subunit is selective to nascent Rab4 vesicles rather than SIE. Previously it was shown that a dominant negative effect of KIF3A mislocalized the late endosomes (Rab7 positive) or lysosomes (Brown et al., 2005). However, we found that the Rab7 associated endosomes are not selectively enriched at the perinuclear region with the KIF3A and KIF3B stalk-tail fragments in the presence of the linker in a manner similar to that of Rab4 (Fig.3B). Kinesin-2 has been shown to associate with apical endosomes positive for FIP5, a Rab11 interacting molecule (Li et al., 2014). An independent study, however, has shown that Kinesin-2 could interact with Rab11 through Rip11/FIP5 complex only instead of a direct interaction (Schonteich et al., 2008). Our observations are consistent with these results and indicated that individual Kinesin-2 subunits do not interact with Rab11-associated endosomes. These results also suggest that the interaction between heterotrimeric Kinesin-2 and Rab4 marks a different set of compartments which are exclusive of other RabGTPases.

Anterograde transport of Rab4-associated vesicles in axons requires Kinesin-2α tail in vivo

To elucidate the role of Kinesin-2 in trafficking the nascent Rab4-associated vesicles in vivo, we reverted to the *lch5* neurons of *Drosophila* larvae aged for 77 hours after egg-laying. KLP64D (Kinesin-2α/KIF3A), the KIF3A orthologue (Ray et al., 1999), KLP68D (Kinesin-2β/KIF3B) (Doodhi et al., 2012), and DmKAP (Doodhi et al., 2009) constitute the heterotrimeric Kinesin-2 in *Drosophila and* Khc-73, a Kinesin-3 family member, is an ortholog of mammalian KIF13A and KIF13B (Miki et al., 2001). Homozygous $Klp64D^{k1}$ (a null allele of *Klp64D*) larvae bearing a copy of *chaGal4* and *UAS-Rab4-mRFP* elements, respectively, were synthetic lethal. Very few such larvae emerged from the egg, and most of them failed to survive until the third instar stage. Previous studies have shown that the mutation in $Klp64D^{k5}$ disrupts heterotrimeric Kinesin-2-dependent processes in *Drosophila* neurons in a manner similar to that of the null allele (Baqri et al., 2006; Ray et al., 1999; Sarpal et al., 2003). Therefore, we used this allele to further study the role of Kinesin-2 on Rab4-associated vesicle transport in axons. Khc-73 was knocked down in all cholinergic neurons by expressing three different *Khc-73*-specific dsRNA constructs (Huckaba et al., 2011; Siegrist and Doe, 2005; Tsurudome et al., 2010).

Synaptic FRAP in the ventral ganglion had a significantly lower Rab4-mRFP influx in the $Klp64D^{-/-}$ background as compared to the wild-type control (Fig.4A and B, Movie S3). Although the change due to the expression of the $Khc\text{-}73^{36733}$, a hairpin dsRNA construct in cholinergic neurons was not statistically significant, the tendency of a moderate decrease was evident (Fig.4B, Movie S3, S4A and S4B). Loss of Kinesin-2 also reduced the enrichment of Rab4 in the neuropil region (Fig.4D–F). Together, these results suggested that primarily heterotrimeric Kinesin-2 maintains the pre-synaptic influx of Rab4. Analysis of the Rab4-associated vesicle movement in the *lch5* axons revealed a significant loss of anterograde traffic density in the $Klp64D^{-/-}$ background (Fig.4G and H; Movie S4A). Coexpression of the full-length KLP64D-GFP along with the Rab4-mRFP in $Klp64D^{-/-}$

background rescued the defects (Fig. 4H, Movie S4A), and restored the average speed and displacement of the Rab4-associated vesicle movements to wild-type levels (Fig.4I, J). Thus, heterotrimeric Kinesin-2 appeared to play a prominent role in the movement of Rab4-associated vesicles.

To test the involvement of the C-terminal tail domains in the transport, we expressed the tail-less KLP64DΔT-GFP along with Rab4-mRFP in the *Klp64D*$^{-/-}$ background. Although KLP64DΔT-GFP was expressed to the same levels and had similar motility to that of the full-length KLP64D-GFP, it failed to restore the traffic density of Rab4 vesicles (Fig. 4H–J; S3, Movie S4A). Previous reports showed that the KLP64DΔT forms a functional heterotrimeric motor complex (Sadananda et al., 2012). The presence of KLP64DΔT-GFP marginally improved the anterograde traffic as compared to the homozygous *Klp64D*$^{-/-}$ background. Analysis of the kymographs revealed a few occasional long runs. Thus, along with the previous results, this observation implies that an interaction between Rab4 and the C-terminal tail domain of Kinesin-2α (KLP64D/KIF3A) could induce long range, anterograde trafficking of a majority of the Rab4-associated vesicles in axons.

Khc-73 contributes to enhanced anterograde runs of the Rab4-associated vesicles in axons

The FRB-FKBP screen indicated that along with Kinesin-2, KIF13A/B also bind to the Rab4-associated vesicles, and to Rab5-positive early endosomes in REF cells (Bentley et al., 2015). Similarly, Khc-73 was shown to bind Rab5-associated, early endosomes through its tail domain in *Drosophila* cells (Huckaba et al., 2011). Using a pixel colocalization analysis, we found that around 5% of Rab4-mRFP-positive vesicles were marked by anti-Rab5 in *lch5* axons (Fig.S5). Although expression of Khc-73 dsRNAs did not affect the pre-synaptic influx of Rab4-mRFP, the kymographs were visibly different (Fig.5A, S4).

Further analysis of the runs indicated a marked change in the distribution of traffic density (Fig.5B). Although the average values were not significantly different, the incidences of the traffic densities higher than the wild-type median were negligible in the *Khc-73*^{SNAP} and *Khc-73*^{36733} backgrounds (Fig.5B). The Rab4-associated vesicles moved with a unimodal speed of 1.06 μm/sec (Fig.5C), which was significantly reduced in the *Klp64D*$^{-/-}$ and remained unaltered in the *Khc-73*^{dsRNA} backgrounds (Fig.S4). The displacement in the wild-type condition was distributed in three distinct modes (Fig.5D). Expression of *Khc-73* dsRNAs eliminated both the shorter and longer displacements (Fig.5D). The longer runs were also reduced in *Klp64D*$^{-/-}$ background. Together, these results may suggest that both these motors are likely to participate in the anterograde movement of a subset of the Rab4-associated vesicles in axons. Rab4 is known to associate with a multitude of vesicles consisting of both nascent Rab4-vesicles and Rab5-positive early endosomes (Sönnichsen et al., 2000). Hence, there is a finite possibility that a collaboration between Khc-73 and Kinesin-2 could propel the longer runs of a fraction of vesicles that are associated with both Rab4 and Rab5.

Rab4 activation induces the transport of associated vesicles towards synapses

Rab4 activity is essential for many neuronal processes such as the extension of axonal growth cone in *Xenopus* and maintenance of the dendritic spines in rat hippocampal cells (Brown et al., 2007; Falk et al., 2014). Also, elevated levels of Rab4 have been correlated with the endosomal abnormalities in the cholinergic basal forebrain of patients who have the Alzheimer's disease (Cataldo et al., 1997). Therefore, to understand the role of the Rab4-associated vesicle transport into the synapses, we expressed constitutively active (Q67L, CA) and dominant negative (S22N, DN) forms of YFP-Rab4 in cholinergic neurons using the *chaGal4* driver, which induces expression towards the final stage of neuronal development (Salvaterra and Kitamoto, 2001).

Compared to the YFP-Rab4, YFP-Rab4^{CA} was visibly more enriched in the neuropil region of the ventral ganglion (Fig.6A). A quantitation of the total YFP fluorescence in the neuropil region further revealed a significantly higher enrichment of YFP-Rab4^{CA} as compared to the YFP-Rab4 (Fig.6B). In contrast, the YFP-Rab4^{DN} levels were significantly lower than the wild-type control (Fig.6B). Similarly, the turnover rates of the YFP-Rab4^{CA} was significantly higher than that of YFP-Rab4, and the turnover rates of YFP-Rab4^{DN} was marginally lower (Fig. 6C, D and Movie S5A). Altogether, these results indicated that activation of Rab4 increases the propensity for anterograde transport of the associated vesicles. In the proximal axons of *lch5* neurons, very few vesicles were labeled with YFP-Rab4^{CA} and none with YFP-Rab4^{DN} (Fig. S6). Also, particulate structures labeled by the YFP-Rab4^{CA} were almost absent from the cortical region and axons (Fig.6A). The YFP-Rab4^{CA}–associated vesicles are likely to move highly processively because the availability of a relatively less number of YFP-Rab4^{CA} on a vesicle could ensure its stable association with the motors. Therefore, it would reduce the chance of detecting motile vesicles labeled with the YFP-Rab4^{CA} in the axon. The YFP-Rab4^{DN} is unlikely to associate with vesicles or bind to the motor, and we found a distributed YFP fluorescence all along the neuron expressing the transgene.

The activation of Rab4 through the phospho-inositol-3-kinase (PI3K) and protein kinase C (PKC) is thought to induce the association with Kinesin-2 downstream of insulin signaling (Imamura et al., 2003). Hence, we investigated whether an ectopic Pi3K activation in the neurons can influence the Rab4 transport in neurons. Coexpression of the regulatory subunit of PI3K, as well as the PI3K^{CA}, did not alter the Rab4-mRFP turnover at the synaptic boutons whereas that of the PI3K^{DN} significantly decreased the inflow (Fig.6E, F and Movie S5B). This observation suggests that PI3K is required for the activation of Rab4 and thereby its association with Kinesin-2. The latter part of the observation is consistent with the low turnover recorded for YFP-Rab4^{DN} at the synapse. Together, these two results also indicate that Rab4 activation is necessary for the anterograde transport of associated vesicles. Since the overexpression of PI3K^{CA} did not enhance the flow of Rab4-mRFP, we concluded that the type-I PI3K is necessary but not sufficient to facilitate the axonal transport of Rab4-associated vesicles.

Overexpression of dominant-negative Rab4 increases synapse density in the ventral ganglion

To further understand the effects of Rab4-dependent transport in the nervous system, we estimated the volume of the synaptic region marked by 'Bruchpilot' in a neuromere hemisegment. The expression of YFP-Rab4^{CA} in cholinergic neurons significantly reduced the volume (Fig. 7A). In comparison, the YFP-Rab4^{DN} expression increased the volume by almost 1.5 folds (Fig.7B). In addition, levels of the Bruchpilot (Brp) and Choline acetyltransferase (ChAT) at the neuropil region the ventral ganglia of the larvae expressing the YFP-Rab4^{DN} were significantly higher than that of the control (Fig.7C). Bruchpilot, homologous to ELKS/CAST/ERC protein of active zone, marks active synaptic contact in *Drosophila* (Wagh et al., 2006), and ChAT is an essential enzyme involved in acetylcholine synthesis and recycling (Greenspan, 1980). Therefore, increased localization of these two proteins at the axonal termini may indicate the formation of additional synapses. Hence, the above observation suggests that the function and localization of Rab4 at the pre-synaptic compartment is likely to regulate the synapse assembly.

We also estimated the larval motility to assess the effect of the expanded synaptic regions in the larval CNS due to YFP-Rab4^{DN} overexpression. Consistent with the expanded synaptic neuromere, the larvae overexpressing the YFP-Rab4^{DN} moved at a significantly higher speed (Fig.7D), indicating that the enhanced localization of the pre-synaptic markers in the ventral ganglion stimulated the larval locomotion. Together with the previous results, this observation indicated that the Rab4-dependent anterograde transport in the axons could maintain a homeostatic balance suppressing the synapse assembly.

The overexpression of YFP-Rab4^{DN} is expected to disrupt interactions between Rab4-associated vesicles and the motors, increasing the local availability of both Kinesin-2 and Khc-73. The latter is implicated in the assembly of T-bars marked by Bruchpilot at the presynaptic compartment (Tsurudome et al., 2010). The observed increase in the ChAT enrichment at the neuropile, which is transported by Kinesin-2 (Sadananda et al., 2012), is also consistent with this hypothesis. Further, the contents of the nascent Rab4-associated vesicles could regulate the membrane recycling at the nerve termini. An experimental analysis in future would be needed to unravel the true underlying mechanism.

Discussion

Kinesin-2 binds to Rab4-associated vesicles through the C-terminal Tail domain of the α subunit

The heterotrimeric Kinesin-2, implicated in the trafficking of apical endosomes and late endosomal vesicles (Bananis et al., 2004; Brown et al., 2005; Li et al., 2014) was initially indicated to bind to its cargoes through the accessory subunit, KAP3 (Hirokawa et al., 2009). Recent studies have, however, stated that the motor subunits could independently interact with soluble proteins through the tail domain (Girotra et al., 2016; Sadananda et al., 2012). Here, we showed that the Kinesin-2α tail could also bind to a membrane-associated protein, Rab4. A preliminary investigation in the lab suggests that the interaction in not direct (*Sil R, Dey S and Ray K, unpublished data*). The RabGTPases are known to recruit a variety of

different motors to the membrane, directing the intracellular trafficking (Horgan and McCaffrey, 2011; Ueno et al., 2011). Although a previous report indicated that Rab4 could bind to Kinesin-2 (Imamura et al., 2003), we found that such an interaction leads to the anterograde trafficking of Rab4-associated vesicles in the axon. Rab4 associates with early and recycling endosomes carrying a diverse range of proteins (Sönnichsen et al., 2000). The FRB-FKBP assay indicated that a particular subset of Rab4-only vesicles associates with Kinesin-2. These vesicles predominantly move towards the synapse in axons. Although Kinesin-2 is also found to bind and transport vesicles containing Acetylcholinesterase in the same axon (Kulkarni et al., 2016), we observed that Rab4 does not mark them. Similarly, the post-Golgi vesicles carrying N-Cadherin and β-Catenin, which are known to associate with Kinesin-2 (Hirokawa et al., 2009), exclude Rab4. Therefore, the Rab4-associated vesicles transported in the axon is likely to contain a unique set of proteins.

Kinesin-2 together with a Kinesin-3 family motor (Khc-73/KIF13) could bind to a subset of the Rab4-associated vesicles

Association of RabGTPases with putative transport proteins were identified using coimmunoprecipitation, pulldown and Yeast two-hybrid assays (Jordens et al., 2005). While these assays provide information on the possible interactions, their nature in a cellular or physiological scenario is unknown. Long range transport of Rab27-CRMP2 complex by Kinesin-1 and Rab3-DENN MADD complex by KIF1A/1Bβ are substantiated by genetic perturbations and imaging (Arimura et al., 2009; Niwa et al., 2008). The FKBP-FRB inducible interaction system used in this study identified Kinesin-2α and KIF13A/B as the key motors for the nascent Rab4-assocaited vesicles. The latter was also shown to associated with Rab5-marked early endosomes (Bentley et al., 2015). The results excluded the possibility that the Rab4 effectors involved in this interaction could bind to Rab5 and Rab11. The *in vivo* analysis of Rab4-associated vesicular traffic further confirmed that the heterotrimeric Kinesin-2 plays a dominant role in the trafficking of nascent Rab4-vesicles towards the synapse.

Kinesin-2 and -3 together contribute longer anterograde runs of the Rab4-associated vesicles

Anterograde axonal movement of Rab4 vesicles is mostly mediated by Kinesin-2 which transports the nascent Rab4 vesicles, whereas Kinesin-3 transports the sorting endosomes. In a recent study, it was shown that combined activities of Kinesin-1 and 2 steers the AChE containing vesicles in the axon (Kulkarni et al., 2016). A simultaneous association of these two motors was suggested to induce longer runs of the AChE containing vesicles. A similar collaboration between Khc-73 and Kinesin-2 could propel the longer runs of the Rab4 vesicles. Khc-73 has been implicated in neuronal functions earlier (Siegrist and Doe, 2005; Tsurudome et al., 2010). Khc-73 knockdown in *Ich5* neurons decreased the motility of Rab4 associated vesicles to a limited extent, which suggests that the motor is only engaged in transporting a relatively small fraction of the Rab4 associated vesicles in the axons. Khc-73 is a processive motor with an in vitro velocity of 1.54 ± 0.46 μm/s (Huckaba et al., 2011) and we found that the Khc-73 RNAi particularly affected the longer runs. Thus, it also suggests that Khc-73 could support the relatively longer runs (~3.05 μm) of the Rab4 vesicles in axons. It was shown that the Rab5-associated vesicles bind to KIF13A/B/Khc-73

(Bentley et al., 2015; Huckaba et al., 2011). Hence, we inferred that the sorting endosomes, positive for both Rab4 and Rab5, can engage both Kinesin-2 and KIF13A/Khc-73 through the cognate adaptors of these two Rabs. However, one needs suitably tagged reagents to establish this conjecture through direct observation *in vivo*.

The turnover of Rab-associated vesicles in the pre-synaptic region negatively influences synapse assembly

Local synaptic vesicle recycling mediated by RabGTPases is critical for the availability of fusion-ready synaptic vesicles to sustain neurotransmission. Translocation mechanisms of these RabGTPases to the synapses are poorly understood. Certain RabGTPases like Rab3 and Rab27 are known to associate with Kinesin-3 and Kinesin-1, respectively, for their anterograde axonal transport (Arimura et al., 2009; Niwa et al., 2008). Rab4 is a protein present on the endosomal sorting intermediates and allows transfer of cargoes from early endosomes to recycling endosomes. As Rab4 is present on both early and recycling endosomal population, logistics of Rab4 transport into the presynaptic terminal was unclear (Sönnichsen et al., 2000). Our results show that Rab4 associated vesicles are present throughout the neuron and moderately enriched in the synapse, which is a consequence of a small anterograde bias in their transport.

The activity of Rab4 is critical for the physiology of the neuron-like extension of the growth cone (Falk et al., 2014). Overexpressed levels and activity of Rab4 found in conjunction with neuropathy, such as the Alzheimer's disease, suggest that overactivation of the endosomal system could influence the disease progression (Cataldo et al., 2000). In *Drosophila*, Rab4 is expressed in both neuronal and non-neuronal tissues throughout the development except in optic lobe neurons of the pupal brain (Jin et al., 2012). These studies project the idea that expression of Rab4 and its activity are crucial for maintenance of neuronal physiology. We showed that the synaptic Rab4 is maintained through axonal transport by heterotrimeric Kinesin-2 and Kinesin-3. As the synaptic localization of Rab4 is dependent on its active form, other factors like PI3-Kinase are also implicated in this trafficking which is consistent with previous observation (Imamura et al., 2003). The results further suggested that the function and localization of Rab4 at axon termini are critical for the maintenance of synaptic balance at ventral ganglion. Overactivation of Rab4 in cholinergic neurons reduced the synapse-bearing region of the ventral ganglion, although the level of the marker, Bruchpilot, remained unchanged. This observation could suggest that the local Rab4 activity at the axon termini suppresses synapse formation. Consistent with this conjecture, we found that the synapse-bearing zone at the neuropil expands when $Rab4^{DN}$ is overexpressed. Although the current data is insufficient to explain the underlying mechanism, it indicated that alteration of the trafficking logistics driven by the heterotrimeric Kinesin-2 and a Kinesin-3 family motors might play an essential role in the development of the defect. Further analysis of the larval behavior and neuronal stability in the ventral ganglion with aging would be useful in unraveling the mechanism.

Experimental Procedures

All the work in mammalian cells was done in the G.B. lab at Jungers Center for Neurosciences, Oregon Health and Science University (OHSU) and overseen by its Institutional Animal Care and Use Committee (IACUC). Drosophila experiments at TIFR were conducted under the guidelines of its Institutional Bio-Safety Committee (IBSC).

Cloning

All cDNA constructs were cloned into a pCAG vector having Chicken β-Actin promoter with Cytomegalovirus-Immediate Early enhancer to ensure high copy number transfectants and steady expression. Details of all constructs are mentioned in the Supplemental material (Table S1).

Mammalian cell culture and Vesicle interaction assay

Vesicle interaction assay using inducible FRB-FKBP conjugation was performed on Rat embryonic fibroblast (REF) cells as described previously (Bentley et al., 2015). Details are mentioned in the supplemental experimental procedures.

Drosophila stocks, rearing and sample preparation

All fly stocks (Table S2) were reared at 25°C on standard corn agar meal with 12-hour light-dark cycle. Unless otherwise mentioned, the eggs were collected for an hour and kept at 25°C for 78 hours. Larvae were filleted in Jan and Jan Buffer (pH=7.2, 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2.2H_2O$, 35.3 mM Sucrose and 5 mM HEPES) through a dorsal incision longitudinally. Fillet preps were either placed on coverslips for immediate live imaging or fixed and immunostained before imaging (see the Supplemental Experimental Procedures for further details).

Imaging

Mammalian cells were imaged using LCI Plan Apochromat 60X/1.4 NA objective on Axio-Observer® epifluorescence setup. Images were acquired using AxioCam® and AxioVision® software (Carl Zeiss GmBH) with a pixel resolution of 0.1 × 0.1 μm^2. Larval ventral ganglia were imaged using 40X /1.35 NA objective at a pixel resolution of 0.6 × 0.6 μm^2 while Lateral Chordotonal (*lch5*) neurons on 60X /1.4 NA at a scaling of 0.115 × 0.115 μm^2 on Olympus FV1000SPD laser scanning confocal microscope.

For the larval locomotion assay video recordings were made using Canon EOS 750D DSLR camera at the rate of 25 frames per second for a duration of 1 min in standard light conditions (described in detail in the Supplemental experimental procedures).

Statistical procedures

All the data is analyzed and depicted with the help of Origin® (http://www.originlab.com/) software. The data is represented as box and whisker plots indicating 75 percentile of the distribution with a coefficient of 1.5. In the box plots, the horizontal bar and small square represent the median and mean respectively. Other plots are represented as histograms or scatter plots of mean values with their standard deviations. The peaks in the frequency

distribution plots are assessed by the “peak finder” module of Origin® (http://www.originlab.com/) using the first derivative method. The pairwise comparisons are made using one-way ANOVA and Bonferroni’s test and the significance values (*p<0.05, **p<0.01, ***p<0.001) are represented on the plots.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all the KR lab members, especially Akanksha Jain, Aparna Sadananda and Dipti Rai, for support, technical help and reagents. Special thanks to Boby KV for maintaining the confocal microscopy facility at DBS, TIFR. All the work in mammalian cells was done in the GB lab at Jungers Center for Neurosciences, Oregon Health and Science University (OHSU). We thank Julie Luisi for her technical help and other Banker lab members for their assistance. The research work at OHSU was supported by NIH grant MH066179 (to GB) and was facilitated by a Fulbright-Nehru Doctoral Fellowship 2014–15 to SD. We also thank Chris Doe for providing fly stocks. The overall research was funded by an intramural grant to KR and a fellowship to SD from TIFR, DAE, Govt. of India.

References

Arimura N, Kimura T, Nakamuta S, Taya S, Funahashi Y, Hattori A, Shimada A, Ménager C, Kawabata S, Fujii K, Iwamatsu A, Segal RA, Fukuda M, Kaibuchi K. Anterograde transport of TrkB in axons is mediated by direct interaction with Slp1 and Rab27. Dev Cell. 2009; 16:675–86. DOI: 10.1016/j.devcel.2009.03.005 [PubMed: 19460344]

Bananis E, Nath S, Gordon K, Satir P, Stockert RJ, Murray JW, Wolkoff AW. Microtubule-dependent movement of late endocytic vesicles in vitro: requirements for Dynein and Kinesin. Mol Biol Cell. 2004; 15:3688–97. DOI: 10.1091/mbc.E04-04-0278 [PubMed: 15181154]

Baqri R, Charan R, Schimmelpfeng K, Chavan S, Ray K. Kinesin-2 Differentially Regulates the Anterograde Axonal Transports of Acetylcholinesterase and Choline Acetyltransferase in Drosophila. J Neurobiol. 2006; :378–392. DOI: 10.1002/neu [PubMed: 16408306]

Bentley M, Decker H, Luisi J, Banker G. A novel assay reveals preferential binding between Rabs, kinesins, and specific endosomal subpopulations. J Cell Biol. 2015; 208:273–281. DOI: 10.1083/jcb.201408056 [PubMed: 25624392]

Brown CL, Maier KC, Stauber T, Ginkel LM, Wordeman L, Vernos I, Schroer TA. Kinesin-2 is a motor for late endosomes and lysosomes. Traffic. 2005; 6:1114–24. DOI: 10.1111/j.1600-0854.2005.00347.x [PubMed: 16262723]

Brown TC, Correia SS, Petrok CN, Esteban JA. Functional compartmentalization of endosomal trafficking for the synaptic delivery of AMPA receptors during long-term potentiation. J Neurosci. 2007; 27:13311–5. DOI: 10.1523/JNEUROSCI.4258-07.2007 [PubMed: 18045925]

Campos-Ortega, JA., Hartenstein, V. The Embyronic Development of Drosophila melanogaster. Springer; Berlin Heidelberg: 1985. Histogenesis and Organogenesis; p. 85-164.

Cataldo AM, Barnett JL, Pieroni C, Nixon RA. Increased neuronal endocytosis and protease delivery to early endosomes in sporadic Alzheimer’s disease: neuropathologic evidence for a mechanism of increased beta-amyloidogenesis. J Neurosci. 1997; 17:6142–6151. [PubMed: 9236226]

Cataldo AM, Peterhoff CM, Troncoso JC, Gomez-Isla T, Hyman BT, Nixon RA. Endocytic pathway abnormalities precede amyloid beta deposition in sporadic Alzheimer’s disease and Down syndrome: differential effects of APOE genotype and presenilin mutations. Am J Pathol. 2000; 157:277–86. [PubMed: 10880397]

de Renzis S, Sönnichsen B, Zerial M. Divalent Rab effectors regulate the sub-compartmental organization and sorting of early endosomes. Nat Cell Biol. 2002; 4:124–33. DOI: 10.1038/ncb744 [PubMed: 11788822]

Doodhi H, Ghosal D, Krishnamurthy M, Jana SC, Shamala D, Bhaduri A, Sowdhamini R, Ray K. KAP, the accessory subunit of kinesin-2, binds the predicted coiled-coil stalk of the motor subunits. Biochemistry. 2009; 48:2248–60. DOI: 10.1021/bi8018338 [PubMed: 19161286]

Doodhi H, Jana SC, Devan P, Mazumdar S, Ray K. Biochemical and molecular dynamic simulation analysis of a weak coiled coil association between kinesin-II stalks. PLoS One. 2012; 7:e45981.doi: 10.1371/journal.pone.0045981 [PubMed: 23029351]

Falk J, Konopacki FA, Zivraj KH, Holt CE. Rab5 and Rab4 regulate axon elongation in the Xenopus visual system. J Neurosci. 2014; 34:373–91. DOI: 10.1523/JNEUROSCI.0876-13.2014 [PubMed: 24403139]

Gindhart JG, Chen J, Faulkner M, Gandhi R, Doerner K, Wisniewski T, Nandlestadt A. The kinesin-associated protein UNC-76 is required for axonal transport in the Drosophila nervous system. Mol Biol Cell. 2003; 14:3356–65. DOI: 10.1091/mbc.E02-12-0800 [PubMed: 12925768]

Girotra M, Srivastava S, Kulkarni A, Barbora A, Bobra K, Ghosal D, Devan P, Aher A, Jain A, Panda D, Ray K. The C-terminal tails of heterotrimeric kinesin-2 motor subunits directly bind to α-tubulin1: possible implications for cilia-specific tubulin entry. Traffic. 2016; doi: 10.1111/tra.12461

Greenspan RJ. Mutations of choline acetyltransferase and associated neural defects. J Comp Physiol. 1980; 137:83–92. DOI: 10.1007/BF00656920

Gurkan C, Lapp H, Alory C, Su AI, Hogenesch JB, Balch WE. Large-scale profiling of Rab GTPase trafficking networks: the membrome. Mol Biol Cell. 2005; 16:3847–64. DOI: 10.1091/mbc.E05-01-0062 [PubMed: 15944222]

Hirokawa N, Noda Y, Tanaka Y, Niwa S. Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol. 2009; 10:682–96. DOI: 10.1038/nrm2774 [PubMed: 19773780]

Hoogenraad CC, Popa I, Futai K, Martinez-Sanchez E, Sanchez-Martinez E, Wulf PS, van Vlijmen T, Dortland BR, Oorschot V, Govers R, Monti M, Heck AJR, Sheng M, Klumperman J, Rehmann H, Jaarsma D, Kapitein LC, van der Sluijs P. Neuron specific Rab4 effector GRASP-1 coordinates membrane specialization and maturation of recycling endosomes. PLoS Biol. 2010; 8:e1000283.doi: 10.1371/journal.pbio.1000283 [PubMed: 20098723]

Hoopmann P, Punge A, Barysch SV, Westphal V, Bückers J, Opazo F, Bethani I, Lauterbach MA, Hell SW, Rizzoli SO. Endosomal sorting of readily releasable synaptic vesicles. Proc Natl Acad Sci U S A. 2010; 107:19055–60. DOI: 10.1073/pnas.1007037107 [PubMed: 20956291]

Horgan CP, McCaffrey MW. Rab GTPases and microtubule motors. Biochem Soc Trans. 2011; 39:1202–6. DOI: 10.1042/BST0391202 [PubMed: 21936789]

Huckaba TM, Gennerich A, Wilhelm JE, Chishti AH, Vale RD. Kinesin-73 is a processive motor that localizes to Rab5-containing organelles. J Biol Chem. 2011; 286:7457–67. DOI: 10.1074/jbc.M110.167023 [PubMed: 21169635]

Hunt SD, Stephens DJ. The role of motor proteins in endosomal sorting. Biochem Soc Trans. 2011; 39:1179–1184. [PubMed: 21936785]

Imamura T, Huang J, Usui I. Insulin-induced GLUT4 translocation involves protein kinase C-λ-mediated functional coupling between Rab4 and the motor protein kinesin. Mol Cell Biol. 2003

Jin EJ, Chan CC, Agi E, Cherry S, Hanacik E, Buszczak M, Hiesinger PR. Similarities of Drosophila rab GTPases based on expression profiling: completion and analysis of the rab-Gal4 kit. PLoS One. 2012; 7:e40912.doi: 10.1371/journal.pone.0040912 [PubMed: 22844416]

Jordens I, Marsman M, Kuijl C, Neefjes J. Rab proteins, connecting transport and vesicle fusion. Traffic. 2005; 6:1070–7. DOI: 10.1111/j.1600-0854.2005.00336.x [PubMed: 16262719]

Jovic M, Sharma M, Rahajeng J, Caplan S. The early endosome: a busy sorting station for proteins at the crossroads. Histol Histopathol. 2010; 25:99–112. [PubMed: 19924646]

Kulkarni A, Khan Y, Ray K. Heterotrimeric kinesin-2, together with kinesin-1, steers vesicular acetylcholinesterase movements toward the synapse. FASEB J. 2016; fj.201600759RRR. doi: 10.1096/fj.201600759RRR

Lasiecka ZM, Yap CC, Vakulenko M, Winckler B. Compartmentalizing the neuronal plasma membrane from axon initial segments to synapses. Int Rev Cell Mol Biol. 2009; 272:303–89. DOI: 10.1016/S1937-6448(08)01607-9 [PubMed: 19121821]

Lazzarino DA, Blier P, Mellman I. The monomeric guanosine triphosphatase rab4 controls an essential step on the pathway of receptor-mediated antigen processing in B cells. J Exp Med. 1998; 188:1769–74. [PubMed: 9815254]

Li D, Kuehn EW, Prekeris R. Kinesin-2 mediates apical endosome transport during epithelial lumen formation. Cell Logist. 2014; 4:e28928.doi: 10.4161/cl.28928 [PubMed: 24843830]

McCaffrey MW, Bielli A, Cantalupo G, Mora S, Roberti V, Santillo M, Drummond F, Bucci C. Rab4 affects both recycling and degradative endosomal trafficking. FEBS Lett. 2001; 495:21–30. DOI: 10.1016/S0014-5793(01)02359-6 [PubMed: 11322941]

Miki H, Setou M, Kaneshiro K, Hirokawa N. All kinesin superfamily protein, KIF, genes in mouse and human. Proc Natl Acad Sci U S A. 2001; 98:7004–11. DOI: 10.1073/pnas.111145398 [PubMed: 11416179]

Niwa S, Tanaka Y, Hirokawa N. KIF1Bbeta- and KIF1A-mediated axonal transport of presynaptic regulator Rab3 occurs in a GTP-dependent manner through DENN/MADD. Nat Cell Biol. 2008; 10:1269–79. DOI: 10.1038/ncb1785 [PubMed: 18849981]

Okada Y, Yamazaki H, Sekine-Aizawa Y, Hirokawa N. The neuron-specific kinesin superfamily protein KIF1A is a unique monomeric motor for anterograde axonal transport of synaptic vesicle precursors. Cell. 1995; 81:769–80. [PubMed: 7539720]

Ray K, Perez SE, Yang Z, Xu J, Ritchings BW, Steller H, Goldstein LS. Kinesin-II is required for axonal transport of choline acetyltransferase in Drosophila. J Cell Biol. 1999; 147:507–18. [PubMed: 10545496]

Rizzoli SO. Synaptic vesicle recycling: steps and principles. EMBO J. 2014; 33:788–822. DOI: 10.1002/embj.201386357 [PubMed: 24596248]

Sadananda A, Hamid R, Doodhi H, Ghosal D, Girotra M, Jana SC, Ray K. Interaction with a kinesin-2 tail propels choline acetyltransferase flow towards synapse. Traffic. 2012; 13:979–91. DOI: 10.1111/j.1600-0854.2012.01361.x [PubMed: 22486887]

Salvaterra PM, Kitamoto T. Drosophila cholinergic neurons and processes visualized with Gal4/UAS–GFP. Gene Expr Patterns. 2001; 1:73–82. DOI: 10.1016/S1567-133X(01)00011-4

Sarpal R, Todi SV, Sivan-loukianova E, Shirolikar S, Subramanian N, Raff EC, Erickson JW, Ray K, Eberl DF. Drosophila KAP Interacts with the Kinesin II Motor Subunit KLP64D to Assemble Chordotonal Sensory Cilia, but Not Sperm Tails. 2003; 13:1687–1696. DOI: 10.1016/j [PubMed: 14521834]

Schonteich E, Wilson GM, Burden J, Hopkins CR, Anderson K, Goldenring JR, Prekeris R. The Rip11/Rab11-FIP5 and kinesin II complex regulates endocytic protein recycling. J Cell Sci. 2008; 121:3824–33. DOI: 10.1242/jcs.032441 [PubMed: 18957512]

Schuster M, Lipowsky R, Assmann MA, Lenz P, Steinberg G. Transient binding of dynein controls bidirectional long-range motility of early endosomes. Proc Natl Acad Sci U S A. 2011; 108:3618–23. DOI: 10.1073/pnas.1015839108 [PubMed: 21317367]

Shirakawa R, Yoshioka A, Horiuchi H, Nishioka H, Tabuchi A, Kita T. Small GTPase Rab4 regulates Ca2+-induced alpha-granule secretion in platelets. J Biol Chem. 2000; 275:33844–9. DOI: 10.1074/jbc.M002834200 [PubMed: 10938270]

Siegrist SE, Doe CQ. Microtubule-Induced Pins/Gαi Cortical Polarity in Drosophila Neuroblasts. Cell. 2005; 123:1323–1335. DOI: 10.1016/j.cell.2005.09.043 [PubMed: 16377571]

Sönnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M. Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol. 2000; 149:901–14. [PubMed: 10811830]

Soppina V, Rai AK, Ramaiya AJ, Barak P, Mallik R. Tug-of-war between dissimilar teams of microtubule motors regulates transport and fission of endosomes. Proc Natl Acad Sci U S A. 2009; 106:19381–6. DOI: 10.1073/pnas.0906524106 [PubMed: 19864630]

Tsurudome K, Tsang K, Liao EH, Ball R, Penney J, Yang JS, Elazzouzi F, He T, Chishti A, Lnenicka G, Lai EC, Haghighi AP. The Drosophila miR-310 Cluster Negatively Regulates Synaptic Strength at the Neuromuscular Junction. Neuron. 2010; 68:879–893. DOI: 10.1016/j.neuron.2010.11.016 [PubMed: 21145002]

Ueno H, Huang X, Tanaka Y, Hirokawa N. KIF16B/Rab14 Molecular Motor Complex Is Critical for Early Embryonic Development by Transporting FGF Receptor. Dev Cell. 2011; 20:60–71. DOI: 10.1016/j.devcel.2010.11.008 [PubMed: 21238925]

Vitale G, Rybin V, Christoforidis S, Thornqvist P, McCaffrey M, Stenmark H, Zerial M. Distinct Rab-binding domains mediate the interaction of Rabaptin-5 with GTP-bound Rab4 and Rab5. EMBO J. 1998; 17:1941–51. DOI: 10.1093/emboj/17.7.1941 [PubMed: 9524117]

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E. Bruchpilot, a Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of Synaptic Active Zones in Drosophila. Neuron. 2006; 49:833–844. DOI: 10.1016/j.neuron.2006.02.008 [PubMed: 16543132]

Highlights

- Live imaging enables quantitative study of transport of Rab4 vesicles in *Drosophila*.
- Rab4 vesicles selectively interact with Kinesin-2α and KIF13A/B subunits.
- Heterotrimeric Kinesin-2 drives most of the anterograde Rab4 traffic in axons.
- Reduced Rab4 inflow increases synapse density and enhances larval motility.

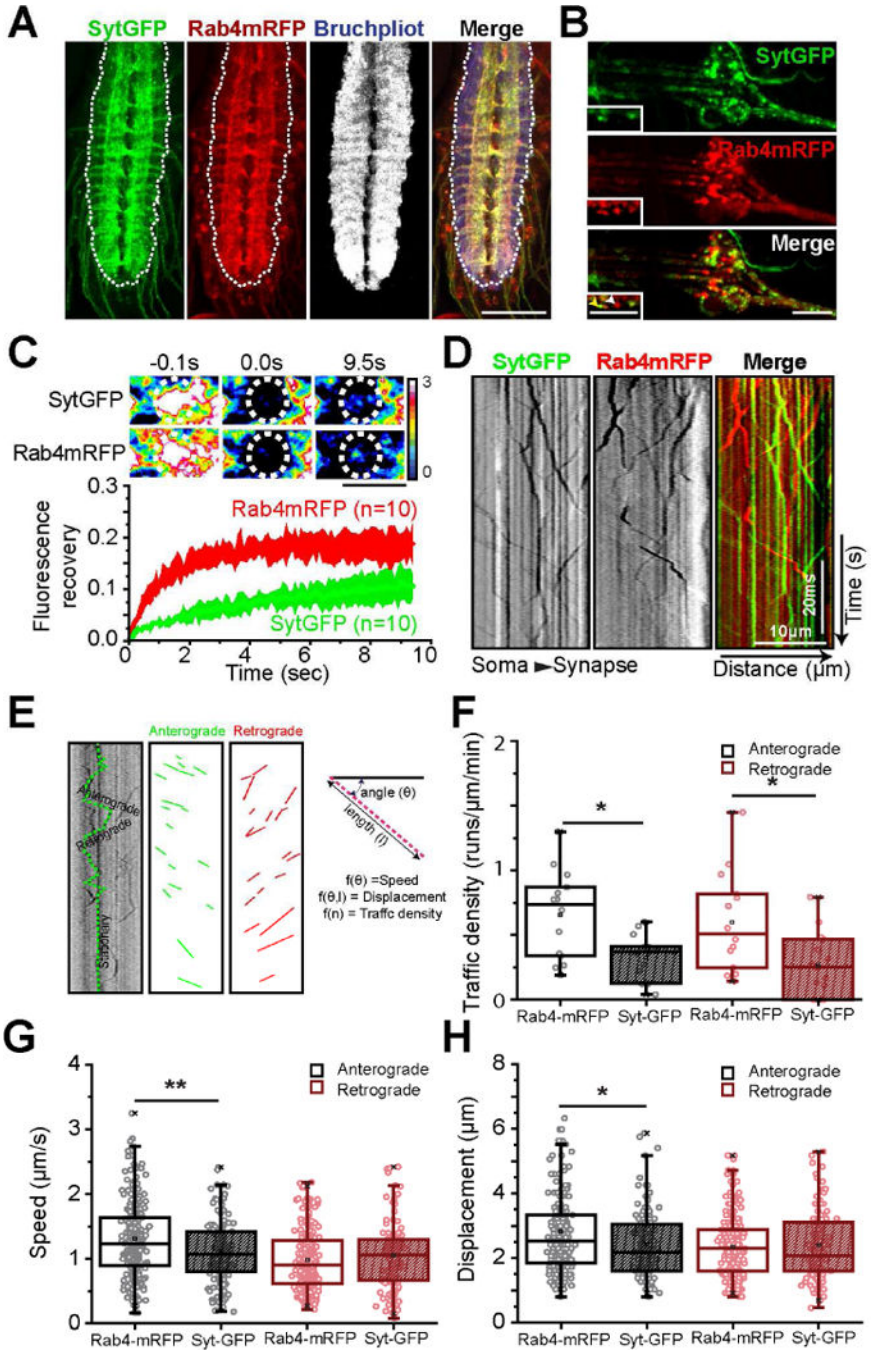


Fig.1.

Transport of Rab4-associated vesicles in the sensory axons of *Drosophila*.

A–B) Coexpression of Rab4-mRFP and Syt-GFP by *chaGal4* show localization of Rab4-mRFP and Syt-GFP-marked structures in the ventral ganglion (A) and *lch5* neurons (B). Inset in (B) shows Rab4 positive vesicles overlapping (yellow arrowhead) and not overlapping (red arrowhead) with Syt-GFP vesicles. Scale bars indicate 10 µm and 5 µm (inset), respectively.

C) Fluorescence recovery after photobleaching (FRAP) profiles of Rab4-mRFP and Syt-GFP in the synaptic boutons of the ventral neuropil. Data is represented as Mean ± S. D. and n values are indicated on the plots. Scale bar indicates 5 μm.

D) Representative kymographs obtained from time-lapse imaging of Rab4-mRFP and Syt-GFP particles in the *lch5* axons.

E) Kymograph illustrates the movement of a Rab4-associated vesicle (marked by green dotted line). The panels on the right side indicate anterograde (green lines) and retrograde (red lines) run segments extracted from the kymograph.

F–H) Anterograde and retrograde transport parameters of Rab4-mRFP and Syt-GFP vesicles quantified as traffic density (F), speed (G), and displacement (H). The pairwise comparison of means, estimated using ANOVA, are indicated (*$p<0.05$, **$p<0.01$) on the plots.

(Movies S1 and S2).

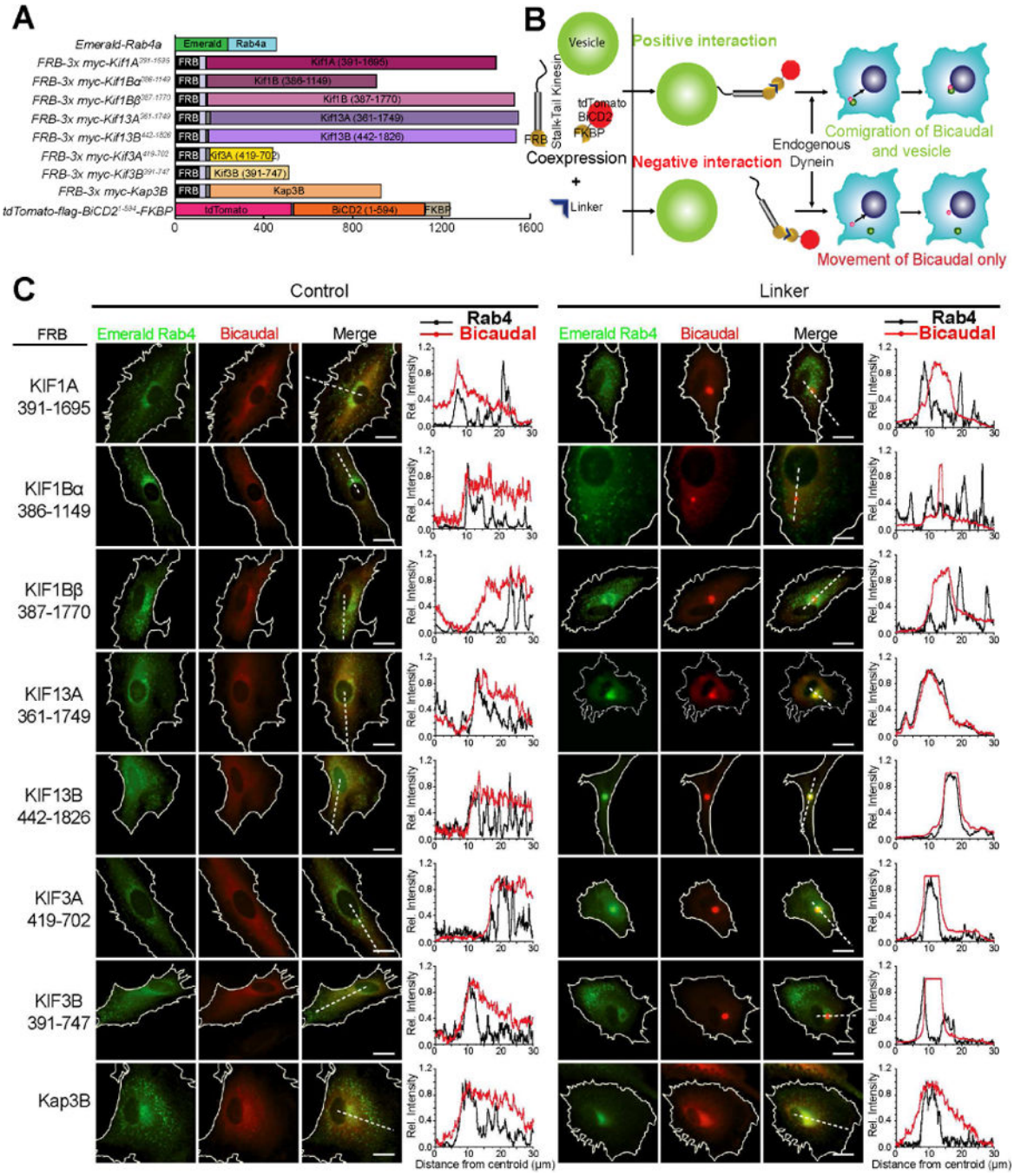


Fig.2.

Evaluation of interaction between the Rab4-assocated vesicles and Kinesin motors in tissue cultured cells using the FRB-FKBP assay.

A) Constructs used in the FRB-FKBP inducible interaction assay to assess the interaction between Rab4 associated vesicles and the stalk-tail domains of different kinesins.

B) Schematic of the FRB-FKBP interaction assay with expected readouts in the case of a positive or negative interaction.

C) The localization of Emerald-Rab4, FRB bait proteins, and tdTomato-BicD2-FKBP in control fibroblasts and in the fibroblasts following the addition of the linker. Linescans from the centroid of the nucleus (dashed lines in Merge) show the degree to which the intensity profiles of Bicaudal and Rab4 overlap. See also Figure S2.

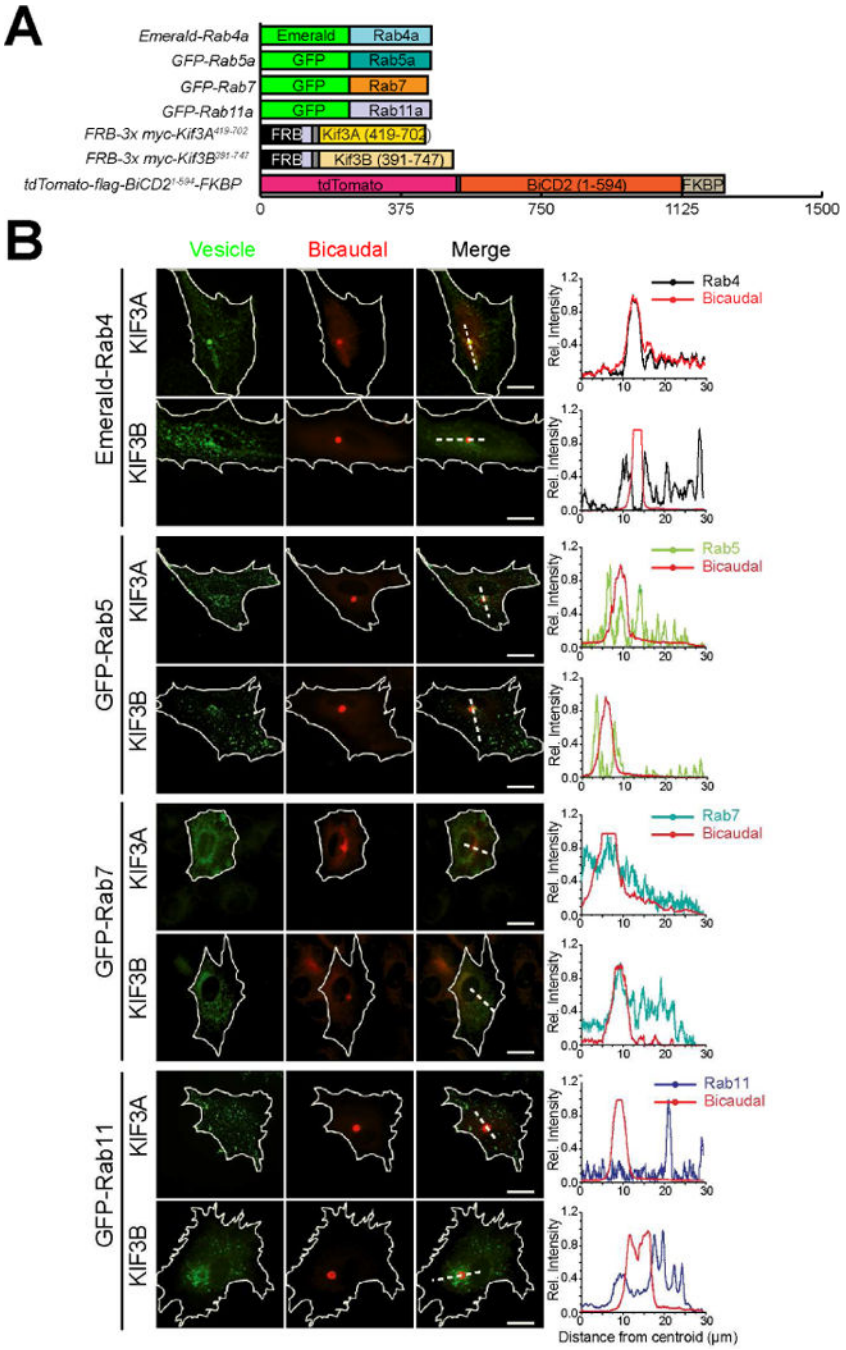


Fig.3.

Evaluation of interaction between Kinesin-2 and vesicles labeled with different Rab.

A) Constructs used in the assay to estimate the interaction of Kinesin-2 stalk-tail domains with endosomes labeled by Rab4, Rab5, Rab7, or Rab11.

B) Fluorescently labeled vesicular markers Emerald-Rab4, GFP-Rab5, GFP-Rab7, and GFP-Rab11, were coexpressed with tdTomato-BicD2-FKBP and FRB-Kif3A$^{419-702}$ or FRB-Kif3B$^{391-747}$. Line scans (dashed lines in Merge) show the degree of overlap between the vesicular markers and Bicaudal.

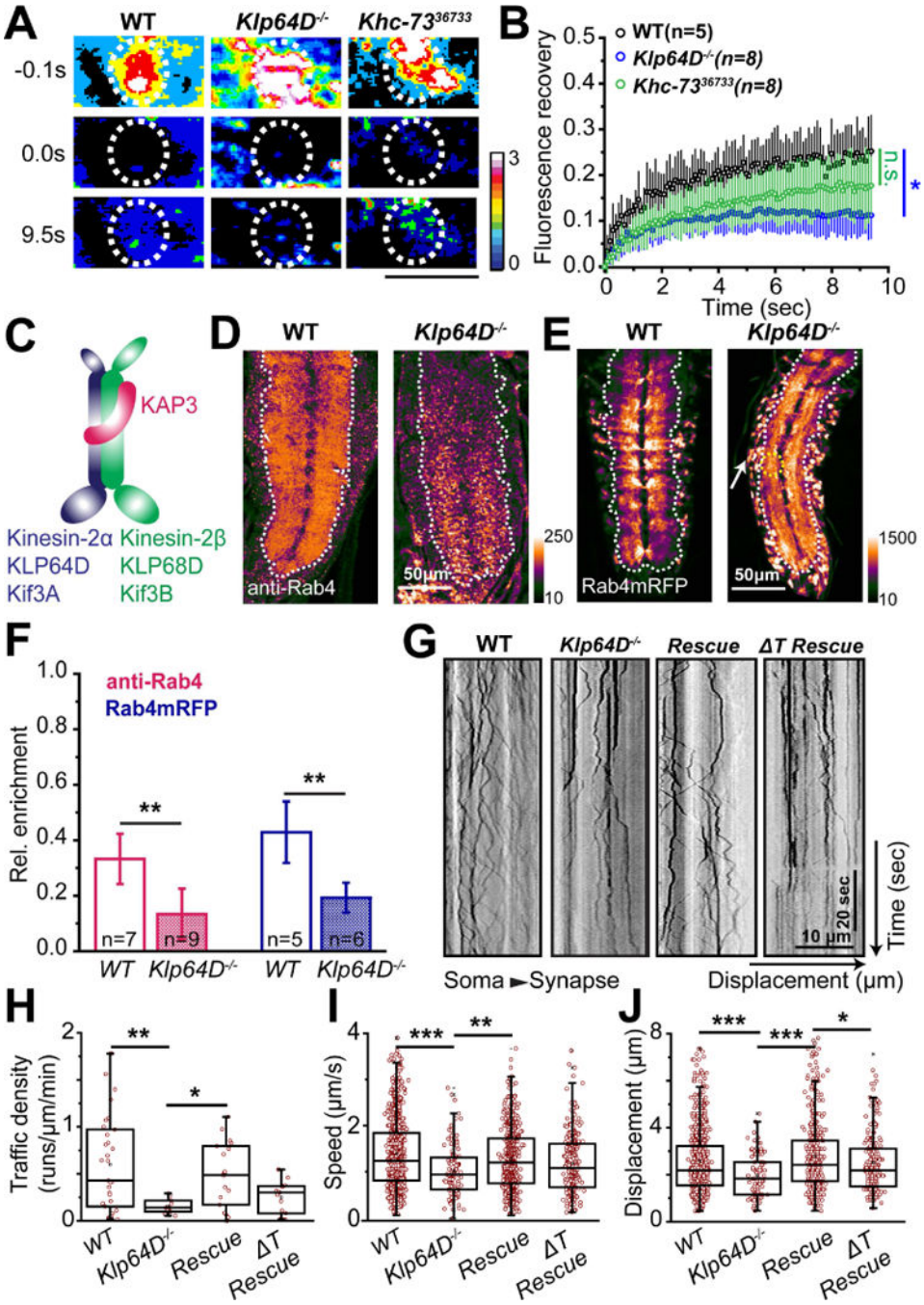


Fig.4.

Role of Kinesin-2α (KLP64D) tail in the anterograde axonal transport of Rab4-associated vesicles.

A, B) Synaptic FRAP profiles of Rab4-mRFP in the wild-type (WT), homozygous *Klp64D*^{k5} (*Klp64D*$^{-/-}$), and *Khc-73*^{36733} dsRNA expressing backgrounds. (A) and (B) depict a select set of time-lapse images in a pseudo color scheme (A), and the recovery of intensity values (average ± SD) with respect to the post-bleach intensities (B). Scale bar indicates 5 μm.

C) Schematic illustrates the composition of the heterotrimeric Kinesin-2 in *Drosophila* and mammals.

D–F) Localization of Rab4 in the ventral ganglion of third instar larvae in wild-type and homozygous *Klp64D*^{k5} (*Klp64D*$^{-/-}$) backgrounds. Pseudocolored images of the representative ventral ganglia show relative levels of the endogenous Rab4 (D) and ectopically expressed Rab4-mRFP (E). Cell bodies show accumulated Rab4 in the cortex (white arrow) and decreased intensity in the neuropil (yellow circle) of the *Klp64D* mutant (E). The histograms depict relative enrichment (mean ± S.D.) of the endogenous Rab4 (magenta) and ectopically overexpressed Rab4-mRFP (indigo) in the wild-type and mutant backgrounds (F).

G) Kymographs of the Rab4-mRFP movement in *lch5* axons. Rab4mRFP was expressed by *chaGal4* in wild-type, the *Klp64D*^{k5}, and in two different transgenically rescued backgrounds (*Klp64D*^{k5} *Rescue - chaGal4>UAS-Klp64D-GFP/chaGal4>UAS-Rab4-mRFP; Klp64D*^{k5}; and *Klp64D*^{k5} *ΔT-Rescue - chaGal4>UAS-Klp64DΔT-GFP/chaGal4>UAS-Rab4-mRFP; Klp64D*^{k5}).

H–J) Traffic density (H), speed (I) and displacement (J) of Rab4-mRFP in *lch5* axons. The p-values (*p<0.05, **p<0.01, ***p<0.001) were estimated using ANOVA.

(Movies S3 and S4A)

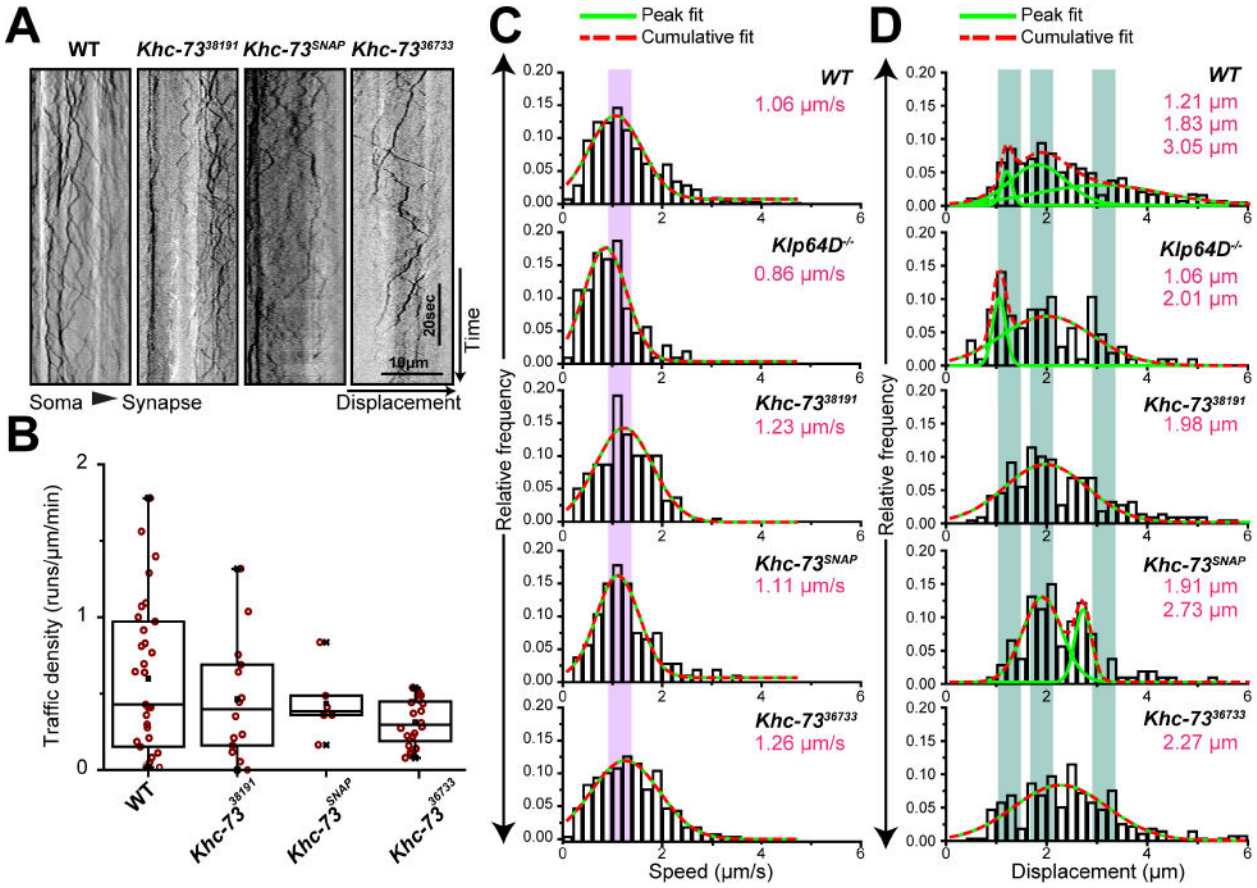


Fig 5.

Role of Khc-73 in the axonal transport of Rab4-associated vesicles.

A) Kymographs depict the Rab4-mRFP movement in the presence of *Khc-73* (38191, SNAP, and 36733) dsRNA in *lch5* neurons.

B) Traffic density in different *Khc-73* RNAi backgrounds. Note that the spread of the distribution reduces considerably in the presence of *Khc-73*^{36733}.

C, D) The speed (C) and displacements (D) of Rab4 associated vesicles in different *Khc-73* RNAi backgrounds. Red dotted lines indicate a multimodal curve fit of the entire distribution. The green lines indicate individual Gaussian distribution modes that could be fitted to each multimodal curve fits (dotted red). Modal values of individual distribution (X-coordinate of the peak) are indicated on each plot. Note that the frequency of long runs is absent in the *Khc-73* RNAi backgrounds.

(Figure S4 and Movie S4B)

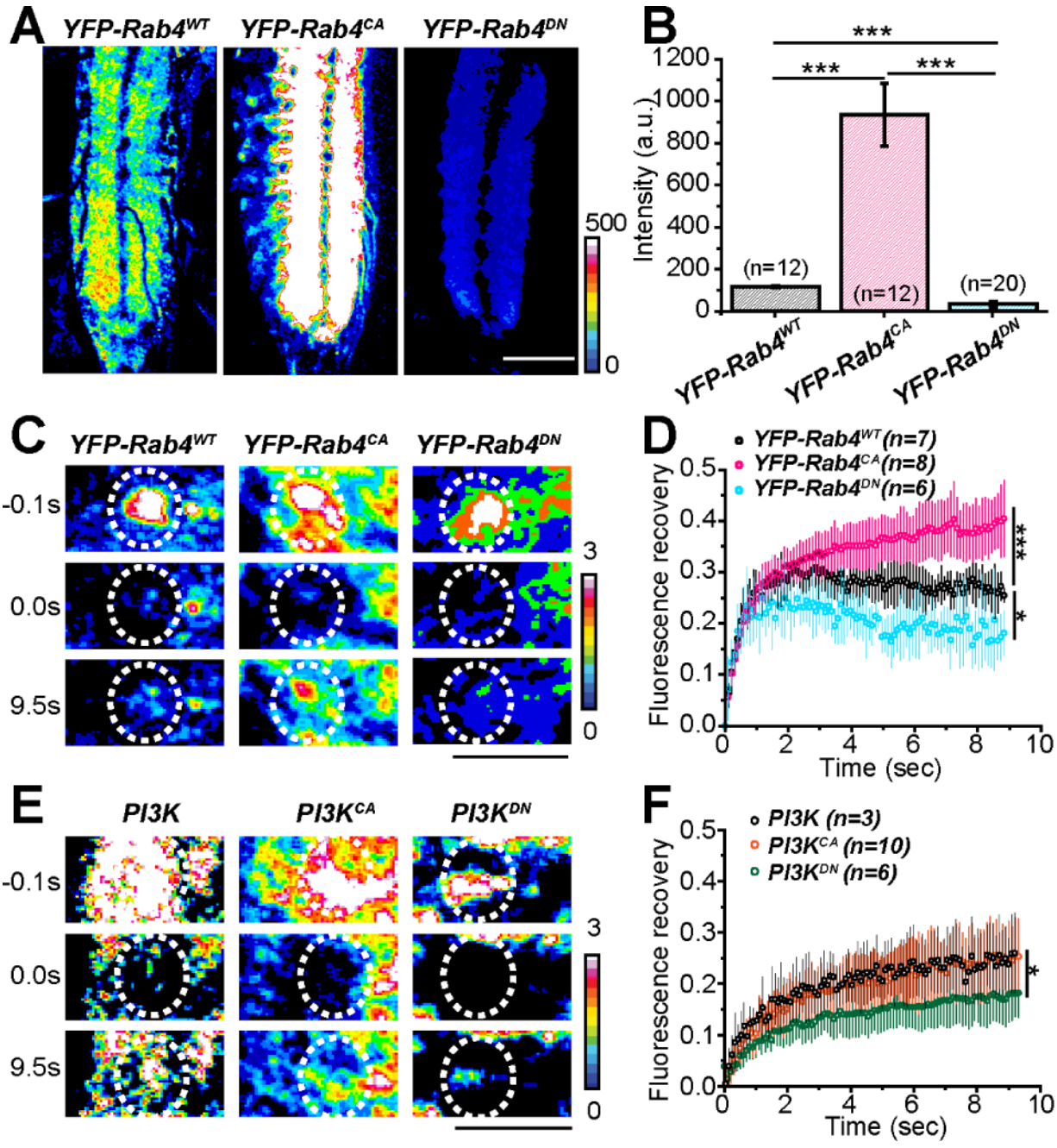


Fig 6.

Regulation of Rab4 transport.

A) Distribution of YFP fluorescence, presented in a pseudocolored intensity scheme (right margin), in the ventral ganglion region of third instar larvae expressing different Rab4 variants.

B) Estimation of the YFP intensity at the neuropil region of larvae expressing different Rab4 variants. Data are represented as mean ± SD. Scale bars indicate 50 μm.

C) Synaptic FRAP profiles, presented in a pseudocolored intensity scheme (right margin), before (−0.1 sec), immediately after (0.0 sec) and at 9.5 seconds after the photobleach. Scale bar indicates 5 μm.

D) YFP Fluorescence recovery (mean ± S.D.) after the photobleach.

E, F) Synaptic FRAP profiles of Rab4-mRFP in neurons coexpressing the wild-type regulatory subunit of PI3K (*PI3K*) and two different forms of *Pi3k92E* (type I) catalytic subunits – the constitutive active $Pi3K92E^{CAAX}$ ($Pi3K^{CA}$) and dominant negative $Pi3K92E^{A2860C}$ ($Pi3K^{DN}$), respectively. (E) and (F) depict a select set of time-lapse images in pseudocolor scheme (E), and the recovery of intensity values (average ± SD) with respect to the post-bleach intensities (F). Scale bar indicates 5 μm.

(Figure S6 and Movies S5).

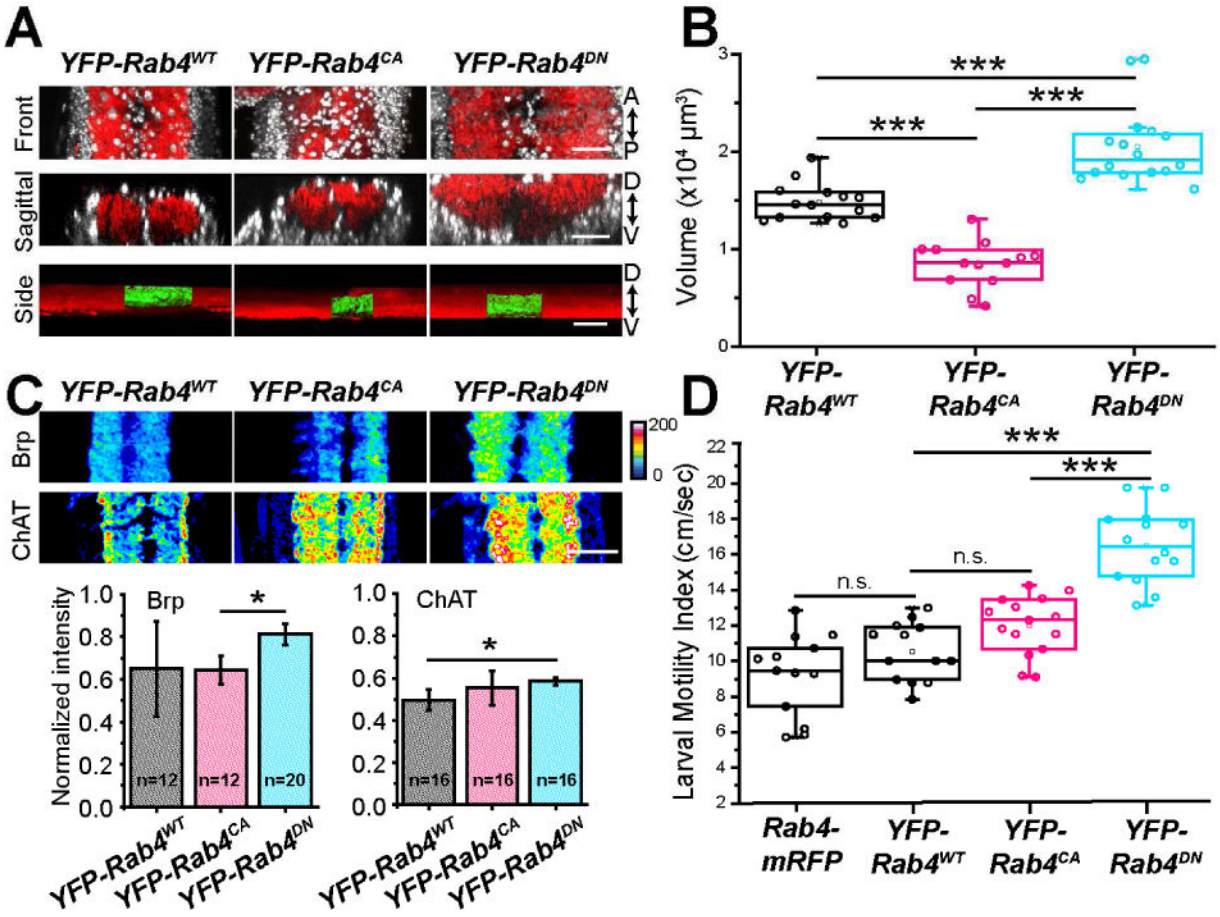


Fig 7.

Role of Rab4 transport in the maintenance of synaptic organization and larval locomotion.

A) Front, transverse and side views of the ventral ganglia (abdominal segments A4–6) from larvae expressing different Rab4 variants in cholinergic neurons. There was a marked increase in the synaptic region marked by Bruchpilot (red) due to the expression of *YFP-Rab4^{DN}*. Nuclei marked by the Hoechst dye are shown in white. The bottom panel shows Brp-stained neuromere hemisegment surface (green) rendered using the "Surface" module of Imaris (Scale bar, 20 µm) in a montage.

B) Estimation of the neuromere volumes in the larvae expressing different Rab4 variants.

C) Relative enrichment of pre-synaptic markers, Bruchpilot (Brp) and Choline Acetyltransferase (ChAT), in the neuropil (Scale bar, 50 µm). Histograms depict relative intensity (mean ± S.D).

D) Motility indices of larvae expressing different Rab4 variants.