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# Two Clathrin Adaptor Protein Complexes Instruct Axon-Dendrite Polarity

## Highlights

- AP-3 and AP-1 sort transmembrane proteins to the axon and the dendrite, respectively
- AP-3 strongly binds to axonal sorting motifs and select cargos to axonal vesicles
- AP-3 and AP-1 are localized and functioning at different domains of Golgi apparatus

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## In Brief

Li et al. studied how axonal and dendritic proteins are targeted to their destined compartments from Golgi apparatus in *C. elegans*. AP-3 strongly binds to axonal sorting motifs and select cargos to the axon. Dendritic targeting is mediated by AP-1.

Li et al., 2016, Neuron *90*, 564–580
May 4, 2016 
http://dx.doi.org/10.1016/j.neuron.2016.04.020

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# Two Clathrin Adaptor Protein Complexes Instruct Axon-Dendrite Polarity

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http://dx.doi.org/10.1016/j.neuron.2016.04.020

## SUMMARY

**The cardinal feature of neuronal polarization is the establishment and maintenance of axons and dendrites. How axonal and dendritic proteins are sorted and targeted to different compartments is poorly understood. Here, we identified distinct dileucine motifs that are necessary and sufficient to target transmembrane proteins to either the axon or the dendrite through direct interactions with the clathrin-associated adaptor protein complexes (APs) in *C. elegans*. Axonal targeting requires AP-3, while dendritic targeting is mediated by AP-1. The axonal dileucine motif binds to AP-3 with higher efficiency than to AP-1. Both AP-3 and AP-1 are localized to the Golgi but occupy adjacent domains. We propose that AP-3 and AP-1 directly select transmembrane proteins and target them to axon and dendrite, respectively, by sorting them into distinct vesicle pools.**

## INTRODUCTION

Neurons are highly polarized cells with morphologically and functionally distinct neurites: dendrites and axons, which contain different proteins for specialized functions. Neurotransmitter receptors, specific ion channels, and signaling molecules are localized at the postsynaptic membrane in the dendrite, while synaptic vesicles (SVs), certain ion channels, and neuromodulators are targeted to the axon (Horton and Ehlers, 2003).

Studies in polarized non-neuronal cells have provided substantial knowledge of the establishment of the cell polarity (Guo et al., 2014; Horton and Ehlers, 2003). Proteins destined to apical and basolateral plasma membranes are thought to take different post-Golgi trafficking routes. In MDCK (Madin-Darby canine kidney) cells, apical and basolateral cargos are segregated at the *trans*-Golgi network (TGN) and exit via separate transport carriers (Keller et al., 2001). The basolateral epithelial proteins are targeted to the somatodendritic compartment in cultured hippocampal neurons (Jareb and Banker, 1998; Parton et al., 1992), suggesting that dendritic protein sorting might adopt a similar mechanism. Additional post-sorting mechanisms also contribute to the establishment and maintenance of neural polarity. First, selective vesicular trafficking by the microtubule motor system transports specific cargo proteins to distinct compartments (Huang and Banker, 2012; Nakata and Hirokawa, 2003; Namba et al., 2011; Setou et al., 2000). Second, a diffusion barrier at the axon initial segment participates in both establishing and maintaining the neural polarity (Al-Bassam et al., 2012; Burack et al., 2000; Sampo et al., 2003). A recent study further showed that most dendritic vesicles are excluded from the axon at a more proximal region, the preaxonal exclusion zone (PAEZ). This polarized sorting at the PAEZ is dependent on axonal and dendritic vesicles binding to different microtubule motors (Farías et al., 2015). Although it has been demonstrated that axonal and dendritic proteins take different trafficking routes, little is known about how axonal and dendritic proteins are distinguished and sorted in the TGN.

One of the major sorting mechanisms in cell biology requires the family of clathrin-associated adaptor protein (AP) complexes, including AP-1 and AP-3. Both AP-1 and AP-3 are localized at the TGN and endosomes and mediate different sorting processes in polarized epithelial cells (Nakatsu and Ohno, 2003). Specifically, AP-1 targets cargos to the basolateral surface (Fölsch et al., 1999, 2001). In contrast, AP-3 is involved in sorting proteins from early endosomes to lysosomes (Le Borgne and Hoflack, 1998; Le Borgne et al., 1998). AP-3 also promotes protein sorting into the regulatory secretory pathway from the TGN (Asensio et al., 2010). Loss of AP-3 causes multiple cargos to be missorted into the constitutive secretory pathway.

In recent years, the roles of AP complexes in neurons have begun to be described. In particular, AP-1 at the Golgi apparatus participates in sorting multiple transmembrane proteins into the somatodendritic compartment (Farías et al., 2012; Jain et al., 2015). In *C. elegans*, AP-1 is implicated in sorting multiple receptors and transporters to dendrites (Dwyer et al., 2001; Margeta et al., 2009; Zhou et al., 2016). In contrast, AP-3 is involved in localizing several SV proteins (Nakatsu et al., 2004; Salazar et al., 2004a, 2005). Moreover, a role for neuronal AP-3 in generating SVs by budding synapse-like microvesicles (SLMVs) from endosomes has also been observed by both in vitro and in vivo assays (Blumstein et al., 2001; Faúndez et al., 1998; Lichtenstein et al., 1998), suggesting that AP-3 recognizes and sorts SV proteins into particular vesicles. Together, these studies support the notion that AP-1 and AP-3 are necessary for the sorting and correct targeting of subsets of membrane proteins in neurons. However, several key questions in neuronal protein sorting remain unanswered. For example, where and how are axonal

and dendritic cargos distinguished during the sorting processes? Are AP-3 and AP-1 instructive in guiding axonal and dendritic proteins to the correct subcellular compartments?

AP complexes mediate vesicle formation and cargo selection by recognizing and binding to signal motifs located in the cytoplasmic domains of the cargo proteins (Robinson, 2004). Each AP complex is composed of four subunits, with each subunit serving a specific role (Bonifacino, 2014). Specifically, the $\alpha$-$\sigma$ hemicomplex of AP-2 binds with another protein sorting motif, the dileucine motif [D/E]xxxL[L/I] (Doray et al., 2007; Kelly et al., 2008). The role of the three residues in the middle of the dileucine motif has not been well studied, although one in vitro study indicates that these three amino acids might be important for conferring different affinities to AP-1 and AP-3 (Rodionov et al., 2002).

Here we show that AP-3 and AP-1 sort axonal and dendritic proteins, respectively, in the soma. Both AP-3 and AP-1 reside at the Golgi apparatus and occupy adjacent, but not overlapping, domains. Axonal and dendritic cargos are sorted into different types of post-Golgi vesicles and transported to their respective destinations. This polarized targeting is mediated by specific dileucine motifs having different binding affinities to AP-3 and AP-1. An axonal dileucine motif binds to AP-3 with high affinity and is required for targeting membrane proteins to the axon. Dendritic motifs with low AP-3 binding efficiency bind AP-1 and are targeted to the dendrite. We propose a sorting model in the soma that AP-3 binds and sorts axonal cargos, while AP-1 sorts dendritic cargos into the dendrite. Based on their different affinities to AP-3 and AP-1, cytosolic sorting motifs instruct the binary choice of targeting to the axon or the dendrite.

## RESULTS

### AP-3 Is Required for the Axonal Localization of Multiple Proteins

RIA neurons are a pair of bilaterally symmetric interneurons located in the head of the worm. Each RIA has a single process that acts as both an axon and a dendrite (Figure 1A). Electron microscopy reconstruction studies have revealed that the single RIA neurite is compartmentalized into a proximal dendritic segment and a distal axonal segment based on characterizations of pre- and postsynaptic structures (White et al., 1986) (Figure 1A). RIA neurons express several neurotransmitter receptors and transporters, including AMPA glutamate receptor subunit/GLR-1, G protein-coupled serotonin receptor/SER-1, glutamate transporter/GLT-4, and vesicular glutamate transporter/EAT-4 (Brockie et al., 2001; Dernovici et al., 2007; Mano et al., 2007; Ohnishi et al., 2011). We created cell-specific fluorescence-tagged transgenes to study their subcellular localizations. Our previous results showed that GFP-tagged GLR-1, ACR-16, and CAM-1 are localized to the proximal dendritic segment (Figure S1A, available online) (Margeta et al., 2009). Using the same strategy, we found that SER-1, GLT-4, and EAT-4, as well as synaptobrevin/SNB-1, a transmembrane SV protein, were localized stringently to the distal axonal segment (Figures 1B, S1D, S1F, and S1H), in striking contrast to the non-polarized cytoplasmic tdTomato (cyto-tdTMT) (Figures 1B, S1D, S1F, and S1H, middle panel). To make objective comparisons, we quantified the distribution of fluorescence proteins using a "polarity index" (PI), in which a completely axonal localization pattern has PI = 1, a completely dendritic localization pattern has PI = 0, and a non-polarized distribution pattern has PI = 0.5 (Figure 1E). As expected, all axonal proteins that we examined showed PI > 0.75, while dendritic GLR-1 had a PI < 0.2 (Figure 1F). Together, these data suggest that the RIA neurite is compartmentalized and that axonal and dendritic proteins are targeted to respective compartments.

Our previous study revealed that AP-1 is required for localizing multiple dendritic transmembrane proteins in RIA, but is not required for localizing SV-associated protein RAB-3 (Figure S1C) (Margeta et al., 2009), hinting that axonal and dendritic proteins might be independently targeted. We therefore decided to investigate how axonal membrane proteins are compartmentalized. We focused on SER-1 because it functions cell autonomously in RIA for several olfactory learning-related behaviors (Harris et al., 2009, 2011) and shows axonal localization in several neuronal cell types, such as another head interneuron, AVE, and a bipolar sensory neuron, ASH (Figure S1J; data not shown).

We systematically examined the localization of SER-1 in RIA in mutants deficient for different AP complexes. Strikingly, while SER-1's axonal localization was intact in *unc-101/AP-1*$\mu$ mutant animals, it was severely defective in animals carrying a null mutation in the APB-3/AP-3 $\beta$ subunit (Figures 1C, 1D, and 1F). In *apb-3/AP-3*$\beta$ mutants, SER-1 was diffusely localized to both the axonal and dendritic segments of RIA (Figure 1C). Examination of another axonal plasma membrane protein, GLT-4, and two SV proteins, EAT-4 and SNB-1, revealed phenotypes similar to that of SER-1: they all failed to be restricted to the axon segment in *apb-3/AP-3*$\beta$ mutants (Figures S1E, S1G, S1I, and 1F). Moreover, *apb-3/AP-3*$\beta$ mutation also disrupts the axonal distribution of SER-1 in AVE and ASH (Figure S1K; data not shown), indicating that AP-3 functions in multiple neurons to target axonal proteins. Interestingly, none of the dendritic proteins we tested were affected in *apb-3/AP-3*$\beta$ mutant animals (Figures 1F and S1B; data not shown). These observations indicate that AP-3 and AP-1 are required for targeting axonal and dendritic proteins, respectively, in the RIA neuron.

### Specific Dileucine Motifs Instruct Neuronal Polarity through AP-Dependent Targeting

AP complexes bind to protein cargos with two types of sequence motifs: the YxxΦ motif and the [D/E]xxxL[L/I] dileucine motif (Robinson, 2004; Traub, 2009). To understand how AP-3 distinguishes and targets axonal proteins, we focused on the cytoplasmic domain of the axonal cargo SER-1, from which we identified a dileucine motif, ETKPLI. We created several truncated and mutated forms of SER-1 and tested their subcellular localizations (Figure 2A). Deletion of the entire cytoplasmic domain or most of C-terminal sequences, including the dileucine motif (SER-$1_{1\text{-}450}$ and SER-$1_{1\text{-}519}$), resulted in a loss of the axonal enrichment (Figure 2A; data not shown). A construct with a smaller deletion that left the dileucine motif intact (SER-$1_{1\text{-}605}$) remained restricted to the axon (Figure 2A; data not shown), indicating the importance of dileucine motif for the axonal targeting. Strikingly, simply replacing the "LI" within the motif with "AA" (SER-$1_{ETKPAA}$) resulted in a dramatic loss of axonal enrichment

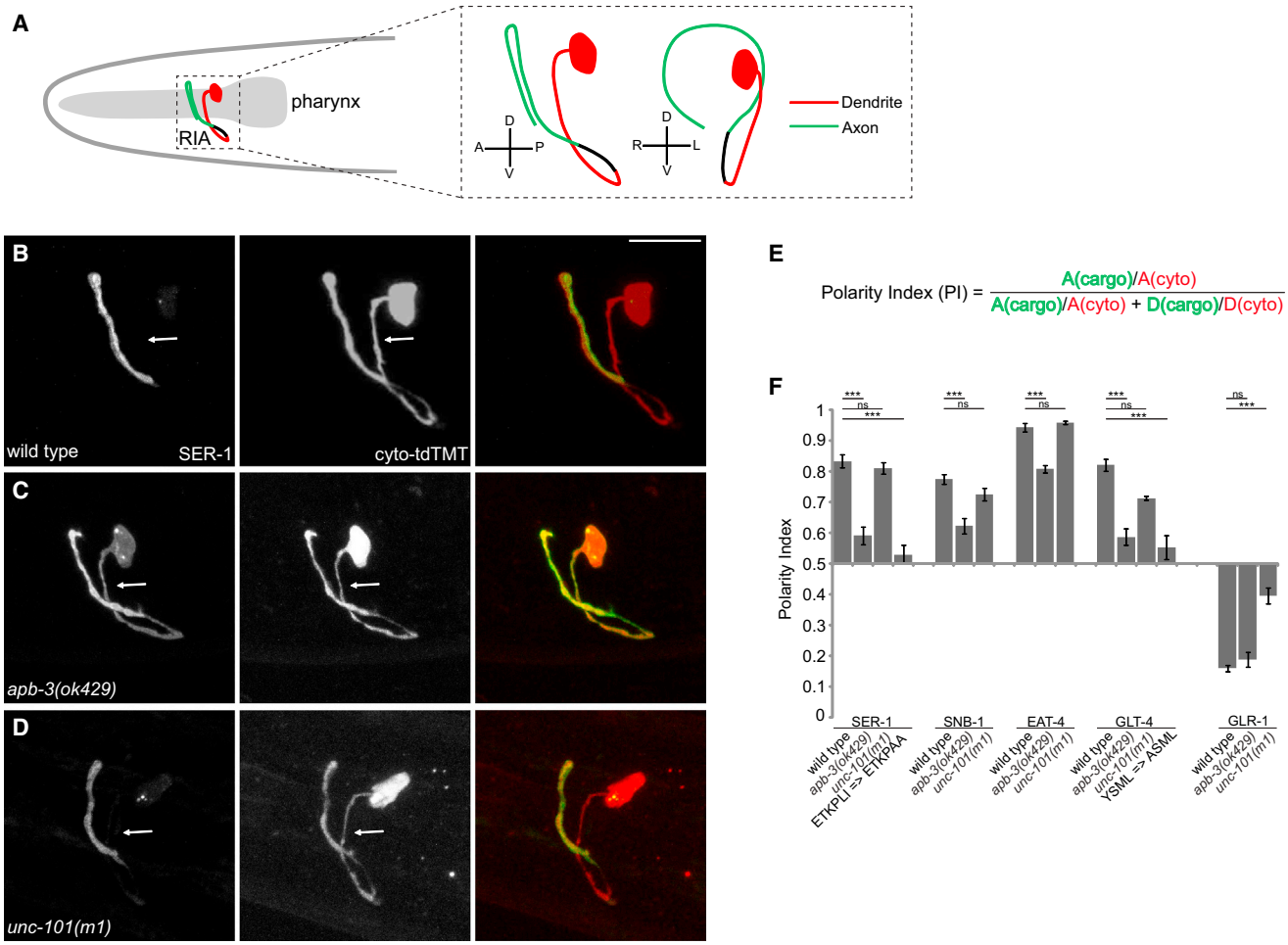



**Figure 1. AP-3 Is Required for Axonal Localization in RIA**

(A) Section of the *C.elegans* head is shown with dashed rectangle showing the lateral and anterior views of the left RIA neuron.

(B–D) GFP-tagged SER-1 is co-expressed with a cytosolic tdTomato in RIA in wild-type (B), *apb-3(ok429)* (C), and *unc-101(m1)* (D) animals. Arrows point to RIA dendrite. Scale bar, 10 μm.

(E) Calculation of the polarity index (PI).

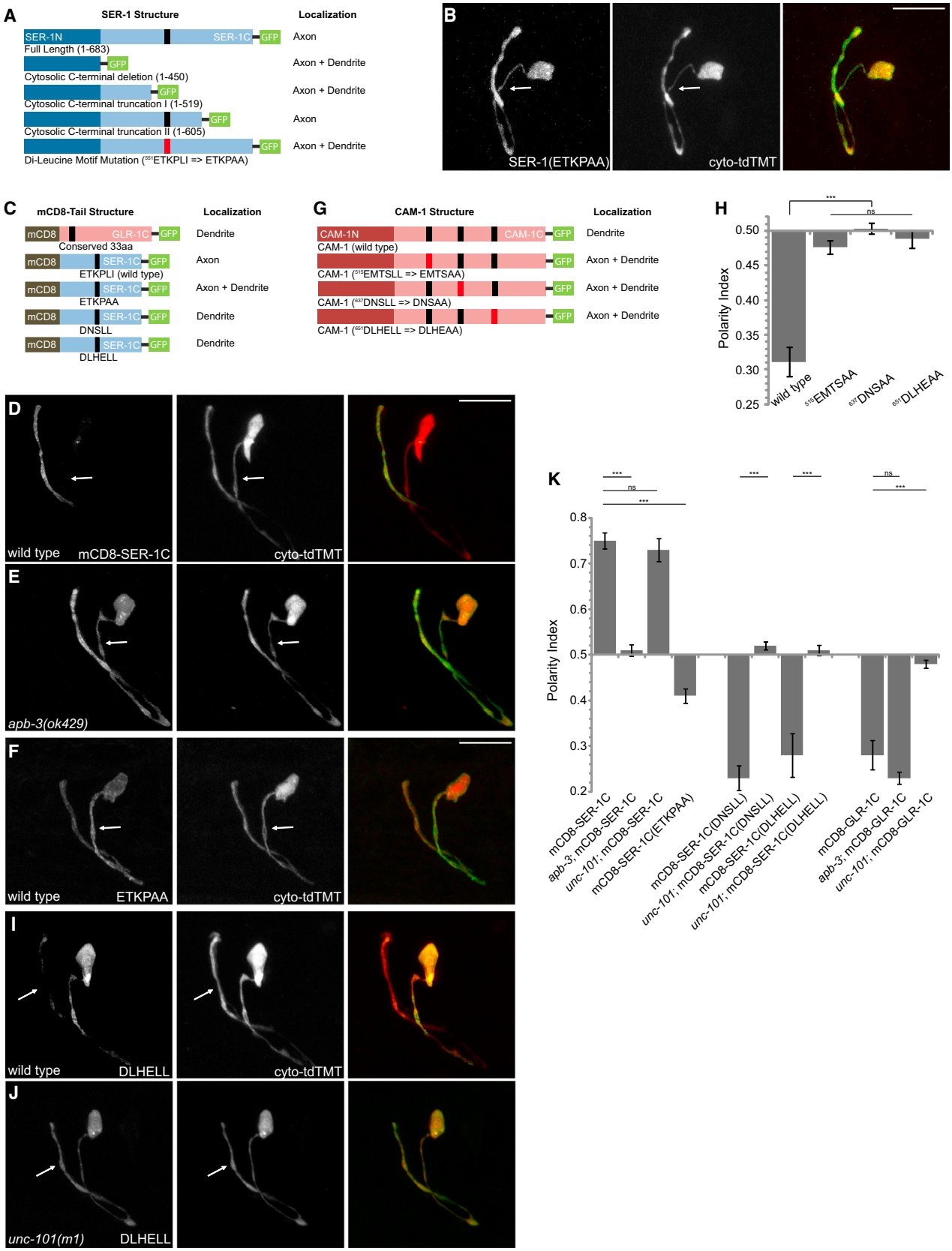
(F) Polarity index (PI) quantification in wild-type, *apb-3(ok429)*, and *unc-101(m1)* animals. ***p < 0.001, mean ± SEM, t test.

(Figures 1F, 2A, and 2B). These results indicate that the putative AP-binding dileucine motif in the cytoplasmic region of SER-1 is required for its polarized localization to the axonal segment.

To further confirm that the dileucine motif mutation affects SER-1 localization by altering its polarized sorting instead of changing the protein conformation and disrupting its insertion into the plasma membrane, we examined SER-1's localization in *C. elegans* intestine cells, which are large polarized epithelial cells with apical and basolateral domains (Figure S2A) (Leung et al., 1999). Wild-type SER-1 is exclusively localized to the basolateral surface of the plasma membrane and some intracellular puncta (Figures S2B and S2C). In contrast, SER-1 containing a mutated dileucine motif (ETKPAA) localizes to both the apical and basolateral surfaces (Figures S2D and S2E). This result suggests that the dileucine motif is not required for membrane insertion of SER-1 but is truly required for polarized targeting.

To test the sufficiency of the dileucine motif for SER-1's localization, we fused SER-1 cytoplasmic domain to a non-polarized transmembrane domain, mCD8 (mCD8-SER-1C). This construct showed a striking axonal enrichment in the RIA neuron that was specifically affected in *apb-3/AP-3β*, but not *unc-101/AP-1μ*, mutants (Figures 2C–2E and 2K). Furthermore, mutating the "LI" in the dileucine motif to "AA" (mCD8-SER-1C$_{ETKPAA}$) abolished the axonal localization (Figures 2F and 2K). Conversely, the cytoplasmic domain of the dendritic protein GLR-1 (mCD8-GLR-1C) was sufficient to target mCD8 to the RIA dendrite in an AP-1-dependent manner (Figures 2C, 2K, S2F, and S2G). Together, these results demonstrate that AP-3 and AP-1 use different signals to sort protein cargos. The dileucine motif in the cytoplasmic domain of SER-1 mediates AP-3-dependent axonal targeting.

Since both AP-1 and AP-3 complexes can bind to their protein cargos through dileucine motifs, we asked whether different

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dileucine sequences support polarized targeting to axons and dendrites. Because we could not find obvious dileucine motifs in the cytoplasmic region of GLR-1, we turned to the dendritically localized transmembrane receptor tyrosine kinase CAM-1/ROR, whose sorting is also dependent on AP-1 (Margeta et al., 2009). Sequence analyses of CAM-1 identified three dileucine motifs in its cytoplasmic domain: $^{515}$EMTSLL, $^{637}$DNSLL, and $^{651}$DLHELL. Mutating any one of these three dileucine motifs caused CAM-1 localizing in both the axon and dendrite (Figures 2G and 2H), suggesting that all three motifs are required for dendritic targeting.

From these observations, we hypothesized that different dileucine motifs may serve as different sorting signals and have distinct properties for axonal versus dendritic targeting. To test this hypothesis, we replaced the dileucine motif in mCD8-SER-1C with each of the three dileucine motifs of CAM-1 while keeping the rest of the SER-1C sequence intact (Figure 2C). Strikingly, substitutions of DNSLL or DLHELL, but not EMTSLL, dramatically reversed the polarization of the chimeric protein from axonal to dendritic (Figures 2C, 2I, 2K, and S2H). Moreover, the dendritic localizations of mCD8-SER-1C$_{DNSLL}$ and mCD8-SER-1C$_{DLHELL}$ were abolished in *unc-101/AP-1μ* mutants, demonstrating that dendritic dileucine motifs are AP-1 dependent (Figures 2J, 2K, and S2I). Together, these results strongly argue that the sorting of axonal and dendritic cargos shares a similar mechanism but utilizes different APs that recognize distinct sorting signals. The exact sequence of these motifs might critically determine the subcellular localization of proteins into axons or dendrites.

For the axonally targeted glutamate transporter, GLT-4, we could not identify a typical dileucine motif in its cytoplasmic domain. Instead, a tyrosine-based sorting motif, $^{473}$YSML, was found. Mutation of its conserved tyrosine led to non-polarized distribution of GLT-4 (Figures S2J and S2K), indicating that both dileucine and tyrosine-based motifs are able to target axonal proteins.

## Distinct Binding Efficiencies between Cargos and AP-3 Determine Axonal and Dendritic Targeting

Interactions between a sorting signal and its corresponding AP complex play important roles in determining cargo specificity. To test if AP-3 mediates SER-1 axonal targeting by directly binding the dileucine motif, we co-expressed GFP-tagged SER-1 cytoplasmic domain (SER-1C-GFP) with FLAG-tagged AP-3 δ/σ hemicomplex trunk domain, whose homologs in AP-1 and AP-2 have been suggested to harbor the dileucine motif recognition site (Doray et al., 2007), in HEK293 cells. Co-immunoprecipitation (coIP) with a GFP antibody showed a strong interaction between SER-1 and AP-3, which was lost when the "LI" in the SER-1 dileucine motif was mutated to "AA" (Figure 3A). This result demonstrates the direct binding between the axonal dileucine motif and AP-3, and suggests that AP-3 may sort the axonal cargo by binding to dileucine motifs.

Since SER-1's axonal localization is dependent on AP-3, but not on AP-1, we next asked if SER-1 cytoplasmic fragment could also bind to AP-1 and whether the binding efficiencies are different for AP-3 and AP-1. We added SER-1C-GFP to different concentrations of AP-1 or AP-3 hemicomplex and pulled down the cell lysates with a GFP antibody. Interestingly, we found that AP-1 could also bind to the SER-1 fragment in a dose-dependent manner (Figure 3B). However, the AP-3 binding is much stronger than the AP-1 binding at all concentrations (Figure 3B). This result shows that axonal dileucine motif binds strongly to AP-3 but weakly to AP-1, and suggests that AP-3′s strong binding to the axonal dileucine motifs enables the selection of axonal cargos.

Since different dileucine sequences determine axonal and dendritic targeting and exhibit different AP dependences, we next tested if different dileucine motifs have distinct binding efficiencies to AP-3 and AP-1, which in turn mediate polarized protein targeting. We asked whether the chimeric SER-1C with the dendritic dileucine motifs prefer AP-1. Using a similar pull-down assay, we found that AP-3 binds to dendritic motifs with significantly lower efficiency compared with the axonal motif (Figure 3C), suggesting that AP-3 can indeed select for axonal dileucine motif over dendritic motifs. In contrast, AP-1 binds to dendritic and axonal motifs with similar efficiencies (Figure 3C), indicating that AP-1 interacts with but cannot select for dendritic motifs. Together, these genetic and biochemistry results suggest the following model: AP-3 and AP-1 are responsible for localizing axonal and dendritic cargos, respectively. AP-3 selects axonal proteins by strongly binding to axonal dileucine motifs (Figure 3D).

If AP-3's strong binding to axonal dileucine motif is deterministic for axonal sorting, we hypothesized that cargos with high AP-3 binding efficiency would localize to the axon while those with low AP-3 binding efficiency would be dendritic. To test this hypothesis, we created SER-1C variants by changing the threonine (T) in the SER-1 dileucine motif to different amino acids

**Figure 2. Dileucine Motif Determines the Polarized Targeting to the Axon or the Dendrite**

(A) Schematic diagrams of the wild-type SER-1 sequence and the truncated variations. Wild-type (black bar) and mutant (red bar) dileucine motifs in the cytoplasmic domains (light blue) are shown.

(B) SER-1(ETKPAA)-GFP is co-expressed with the cytosolic tdTomato in the RIA neuron. Arrow points to RIA dendrite. Scale bar, 10 μm.

(C) Schematic diagrams of mCD8-tagged cytoplasmic domains of GLR-1 and SER-1 variants. Black bars show the sorting motif variants.

(D and E) mCD8-SER-1C-GFP is co-expressed with the cytosolic tdTomato in wild-type (D) and *apb-3(ok429)* (E) animals. Arrows point to RIA dendrites. Scale bar, 10 μm.

(F) mCD8-SER-1C(ETKPAA)-GFP is co-expressed with the cytosolic tdTomato in RIA. Arrow points to RIA dendrite. Scale bar, 10 μm.

(G) Schematic diagrams of wild-type CAM-1 sequence and the mutant variants. Wild-type (black bar) and mutant (red) dileucine motifs in the cytosolic domains (light red) are shown.

(H) Polarity index (PI) quantification for wild-type and mutant variations of CAM-1 in RIA. ***$p < 0.001$, mean ± SEM, t test.

(I and J) mCD8-SER-1C(DLHELL)-GFP is co-expressed with the cytosolic tdTomato in wild-type (I) and *unc-101(m1)* (J) animals. Arrows point to RIA axons.

(K) Polarity index (PI) quantification in wild-type, *apb-3(ok429)*, and *unc-101(m1)* animals. ***$p < 0.001$, mean ± SEM, t test.

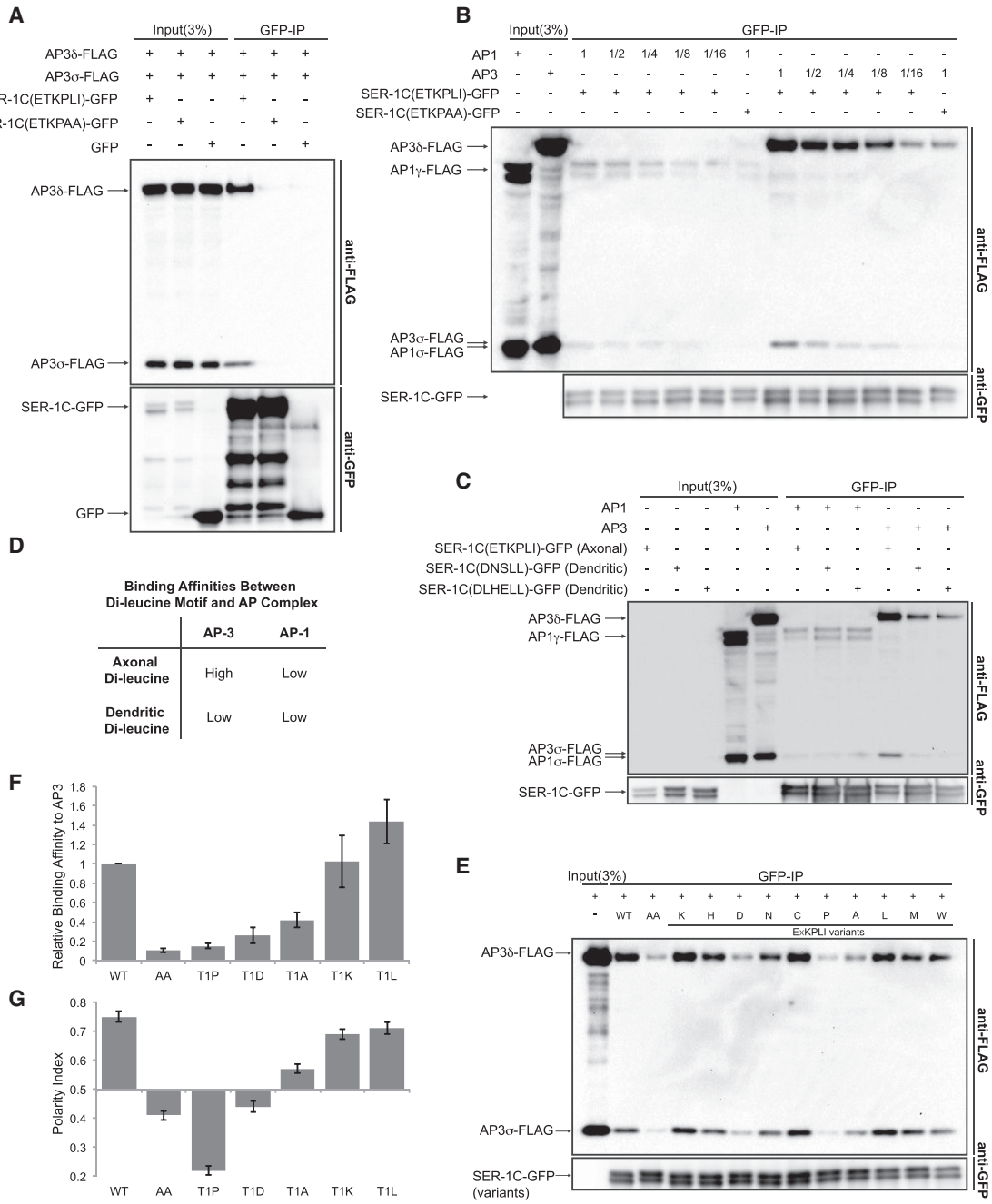



**Figure 3. Different Dileucine Motifs Have Distinct Binding Efficiencies for AP-3 and AP-1**

(A) CoIP of FLAG-tagged AP-3 hemicomplex composed of AP-3δ trunk domain and AP-3σ with wild-type SER-1C-GFP, mutant SER-1C-GFP, and GFP proteins.

(B) Different concentrations of FLAG-tagged AP-3 and AP-1 hemicomplex are pulled down by wild-type or mutant SER-1C-GFP.

(C) FLAG-tagged AP-3 and AP-1 hemicomplex are pulled down by wild-type SER-1C-GFP or dendritic dileucine motif variants.

(D) Summary chart of the binding efficiency between the dileucine motifs and AP complexes.

(E) FLAG-tagged AP-3 hemicomplex is pulled down by SER-1C(ExKPLI)-GFP variants.

(F) Quantification of the relative binding efficiencies. Mean ± SEM.

(G) Polarity index (PI) quantification for SER-1C(ExKPLI)-GFP in RIA. Mean ± SEM.

(ExKPLI), then independently tested their AP-3 binding efficiency and measured their polarized localization in RIA. We found that different SER-1C variants showed distinct localization patterns (Figure 3E). Strikingly, the variants with high AP-3 binding efficiencies (e.g., T1K and T1L) are localized in the axon, while those with low AP-3 binding efficiencies lost axonal enrichment or even

polarized to dendrites (e.g., T1P) (Figures 3E–3G). The correlation between AP-3 binding efficiency and the polarized localization demonstrates that the strong binding between AP-3 and axonal dileucine motif determines cargo's axonal localization.

To understand if AP-3-mediated sorting represents a general mechanism for localizing axonal proteins, we searched for sorting motifs from transmembrane proteins identified in rat SVs in a proteomic study (Takamori et al., 2006). Indeed, out of the 64 confirmed SV transmembrane proteins, 46 and 35 proteins have dileucine-like and tyrosine motifs, respectively, in their putative cytoplasmic domains (Table S1). A total of 105 dileucine-like motifs and 75 tyrosine motifs were identified (Figures S3F and S3G; Table S1). A large portion of mouse axonal and dendritic transmembrane proteins also contain dileucine-like (66.7% and 53.8%) and tyrosine motifs (74.1% and 61.5%), while mitochondria inner membrane (IMM) proteins that localize in both axons and dendrites in neurons contain significantly fewer dileucine-like (13.5%) and tyrosine (31.4%) motifs (Figures S3F and S3G; Table S1). These results hint that AP binding motifs exist widely among vertebrate neuronal proteins, and that the AP-mediated sorting might represent a general mechanism for neuronal polarization.

## AP-3 Competes with AP-1 to Sort Axonal Proteins

Our observations suggest that AP-3 selects axonal motifs by competing against AP-1. To test this hypothesis, we sought for neurons with morphologically separate axons and dendrites and asked whether the mistargeted axonal proteins in AP-3 loss-of-function mutants are sorted into the dendrite by AP-1. DA9 is a bipolar cholinergic motor neuron with distinct axon and dendrite emerging from opposite sides of the soma. En passant synapses form within a well-defined synaptic region along the dorsal axon (Figure 4B) (Klassen and Shen, 2007). A plasma-membrane choline transporter, CHO-1, is predominantly localized to DA9 axon and enriched at presynapses in wild-type animals (Figures 4A and 4F). However, in *apb-3/AP-3β* mutants, the polarized axonal localization of CHO-1 was diminished, with strong CHO-1-GFP present in the DA9 dendrite (Figures 4C, arrow, and 4F). This result demonstrates that AP-3 is also required for polarized targeting of axonal proteins in bipolar neurons. CHO-1 distribution was not altered in *unc-101/AP-1μ* mutants (Figures 4D and 4F), whereas the *unc-101/AP-1μ* mutation completely suppressed the dendritic mistargeting of CHO-1 in *apb-3/AP-3β* mutants (Figures 4E and 4F). These data suggest that the presence of CHO-1 in the dendrite in *apb-3/AP-3β* mutants is dependent on AP-1, and support the model that AP-3 sorts axonal cargos by competing with AP-1 (Figure 4J).

Since AP-3 likely functions broadly in many neurons for sorting multiple axonal proteins, loss of AP-3 may affect neural function and cause abnormal behavior. To assess the functional importance of AP-3-dependent axonal protein sorting, we conducted an aldicarb sensitivity assay to measure the efficacy of cholinergic synaptic transmission (Mahoney et al., 2006). *apb-3/AP-3β* mutants exhibited increased resistance to aldicarb, the acetylcholinesterase inhibitor, indicative of impaired cholinergic synaptic transmission (Figure S3A). This result suggests that AP-3 is widely required for axonal sorting of multiple proteins and important for the presynaptic function.

AP-3 complex is highly conserved between *C. elegans* and vertebrates at the primary sequence level. To ask if the mechanism of AP-3 in sorting axonal proteins we describe here is conserved, we examined the localization of ZnT-3, a vertebrate AP-3 cargo, in worms. GFP-tagged mouse ZnT-3 in DA9 is predominantly targeted to the presynaptic terminals (Figure S3B), indicating that worm neurons can sort vertebrate ZnT-3 protein. Interestingly, the synaptic enrichment of ZnT-3 is dramatically reduced in *apb-3/AP-3β* mutants (Figures S3C and S3E). Faint but consistent fluorescence signal can be observed in the mutant dendrite (Figure S3C, lower panel, arrows). In contrast, the synaptic localization of ZnT-3 is not altered in *unc-101/AP-1μ* mutants (Figures S3D and S3E). Together, these results suggest that AP-3-mediated sorting mechanism is likely conserved.

## AP-3 and AP-1 Sort Axonal and Dendritic Proteins, Respectively, into Distinct Trafficking Vesicles

Our results strongly suggest that the separation of axonal and dendritic proteins is mediated by AP-3 and AP-1. The selectivity is achieved by the strong binding between axonal cargos and AP-3. We next asked where AP-1 and AP-3 function in the polarized sorting of neuronal proteins. The TGN in the soma is a major organelle that directs transmembrane proteins to different subcellular destinations in polarized cells (Guo et al., 2014). Since both AP-1 and AP-3 have been shown to localize and function at the TGN and post-Golgi vesicles (Nakatsu and Ohno, 2003), we speculated that AP-3 and AP-1 distinguish axonal and dendritic proteins and separate them in the soma at the level of Golgi. Indeed, both UNC-101/AP-1μ and APM-3/AP-3μ showed punctate pattern near the nucleus (Figures 5A–5D, left panels). The perinuclear puncta of both AP-1 and AP-3 are highly co-localized with AMAN-2, a Golgi-localized alpha-mannosidase II (Figures 5A, 5B, and 5E), indicating that both AP complexes are enriched at the Golgi apparatus. UNC-101/AP-1μ and APM-3/AP-3μ showed only partial co-localization with an early endosomal marker RAB-5 (Figures 5C–5E), suggesting that they may have additional function at endosomes. An axonal cargo, CHO-1, is also partially co-localized with AMAN-2, but not RAB-5, consistent with the notion that TGN participates in sorting axonal proteins (Figures 4G–4I).

While AP-1 and AP-3 localize to the Golgi and sort proteins into distinct types of vesicles, they may occupy different domains within the Golgi. To directly test this hypothesis, we used super-resolution fluorescence microscopy to image AP-1 and AP-3 in the soma. Strikingly, UNC-101/AP-1μ and APM-3/AP-3μ are localized to adjacent but largely not overlapped perinuclear domains (Figure 5F). Pearson's correlation coefficient between AP-3 and AP-1 is significantly smaller than that in the control group where the same protein is independently imaged by two dyes (Figures 5F–5H). This result suggests that AP-3 and AP-1 reside at distinct domains of Golgi, and strongly supports the argument that AP-3 and AP-1 sort cargo proteins into different vesicles from the Golgi.

To directly examine whether the axonal and dendritic cargos are sorted into distinct post-Golgi carriers in the soma, we simultaneously imaged GLR-1 and RAB-3 vesicles from the most proximal segment of the RIA neuron. Since RIA only has one neurite, the anterograde trafficking vesicles within this segment likely

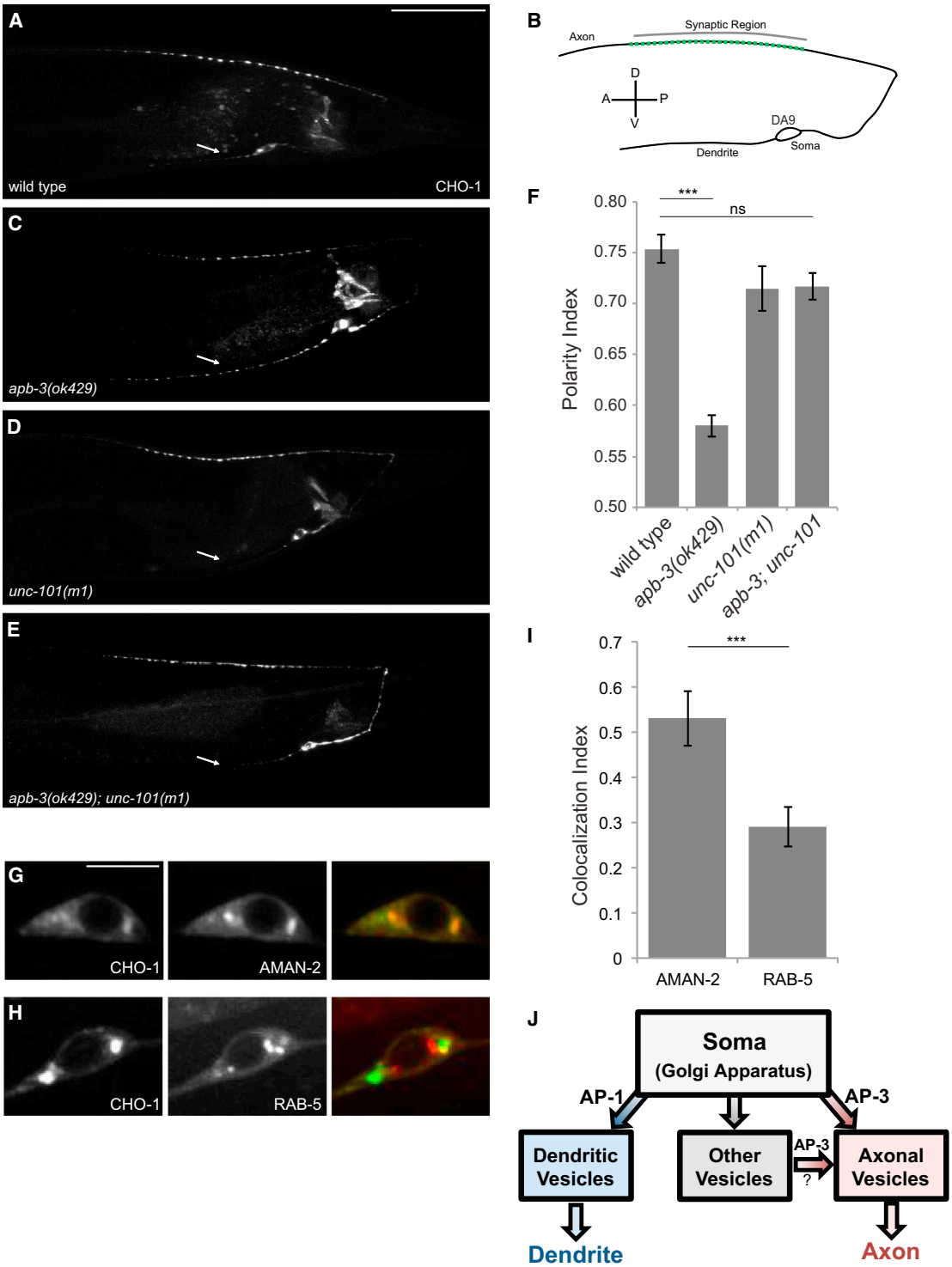



**Figure 4. AP-3 and AP-1 Compete for Axonal Protein Sorting and Trafficking**

(A–E) GFP-tagged CHO-1 in DA9 neuron in wild-type (A), *apb-3(ok429)* (C), *unc-101(m1)* (D), and *apb-3(ok429); unc-101(m1)* (E) animals. Arrows point to DA9 dendrites. Scale bar, 25 μm. (B) shows the schematic diagram.

(F) Polarity index (PI) quantification of the CHO-1 localization. ***$p < 0.001$, mean ± SEM, t test.

(G and H) GFP-tagged CHO-1 is co-expressed with tdTomato-tagged AMAN-2 (G) and RAB-5 (H). Scale bar, 5 μm.

(I) Quantification of the co-localization between CHO-1, AMAN-2, and RAB-5. ***$p < 0.001$, mean ± SEM, t test.

(J) Schematic model of AP-3- and AP-1-dependent protein sorting.

represent vesicles that are directly generated from the TGN. Interestingly, GLR-1 and RAB-3 are present on distinct trafficking carriers and rarely show co-labeling or co-transport (Figure 5I). In fact, GLR-1 and RAB-3 trafficking vesicles move at different speeds, with GLR-1 vesicles being slower than RAB-3 (Figures 5J and 5K). These results indicate that axonal and dendritic proteins are separated into distinct post-Golgi carriers.

We next asked if AP-1 and AP-3 are involved in generating these two types of trafficking vesicles. We therefore imaged the post-Golgi trafficking of GLR-1 and RAB-3 in *apb-3/AP-3β* and *unc-101/AP-1μ* mutants (Figures 6A and 6B). In both mutants, a significant number of trafficking vesicles are co-labeled by both GLR-1 and RAB-3 (Figures 6A, 6B, yellow lines, and 6C), indicating defective separation of axonal and dendritic proteins in the soma.

Notably, mutations in AP-3 and AP-1 specifically affect the sorting of RAB-3 and GLR-1, respectively. In *apb-3/AP-3β* mutants, the average speed of RAB-3 is reduced to that of GLR-1, while the speed of GLR-1 does not change (Figures 6D and 6F). Together with the increased incidence of RAB-3/GLR-1 co-trafficking, these observations strongly suggested that AP-3 is required to select cargo proteins into the fast-moving axonal vesicles. This result is also consistent with the fact that AP-1 does bind axonal proteins, albeit with lower affinity compared with AP-3 (Figure 3B). Interestingly, we did not observe dramatic speed changing of either RAB-3 or GLR-1 in *unc-101/AP-1μ* mutants (Figures 6E and 6F). However, the total number of post-Golgi anterograde trafficking events of GLR-1, but not RAB-3, is reduced (Figure 6G), suggesting that AP-1 is required for efficient sorting of proteins into dendritic vesicles. Indeed, we observed dramatic accumulation of GLR-1 in RIA cell body in *unc-101/AP-1μ* mutants, due to the ineffective sorting of GLR-1 from the Golgi in the soma (Figure 6H; also compare Figures S1A and S1C).

To further test the interplay between AP-1 and AP-3 and whether they compensate for each other in sorting axonal and dendritic proteins, we examined the effect of combined loss of AP-1 and AP-3. Compared with *apb-3/AP-3β* single mutants, *apb-3/AP-3β*; *unc-101/AP-1μ* double mutants showed reduced numbers of anterograde trafficking events of RAB-3 (Figures S4A and 6G), consistent with the notion that RAB-3 in *apb-3/AP-3β* mutants is missorted into dendritic vesicles that are dependent on AP-1. In contrast, neither the speed nor the number of GLR-1 movements was further reduced in the double mutants compared with *unc-101/AP-1μ* single mutants, suggesting that AP-3 cannot efficiently sort dendritic vesicles, even in the absent of AP-1 (Figures 6F and 6G). Notably, in the double mutants we observed further increased incidence of RAB-3/GLR-1 co-trafficking events compared with either single mutants (Figures S4A and 6C). This supports the existence of a third vesicular sorting pathway in addition to AP-1 and AP-3. This backup pathway can generate vesicles but fails to distinguish axonal and dendritic proteins.

## AP-3 Sorts Axonal Proteins from the Soma

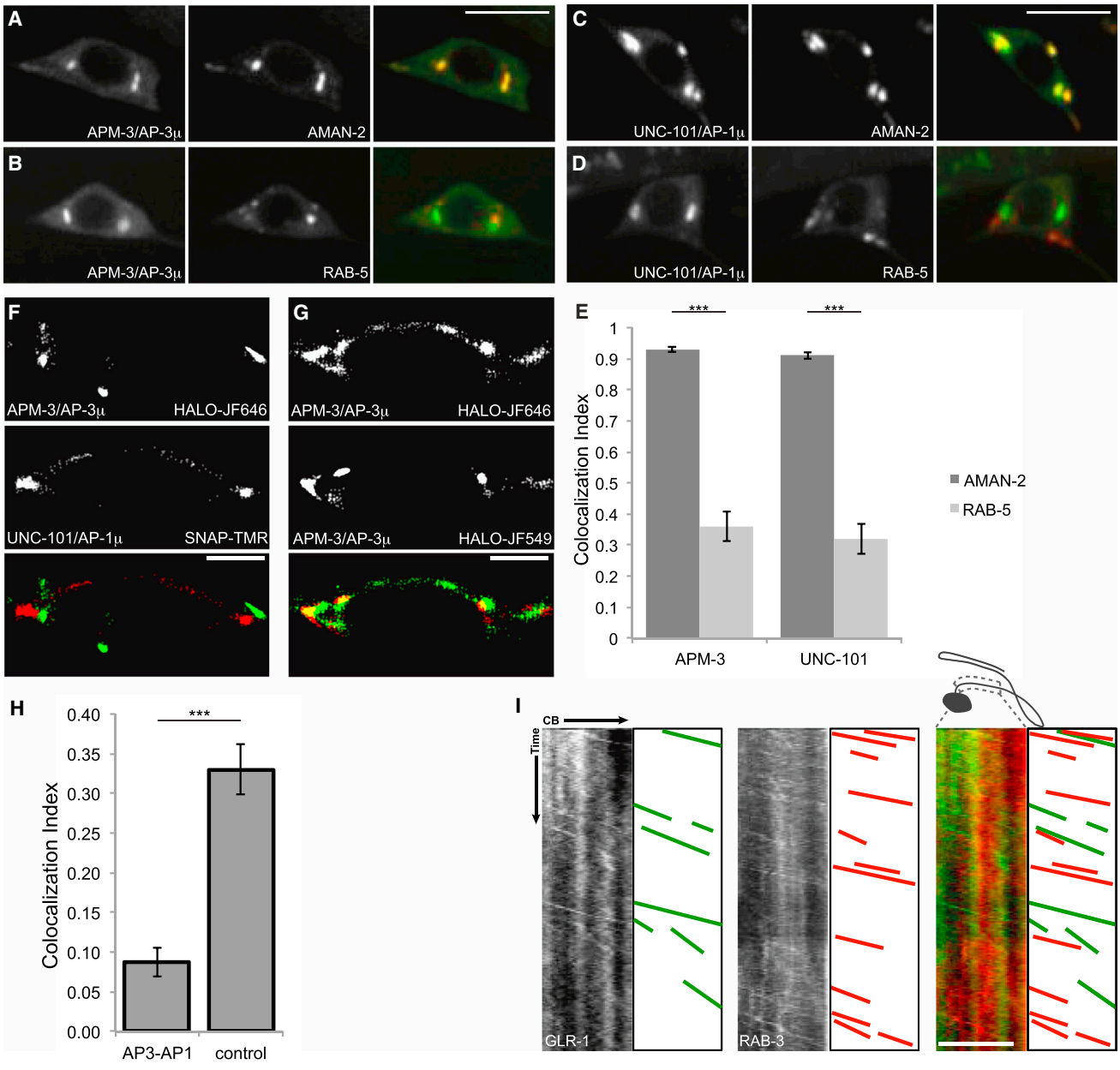
The observations that AP-3 is enriched at Golgi apparatus and plays crucial roles in sorting axonal proteins support the model that AP-3 generates axonal vesicles from the Golgi. To test this model, we labeled vesicles that are freshly released from the TGN using the RUSH system. RUSH system is a method to pulse-label proteins in the secretory pathway by trapping proteins in the endoplasmic reticulum (ER) followed by synchronous release (Boncompain et al., 2012). We fused SNB-1, a transmembrane SV cargo, with a streptavidin-binding peptide (SBP) and a GFP tag (SNB-1-SBP-GFP). This fusion construct was co-expressed with a hook protein, Streptavidin-KDEL, in DA9 bipolar neuron. SNB-1-SBP-GFP was initially retained in ER by Streptavidin-KDEL in the biotin-depleted condition. Treatment of biotin releases SNB-1-SBP-GFP from the ER hook, which allows the tagged protein to travel through Golgi apparatus to post-Golgi vesicles (Figure 7A).

To evaluate the efficiency of the ER retention and the biotin-induced release of the vesicles, we compared the localization of SNB-1-SBP-GFP before and after biotin treatment. SNB-1-SBP-GFP signal was primarily found at ER-like perinuclear regions in the soma and largely absent from the synapses in the biotin-deficient condition (Figures 7B and 7J). After 3 hr treatment of biotin, the majority of cargos were found at the synapses with dramatically reduced level in the soma (Figures 7C and 7J). Therefore, RUSH system allows us to track the vesicles that are generated from the TGN in vivo.

To understand the trafficking of axonal proteins that are generated from the TGN, we monitored the outward-moving SNB-1-SBP-GFP vesicles from the soma into the axon and the dendrite in the biotin-depletion condition and after 1 hr biotin feeding, when the majority of the cargos were still in the soma (Figure 7J). In wild-type animals, we observed significantly increased numbers of SNB-1 vesicles entering the axon, but not the dendrite, after the biotin treatment (5.57/min into axon versus 0.48/min into dendrite; Figures 7D, 7E, and 7H). However, the preference of axonal sorting of SNB-1 is diminished in *apb-3/AP-3β* mutants (2.67/min into axon versus 3.41/min into dendrite; Figures 7F, 7G, and 7H). Moreover, the speed of SNB-1 vesicles is decreased in both the axon and the dendrite in *apb-3/AP-3β* mutants, indicative of the missorting of SNB-1 in the absent of AP-3 (Figure 7I). These data showed that Golgi-derived SNB-1 vesicles selectively enter axons in wild-type animals. In *apb-3/AP-3β* mutants, SNB-1 is missorted into vesicles that preferably enter the dendrite. Together with the co-localization of AP-3 and AMAN-2 in the soma, these results indicate that AP-3 functions at the Golgi in the soma to sort transmembrane proteins into axonal vesicles.

## RAB-35 and RAB-19 Function with AP-3 and AP-1, Respectively, in Sorting Axonal and Dendritic Proteins

We next investigated the additional membrane trafficking machinery involved in the AP-dependent sorting processes. We focused on the RAB small GTPases because of their known roles in many intracellular membrane trafficking events (Zerial and McBride, 2001). We created dominant-negative (DN) constructs of all the 22 RAB proteins in *C. elegans* using their GDP bound forms, expressed them in RIA, and screened for mislocalization phenotype of axonal or dendritic proteins (Gallegos et al., 2012). This candidate screen uncovered two RAB proteins, RAB-35 and RAB-19. RAB-35$^{DN}$ disrupted the subcellular localization of SER-1, but did not affect the dendritic targeting of GLR-1 (Figures S5A–S5C). In contrast, RAB-19$^{DN}$ specifically affected the localization of GLR-1, but not that of SER-1 (Figures S5A, S5D, and S5E). These observations raised the possibility that RAB-35 may function with AP-3 in axonal sorting, while RAB-19

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functions with AP-1 to sort dendritic proteins. To examine this hypothesis, we simultaneously imaged the anterograde movements of RAB-35 and RAB-19 from the proximal RIA neurite (Figures S5G and S5J). Interestingly, RAB-35 moves at the same speed as RAB-3 in wild-type animals (Figures S5F and S5H). More strikingly, the speed of RAB-35 is significantly reduced in *apb-3/AP-3b* mutants, just like RAB-3 (Figures S5F and S5I). On the other side, RAB-19 has a speed profile that closely mimics that of GLR-1 in both wild-type and *unc-101/AP-1μ* mutant animals (Figures S5F, S5K, and S5L). In addition, the total number of anterograde trafficking of RAB-19 is halved in *unc-101/AP-1μ* mutants, similar to the effect on the GLR-1 vesicles (Figure S5M). Together, these results support the hypothesis that RAB-35 and AP-3 may function together to sort axonal proteins, while RAB-19 functions with AP-1 in dendritic sorting.

### Additional Sorting Mechanism May Exist at the Presynaptic Region for Maintaining Axonal Proteins Locally

In addition to the cell body localization, we found that APM-3/AP-3μ is also enriched in the presynaptic region of RIA with striking co-localization with synaptobrevin/SNB-1 (Figure S6A), consistent with the observation in vertebrates (Newell-Litwa et al., 2007; Seong et al., 2005). Since many SV proteins contain dileucine motifs in their cytoplasmic domains (Figure S3F), it is plausible that AP-3 is enriched at presynaptic terminals by binding to these dileucine motifs. To test this possibility, we examined APM-3/AP-3μ and SER-1 localization in three mutant backgrounds, *unc-104/KIF1A*, *syd-2/Liprin-α*, and *ttx-7/IMPase*, in which SVs are mislocalized to the dendrite of RIA by distinct mechanisms including insufficient trafficking, defective presynaptic assembly, and disrupted synaptic organization (Figures S6B–S6D) (Hall and Hedgecock, 1991; Tanizawa et al., 2006; Zhen and Jin, 1999). Remarkably, APM-3/AP-3μ is mislocalized to the dendritic segment of RIA in all three mutants, closely following the distribution of the mislocalized SV clusters (Figures S6E, S6G, and S6I). These results argue strongly that AP-3 is enriched at synapses through its association with SVs. The axonal distribution of SER-1 is also disrupted in all three mutants (Figures S6F, S6H, and S6J), suggesting that the local restriction of AP-3 in the axon might be important for maintaining SER-1 through a local mechanism. Together, these results suggest that AP-3 at presynaptic terminals may also contribute to maintaining axonal proteins in addition to sorting proteins in the soma.

Another adaptor complex, AP-2, is also involved in the presynaptic sorting of axonal proteins. Loss of AP-2 causes defective axonal, but not dendritic protein, localization (Figure S4B) (Margeta et al., 2009). Intriguingly, we did not detect trafficking defects of anterograde GLR-1 and RAB-3 vesicles from the soma in *dpy-23/AP-2μ* mutants. Neither the incidence of the co-trafficking of RAB-3 and GLR-1, nor were the frequency and the speed of the anterograde trafficking altered (Figures S4D–S4F). These results suggest that AP-2 may not function in the soma to sort axonal proteins. Instead, since AP-2 is known to be involved in the endocytosis at the presynaptic terminals (Dittman and Ryan, 2009; Gu et al., 2008, 2013), it is likely that the defective axonal localization in *dpy-23/AP-2μ* mutants is caused by the dysfunction of AP-2 at the presynaptic region. Therefore, protein sorting may also occur at the synapses in addition to the TGN. Both mechanisms contribute to the overall polarity of axonal and dendritic proteins.

## DISCUSSION

Polarized protein distribution is essential for the morphological and functional compartmentalization of the axon and dendrite. Newly synthesized transmembrane proteins traffic through the Golgi apparatus, where they are packaged into distinct carriers and transported to various destinations (Guo et al., 2014). Here we investigated how axonal and dendritic proteins are sorted to correct subcellular compartments. Our results uncover a surprisingly simple sorting mechanism in the soma: AP-3 selects axonal proteins by strongly binding to the axonal dileucine motifs, while AP-1 binds dendritic proteins and sorts them into dendritic carriers (Figure 4J). Together with specific RAB proteins, AP complexes define distinct intracellular trafficking pathways for axonal and dendritic proteins.

### AP-3 Binding Efficiencies to Specific Dileucine Motif Sequences Determine Targeting Preference

We found one dileucine motif (ETKPLI) on the cytoplasmic region of SER-1 that is necessary and sufficient for axonal targeting (Figures 2B, 2D, and 2K). Two other dileucine motifs (DNSLL and DLHELL) present in CAM-1 are crucial for dendritic targeting (Figures 2G and 2H). This preferential targeting is likely mediated by different cargo binding efficiencies to AP-1 and AP-3. ETKPLI strongly binds to AP-3 but only weakly interacts with AP-1 (Figure 3B). Replacing ETKPLI by either DNSLL or DLHELL greatly reduces AP-3 binding (Figure 3C), causing a switched localization from axon to dendrite (Figures 2I, 2K, and S2H). These results indicate that AP-3 specifically binds and selects axonal dileucine motifs. We found a strong correlation between AP-3

**Figure 5. Axonal and Dendritic Proteins Are Sorted into Different Vesicles from the TGN**

(A–D) GFP-tagged APM-3/AP-3 subunit μ (A and B) or UNC-101/AP-1 subunit μ (C and D) is co-expressed with tdTomato-tagged AMAN-2 (A and C) or RAB-5 (B and D) in the soma. Scale bar, 5 μm.

(E) Quantification of the co-localization. ***$p < 0.001$, mean ± SEM, t test.

(F) Super-resolution image of HALO-tagged APM-3/AP-3 subunit μ co-expressed with SNAP-tagged UNC-101/AP-1 subunit μ in the soma. Scale bar, 2 μm.

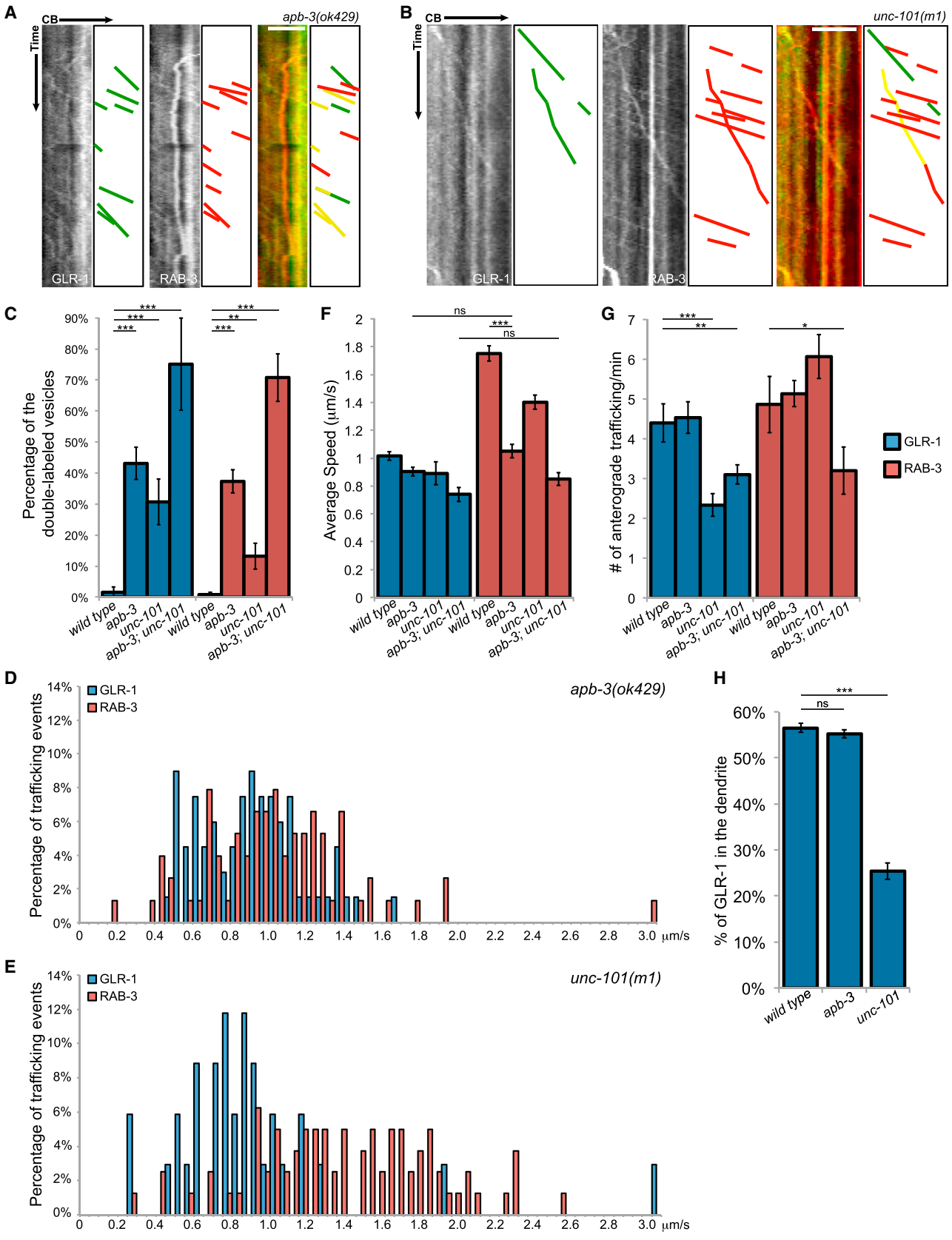
(G) Super-resolution image of HALO-tagged APM-3/AP-3 subunit μ stained by HALO-JF549 and HALO-JF646. Scale bar, 2 μm.

(H) Quantification of the co-localization. ***$p < 0.001$, mean ± SEM, t test.

(I) Kymographs of simultaneous recordings from GFP-tagged GLR-1 (left, green) and RFP-tagged RAB-3 (middle, red) in RIA proximal axon. Anterograde is toward the right. Total recording time is 90 s. Scale bar, 5 μm.

(J) Distributions of the speeds of GLR-1 and RAB-3 movements in wild-type animals.

(K) Quantification of the average speed of GLR-1 and RAB-3 per minute in wild-type animals. ***$p < 0.001$, mean ± SEM, t test.

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binding efficiencies and axonal polarity in vivo (Figures 3F and 3G), indicating that AP-3 binding efficiency determines whether a cargo protein is destined to the axon. Consistent with the biochemistry data, we found that AP-3 outcompetes AP-1 to sort axonal proteins. In the absence of AP-3, the mistargeting of axonal proteins to the dendrite is dependent on AP-1 (Figures 4C, 4E, and 4F). Different sequences of dileucine motifs bind to AP-1 and AP-3 with different affinities in vitro (Rodionov et al., 2002). In particular, dileucine motifs with an immediate upstream proline residue prefer AP-3 over AP-1 (Rodionov et al., 2002). Consistent with this result, the axonal motif ETKPLI in SER-1 contains a proline at the right position.

Distinct sequences of tyrosine-based motifs have also been suggested to bind with various affinities to different APs (Ohno et al., 1996, 1998), suggesting that axonal versus dendritic targeting could also be achieved by AP binding the tyrosine motif. Indeed, we observed that GLT-4 was no longer axonally targeted when a tyrosine motif in its cytoplasmic domain was mutated (Figures S2J and S2K). This notion has also been suggested in neuroendocrine PC12 cells. P-selectin, after being packaged into endosomes, is delivered into either SLMVs or lysosomes dependent on the presence of a tyrosine motif and its binding to AP-3 (Blagoveshchenskaya et al., 1999).

Multiple axonal and dendritic proteins have dileucine motifs or tyrosine-based motifs in their cytoplasmic domains. Dileucine motifs have been found in vesicular transporters, ion channels, and several GPCRs in many species (Colgan et al., 2007; Foss et al., 2013; Garrido et al., 2001; Marchese et al., 2008; Rivera et al., 2003; Tan et al., 1998). We also found that more than 80% of the polarized neuronal transmembrane proteins, including SV-localized, axonal, and dendritic proteins, contain dileucine-like or tyrosine-based motifs (Figures S3F and S3G; Table S1). As an example, ZnT-3, a vertebrate SV-localized protein targeted by AP-3 also localizes to presynaptic region when expressed in worm neurons (Figure S3B). Remarkably, this localization is also dependent on *C. elegans* AP-3 (Figures S3C and S3E), indicating that the AP-3-mediated axonal sorting is a conserved mechanism. Together, AP-dependent, motif-mediated sorting mechanisms might be widely used for multiple axonal and dendritic protein targeting events in both invertebrate and vertebrate neurons.

## The Axonal and Dendritic Proteins Are Sorted into Different Routes in the Soma

We simultaneously imaged the trafficking in the proximal neurite of RIA. We found that the vast majority of RAB-3 and GLR-1 vesicles do not co-localize or co-transport (Figures 5I and 6C). Moreover, the speed profiles of RAB-3 and GLR-1 are distinct (Figures 5J and 5K). These results suggest that axonal and dendritic proteins are sorted into different types of post-Golgi vesicles and are mobilized by different molecular motors in the soma. Consistent with this hypothesis, SV precursors are transported by KIF1A/UNC-104 (Hall and Hedgecock, 1991; Maeder et al., 2014), while GLR-1 has been shown to be trafficked by KIF3/UNC-116 (Hoerndli et al., 2013).

We argue that AP-3 functions in the soma and selectively sorts proteins into axonal vesicles for several reasons. First, knockout of AP-3 specifically affects the polarized localization of axonal, but not dendritic, proteins (Figure 1F). Second, we observed increased incidence of post-Golgi co-transportation of RAB-3 and GLR-1 from the soma in *apb-3/AP-3β* mutants (Figures 6A and 6C), showing that a significant portion of RAB-3 is missorted into dendritic vesicles in the absence of AP-3. In support of this notion, axonal proteins bind to both APs but have higher efficiency to AP-3 (Figures 3A and 3B). Importantly, in bipolar neurons, the newly released axonal protein SNB-1 from the ER is preferentially transported into the axon (Figures 7D, 7E, and 7H). This polarized trafficking is abolished in *apb-3/AP-3β* mutants (Figures 7F–7H). In addition, the speed of SNB-1 vesicles is also reduced in *apb-3/AP-3β* mutants (Figure 7I), suggestive of the missorting of SNB-1 to other types of vesicles in the loss of AP-3. These results strongly argue that AP-3 sorts proteins into the axonal vesicles in the soma.

On the other hand, *unc-101/AP-1μ* mutation leads to only a moderate incidence of co-transportation of RAB-3 and GLR-1, indicative of partial missorting of GLR-1 into axonal vesicles (Figure 6B). More apparently, it causes a reduced amount of outward transportation of GLR-1 and dramatic accumulation of GLR-1 in the soma (Figures 6G and 6H). These results hint that in the absence of AP-1, AP-3 is not sufficient to missort the majority of dendritic proteins, although it also binds to dendritic sorting motifs (Figure 3C). One possibility is that AP-3 is less abundant than AP-1 in the Golgi apparatus. In fact, multiple studies show that AP-1 is concentrated and functioning at the Golgi (Bonifacino, 2014; Robinson, 2004). However, AP-3 is believed to localize to both Golgi and the endosomes (Dell'Angelica et al., 1997; Peden et al., 2004). In non-neuronal cells, Golgi-localized AP-3 facilitates protein sorting into the regulatory secretory pathway from the TGN (Asensio et al., 2010).

Our data also suggest that there are additional sorting pathways in the soma that constitutively sort proteins into trafficking vesicles. First, loss of AP-3 or AP-1 causes mislocalization of their respective targeting proteins into both the axon and the dendrite, indicating that the additional sorting pathways lack polarity information. Second, in *unc-101/AP-1μ* mutants, the speed of GLR-1 vesicles is different from that of RAB-3 vesicles (Figures 6E and 6F), suggesting that GLR-1 is sorted into another type of vesicle in the absence of AP-1. Third, in the combined

**Figure 6. Axonal and Dendritic Proteins Are Missorted in AP-3 and AP-1 Loss-of-Function Mutant Animals, Respectively**

(A and B) Kymographs of simultaneous recordings from GFP-tagged GLR-1 (left, green) and RFP-tagged RAB-3 (middle, red) in RIA proximal axon in *apb-3(ok429)* (A) or *unc-101(m1)* (B) mutants. Anterograde is toward the right. The total recording time is 90 s. Scale bar, 5 μm.

(C) Percentage of the double-labeled vesicles in the total number of GLR-1 or RAB-3 vesicles. ***$p < 0.001$, **$p < 0.01$, mean ± SEM, t test.

(D and E) Distributions of the speeds of GLR-1 and RAB-3 movements in *apb-3(ok429)* (D) or *unc-101(m1)* (E) mutants.

(F) Quantification of the average speed of GLR-1 and RAB-3 per minute. ***$p < 0.001$, mean ± SEM, t test.

(G) Quantification of the total number of anterograde trafficking events of GLR-1 and RAB-3 per minute. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, mean ± SEM, t test.

(H) Quantification of the relative GLR-1 intensity in RIA dendrite. ***$p < 0.001$, mean ± SEM, t test.

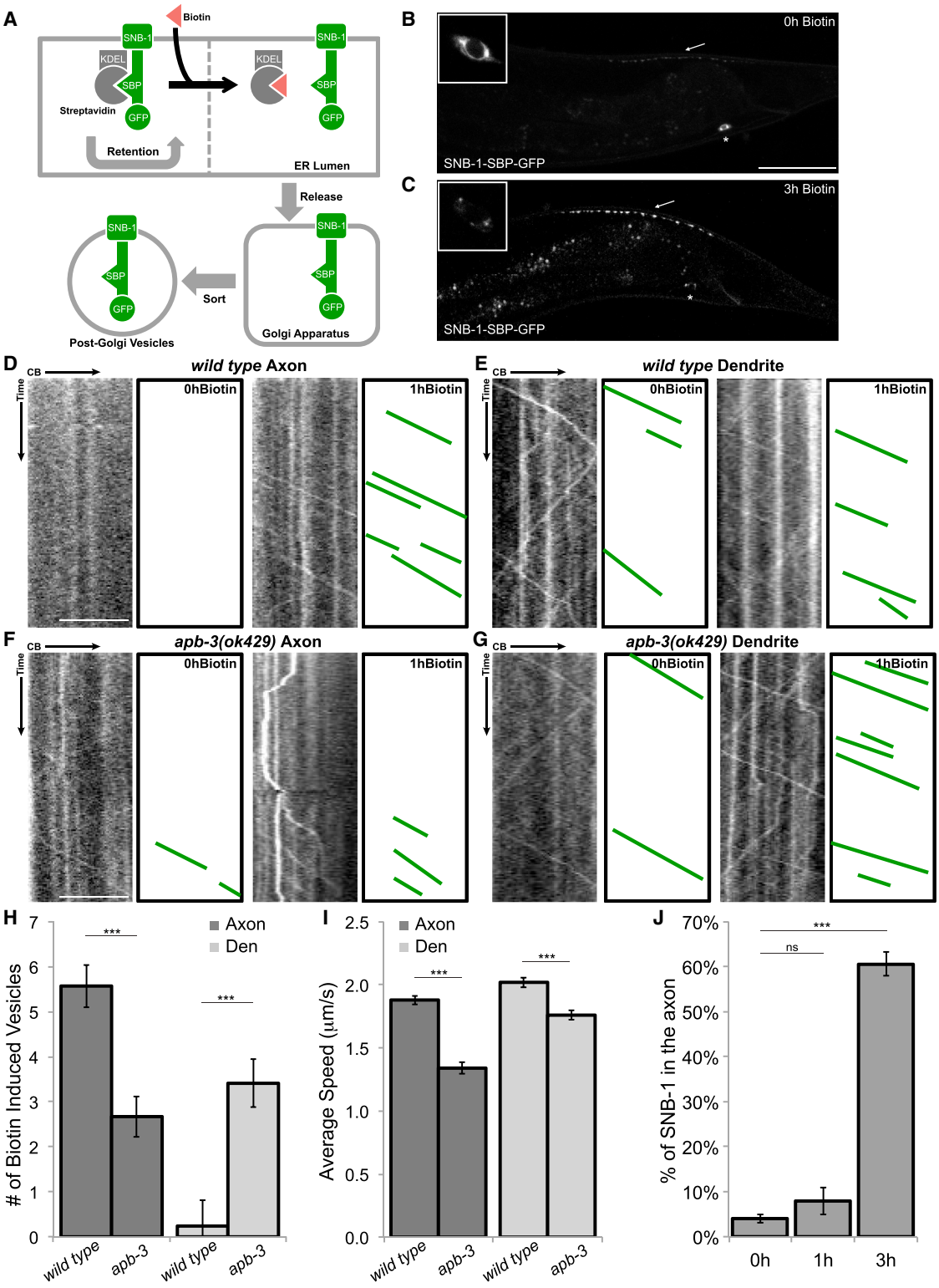


**Figure 7. AP-3 Functions in Sorting Axonal Proteins from the TGN**

(A) Schematic illustration of the RUSH system.

(B and C) SNB-1-SBP-GFP localization in DA9 in biotin-deficient (B) and biotin-sufficient (C) conditions. Scale bar, 25 μm. Enlarged images of DA9 cell body are shown in the corner. Arrows point to DA9 synaptic regions. Asterisks show the DA9 cell bodies.

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loss of AP-1 and AP-3, a small portion of axonal and dendritic proteins are still packaged into post-Golgi trafficking vesicles and are transported to the synaptic region in both monopolar and bipolar neurons (Figures 4E, 6G, and S4A). Notably, in the double mutants the majority of RAB-3 and GLR-1 proteins are co-trafficking within a type of slow-moving vesicles (Figures 6C, 6F, and S4A), strongly arguing that both axonal and dendritic proteins are missorted into other types of vesicles that have no preferential targeting compartment.

### AP-3 May Maintain Synaptic Organizations by Sorting Presynaptic Proteins Locally

We observed AP-3 enriched at synapses in multiple neurons (Figure S6A; data not shown), in line with observations in vertebrates (Blumstein et al., 2001; Newell-Litwa et al., 2007; Seong et al., 2005). We believe that AP-3 is localized at presynaptic terminals by interacting with SV proteins. First, APM-3/AP-3μ is punctate at synapses and co-localizes with SNB-1 (Figure S6A). Second, the detection of AP-3 from purified SVs of rat brains demonstrates the direct interaction of AP-3 and SVs (Takamori et al., 2006; Wilhelm et al., 2014). Moreover, direct binding of AP-3 with SV-localized molecules, such as vGluT1, VAMP-2, and ZnT3, has been reported in vitro (Salazar et al., 2004b, 2005; Salem et al., 1998). Third, in multiple mutants where SVs are mislocalized (Figures S6B–S6D), APM-3/AP-3μ distributions are also altered in an identical manner (Figures S6E, S6G, and S6I), suggesting that AP-3 localization is dependent on SVs.

AP-3 can generate SV-like vesicles in a cell-free synaptic vesicle biogenesis assay. Cytosol extracted from wild-type, but not AP-3, neuronal null mutant mice is able to bud SLMVs in vitro (Blumstein et al., 2001; Faúndez et al., 1998; Lichtenstein et al., 1998). Consistent with these studies, we found that SER-1 is mislocalized to the dendrite in mutants where AP-3 presynaptic localization is disrupted (Figures S6F, S6H, and S6J). It is plausible that AP-3 sorts proteins from early endosomes into SVs at presynaptic terminals. Therefore, besides sorting axonal proteins in the soma, AP-3 also localizes to the presynaptic region through association with SVs and might function locally to maintain the molecular composition of presynaptic terminals.

## EXPERIMENTAL PROCEDURES

### Time-Lapse Imaging

Time-lapse imaging was performed on an inverted Zeiss Axio Observer Z1 microscope equipped with a Plan-Apochromat 63x1.4 objective, a Hamamatsu EM-CCD digital camera, and a Yokogawa CSU-X1 spinning disk. When necessary, 2–6 s UV bleaching was used to reduce the background. Movies and kymographs were generated and analyzed by ImageJ.

### RUSH System

ER hook (Streptavidin-KDEL) and SNB-1-SBP-GFP were expressed in DA9 neuron. The dynamics of the vesicles were analyzed in young adult using the RUSH system (Boncompain et al., 2012). Worms were synchronized at the L1 stage, then fed with an overnight culture of biotin auxotrophic *E. coli* (MG1655bioB:kan, kindly donated by Dr. John E. Cronan, University of Illinois). Release of SNB-1-SBP-GFP was achieved by collecting and feeding young adult worms by 50 mM D-biotin.

### Biochemistry

HEK293T cells were transfected with various GFP-tagged proteins and FLAG-tagged proteins. Two days after transfection, cells were collected, sonicated, and lysed in TNTE lysis buffer. Anti-GFP antibody-coated agarose beads (Chromotek) were used to collect and enrich GFP-tagged proteins. Proteins were eluted using SDS-PAGE sample buffer and detected using western blot with mouse antibody to FLAG (1:2,000, Sigma), rabbit antibody to GFP (1:2,000, Abcam), and HRP-conjugated goat antibodies to mouse or rabbit (1:10,000, Jackson ImmunoResearch).

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.04.020.

## AUTHOR CONTRIBUTIONS

P.L. and K.S. designed and conducted experiments and wrote the paper. S.A.M. and E.M.J performed the super-resolution microscopy experiment. K.S. supervised the project.

## ACKNOWLEDGMENTS

We thank K. Ching, J.Y. Wan, J. Li, and S. Sundaresh for help on experiments; C. Gao for technical assistance; and P. Kurshan, S. Yogev, and M. Kokes for valuable comments and discussions. This work was funded by HHMI grants to K.S. and E.M.J., NIH (R01 NS048392) (K.S.), and NIH (R01 NS034307) (E.M.J.). P.L. was an HHMI International Student Research fellow.

Received: October 20, 2015
Revised: March 3, 2016
Accepted: April 12, 2016
Published: May 4, 2016

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(D–G) Kymographs of recording from SNB-1-SBP in the DA9 proximal axon (D and F) and dendrite (E and G) in wild-type (D and E) and *apb-3(ok429)* (F and G) in biotin-deficient (left) and 1 hr biotin condition (right). Anterograde is toward the right. The total recording time is 70 s. Scale bar, 5 μm.
(H) Quantification of the anterograde trafficking events of SNB-1 per minute induced by 1 hr biotin treatment. ***$p < 0.001$, mean ± SEM, t test.
(I) Quantification of the average speed of SNB-1 per minute. ***$p < 0.001$, mean ± SEM, t test.
(J) Quantification of the percentage of SNB-1 in DA9 axon after 0, 1, and 3 hr biotin treatment. ***$p < 0.001$, mean ± SEM, t test.

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