Regulation of dendrite growth and maintenance by exocytosis

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Abstract

Dendrites lengthen by several orders of magnitude during neuronal development, but how membrane is allocated in dendrites to facilitate this growth remains unclear. Here, we report that Ras opposite (Rop), the Drosophila ortholog of the key exocytosis regulator Munc18-1, is an essential factor mediating dendrite growth. Neurons with depleted Rop function exhibit reduced terminal dendrite outgrowth followed by primary dendrite degeneration, suggestive of differential requirements for exocytosis in the growth and maintenance of different dendritic compartments. Rop promotes dendrite growth together with the exocyst, an octameric protein complex involved in tethering vesicles to the plasma membrane, with Rop-exocyst complexes and exocytosis predominating in primary dendrites over terminal dendrites. By contrast, membrane-associated proteins readily diffuse from primary dendrites into terminals, but not in the reverse direction, suggesting that diffusion, rather than targeted exocytosis, supplies membranous material for terminal dendritic growth, revealing key differences in the distribution of materials to these expanding dendritic compartments.
Introduction

Axons and dendrites lengthen by several orders of magnitude during neuronal development and this lengthening of neurites is essential for establishing the morphological features of a neuron that ultimately determine its connectivity and function. The addition of membrane proteins and lipids via exocytosis plays a fundamental role in this process, but growth in axons and dendrites appear to be regulated by distinct pathways. In *de novo* axon outgrowth, addition of new membrane occurs primarily via exocytosis of plasmalemmal precursor vesicles (PPVs) at the growth cone (Bray, 1970; Craig et al., 1995; Lockerbie et al., 1991), which is mediated, in part, by soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Pfenninger, 2009). PPVs that are synthesized in the cell body are transported to growing axons (Pfenninger and Johnson, 1983), and although the source for material to support dendrite growth is unclear, the polarized localization of satellite secretory pathways in dendrites suggests that PPVs might be locally generated in some dendrites (Horton et al., 2005; Pfenninger, 2009; Ye et al., 2007). While it has been demonstrated that disrupting post-Golgi trafficking can impede dendrite growth (Horton et al., 2005; Ye et al., 2007), little is known about the role that exocytosis specifically plays in dendritic morphogenesis.

Most intracellular membrane fusion events require both SNARE and Sec1/Munc18 (SM) proteins. SM proteins are essential components of the molecular machinery that regulates exocytosis and cooperate closely with SNARE proteins to mediate vesicle fusion (Sudhof and Rothman, 2009). Munc18-1, the neuron-specific isoform of the SM protein family, is a key regulator of neurosecretion and mediates synaptic vesicle release through its interactions with Syntaxin-1 and the SNARE complex (Hata et al., 1993; Verhage et al., 2000). However, Munc18-1 protein is not restricted to axon terminals but is distributed throughout the neuronal membrane, suggesting that Munc18-1 may have other roles in the neuronal membrane that are separable from its function in neurotransmitter release (Garcia et al., 1995).

In yeast, the SM protein Sec1 physically interacts with subunits of the exocyst to facilitate SNARE complex assembly and membrane fusion upon vesicle arrival at the plasma membrane (Morgera et al., 2012; Wiederkehr et al., 2004). The exocyst is an evolutionarily conserved octameric protein complex that was originally identified in yeast based on its role in the spatial targeting and tethering of post-Golgi vesicles to specific sites of membrane expansion (Novick et al., 1980; TerBush et al., 1996). In the absence of a functional exocyst, secretory
vesicles are delivered to the plasma membrane, but SNARE complexes do not form and exocytosis is blocked (Grote et al., 2000). Although the exocyst plays well-established roles in neuronal polarization and the regulation of neurite outgrowth (Hazuka et al., 1999; Lalli and Hall, 2005; Murthy et al., 2003; Vega and Hsu, 2001), a role in dendrite development has not been specifically attributed to the exocyst. Likewise, whether Sec1-exocyst complexes form in higher eukaryotes is unknown.

*Drosophila* class IV dendritic arborization (C4da) neurons elaborate highly complex dendritic arbors that undergo a period of extensive growth during larval development during which time the linear dimensions of dendrites increase more than ten-fold (Grueber et al., 2002; Parrish et al., 2009), necessitating enormous amounts of material to drive the expansion of the plasma membrane. C4da neurons therefore provide an excellent system to investigate how new material is supplied in dendrites to support their growth and maturation. Here, we identify Rop, the *Drosophila* homolog of vertebrate Munc18-1, as a key regulator of dendrite development. In *Rop* mutants, C4da neurons show an initial defect in outgrowth of terminal dendritic branches, followed by progressive loss of terminal dendrites and degeneration of major dendritic branches. We found that Rop mediates dendrite growth, in part, through interaction with the exocyst subunit Sec6. These Rop/Sec6 complexes are enriched in primary dendrites of C4da neurons, suggesting that this dendritic compartment is the predominant site for dendritic exocytosis. Indeed, using a pH-sensitive *in vivo* reporter, we found that exocytosis predominates along major branches during dendrite arbor expansion and that the lateral diffusion of material from primary dendrites to terminal dendritic branches likely supports terminal dendrite growth. Finally, we demonstrate that the balance between exocytosis and endocytosis is not only important for dendrite growth, but is also required for maintenance of the dendritic arbor.

**Results**

**Identification of Rop as a critical regulator of dendrite growth**

To identify regulators of dendrite growth, we performed an RNAi-based screen of phylogenetically conserved genes in class I da (C1da) neurons, which elaborate simple, stereotyped dendrites and are amenable to RNAi-based analysis of dendrite morphogenesis (Foley and O'Farrell, 2004; Parrish et al., 2006). From this screen, we found that dsRNA-mediated knockdown of *Rop* caused severe reductions in dendritic outgrowth of embryonic C1da
neurons (data not shown). To validate the observed RNAi phenotype, we examined C1da neurons in embryos homozygous for an amorphic allele of \textit{Rop} (\textit{Rop}^{G27}) and found that C1da neurons in \textit{Rop}^{G27} homozygous mutant embryos exhibited growth defects comparable to \textit{Rop} knock-down by RNAi, including severe reduction in outgrowth of both the primary dendrite and secondary lateral branches (Fig. 1A,B), demonstrating that \textit{Rop} is required for overall dendritic arbor growth. To explore the generality of \textit{Rop} function in dendrite growth, we assayed for requirements of \textit{Rop} function in the development of the more complex dendritic arbors of C4da neurons. Similar to C1da neurons, C4da neurons exhibited severe deficits in the outgrowth of primary dendrites and terminal dendritic branches in \textit{Rop}^{G27} homozygous mutants, resulting in dramatic reductions in receptive field size (Fig. 1C,D). Thus, \textit{Rop} appears to be generally required to support dendrite growth from early stages of dendrite development in the \textit{Drosophila} embryonic peripheral nervous system (PNS). Consistent with \textit{Rop} functioning cell-autonomously in dendrite growth, we found that \textit{Rop} is highly expressed in multidendritic (md) sensory neurons, including all da neurons, during embryogenesis. However, \textit{Rop} is also highly expressed in other cell types in the body wall, including the epidermis, suggesting its function is not exclusive to neurons (Fig. 1E-G).

\textbf{\textit{Rop} is autonomously required for growth and maintenance of the dendritic arbor}

\textit{Rop}^{G27} mutants fail to develop normally beyond mid-embryogenesis, failing to produce cuticle and exhibiting abnormalities in the underlying epidermis (Harrison et al., 1994). Given the importance of non-autonomous epithelial-derived cues in influencing dendrite growth (Jiang et al., 2014; Parrish et al., 2009) and the broad distribution of \textit{Rop} expression (Fig. 1E-G), we sought to assay for cell-autonomous functions for \textit{Rop} in dendrite development. To this end, we performed mosaic analysis with a repressible cell marker (MARCM) to generate single-cell C4da neuron clones homozygous for the \textit{Rop}^{G27} allele in an otherwise heterozygous background and assayed for effects on dendrite growth (Lee and Luo, 1999). Using SOP-FLP lines to facilitate the generation of C4da MARCM clones and reduce the impact of heat shock on developmental timing (Shimono et al., 2014), we found that dendrite defects were first manifest at 72 h after egg laying (AEL) in \textit{Rop}^{G27} mutant C4da neuron clones (Fig. 2A). Although there was no statistical difference in the number of terminal dendritic branches or total dendrite length between \textit{Rop} mutant and wild-type control neurons (Fig. 2B), a noticeable thinning of terminal dendrites in
Rop mutant C4da neurons was apparent at this time-point. By 96 h AEL, RopG27 C4da MARCM clones exhibited severely reduced dendritic complexity, as indicated by significant reductions in total dendrite length and in the total number of terminal dendritic branches (Fig. 2A,B). This reduction in complexity in the Rop mutant is not due to an overall loss of dendrites, as the numbers of terminal dendrites in Rop mutants did not differ significantly at 72 h and 96 h AEL and is more likely to reflect a failure in dendritic branch formation. Varicosities along some of the major dendritic branches also become apparent by this time (Fig. 2A, arrows). By 144 h AEL, RopG27 C4da MARCM clones showed a profound loss of dendrites and extensive beading along the major dendritic branches. In extreme cases, Rop mutant neurons were completely devoid of terminal dendritic branches and primary dendrites appeared highly fragmented, features characteristic of dendrite degeneration (Fig. 2C). We further monitored the effects of Rop mutations on axon growth by visualizing axon projection patterns of individual da neuron MARCM clones. Whereas RopG27 C4da MARCM clones exhibited severe defects in dendritic morphology at 96 h AEL, there was no obvious abnormality in the axon projections of Rop mutant da neuron clones at this time-point (data not shown). However, axons of Rop mutant neurons appeared thin with varicosities along the terminals by 144 h AEL (supplementary material Fig. S1), suggesting Rop may also be required for some aspects of axon development.

The dendrite phenotypes we observed could reflect a role for Rop in dendrite growth or could be a secondary effect of cell death caused by loss of Rop function. To differentiate between these two possibilities, we stained RopG27 C4da MARCM clones with markers of cell death and found that Rop mutant clones showed no detectable signal for the apoptotic effector Death caspase-1 (Dcp-1), and excluded the vital dye Acridine Orange (supplementary material Fig. S1 and data not shown). To further establish the requirement for Rop function in dendrite patterning, we monitored effects of a second Rop allele (RopA19) on dendrite growth. Similar to what we observed with the RopG27 allele, C4da MARCM clones homozygous for the hypomorphic RopA19 allele showed significant reductions in total dendrite length and in the total number of terminal dendritic branches (Fig. 2D,E). Finally, we found that expressing an epitope-tagged version of Rop was sufficient to rescue the dendrite growth defects of Rop mutants (Fig. 2D,E), further demonstrating that Rop functions cell-autonomously in C4da neurons to promote dendrite growth.
Given the severity of the dendritic growth defect in Rop\textsuperscript{G27} homozygous mutant embryos, we examined whether Rop protein perdurance contributed to the delayed phenotype of Rop\textsuperscript{G27} mutant MARCM clones. Indeed, Rop immunoreactivity was detectable in Rop\textsuperscript{G27} mutant C4da MARCM clones at 72 h AEL, when dendrite defects first become apparent, whereas Rop immunoreactivity was dramatically reduced in Rop\textsuperscript{G27} mutant C4da MARCM clones by 120 h AEL (supplementary material Fig. S2). Similarly, perdurance of maternal Rop function masks embryonic phenotypes in zygotic Rop mutants (Harrison et al., 1994). Thus, the late onset of dendrite defects likely reflects the timing when Rop activity is exhausted in Rop\textsuperscript{G27} MARCM clones, with persistent Rop protein supporting dendrite growth up to 72 h AEL. Interestingly, the growth defects were not uniformly distributed in dendrite arbors of Rop\textsuperscript{G27} mutant C4da clones: terminal dendrites exhibited heightened sensitivity to Rop function, suggesting that terminal dendrites have an increased requirement for exocytosis compared to other regions of the arbor, that exocytic membrane addition preferentially occurs at terminal dendrites, or both. To further explore the generality of the role of exocytosis in dendrite growth, we monitored the effects of RNAi-mediated knockdown of a subset of SNARE proteins important for vesicle trafficking and fusion, including Syntaxin1A, Snap24, Snap25, \(\alpha\)-Snap, and Synaptotagmin 1. Knockdown of these components led to significant reductions in terminal branching (supplementary material Fig. S3), consistent with the notion that terminal dendrites have a heightened requirement for the exocytic machinery. Taken together, our results indicate a role for the exocytic machinery in dendritic morphogenesis and demonstrate a cell-autonomous requirement for Rop in the growth and subsequent maintenance of the dendritic arbor.

\textit{Rop interacts with the exocyst to control dendritic branching}

We next set out to identify genes that act with Rop to control dendritic morphogenesis, focusing our attention on the possible relationship between Rop and the exocyst, which regulates polarized trafficking of membranous material. To determine whether the exocyst is cell-autonomously required for dendrite morphogenesis, we used MARCM to generate C4da MARCM clones that were homozygous mutant for loss-of-function alleles of two core components of the exocyst complex, Sec5 (Sec5\textsuperscript{E10}) and Sec6 (Sec6\textsuperscript{Ex15}) (Murthy et al., 2003; Murthy et al., 2005). We found that both Sec5\textsuperscript{E10} and Sec6\textsuperscript{Ex15} C4da MARCM clones showed significant reductions in total numbers of terminal dendritic branches as well as total dendrite
length (Fig. 3A-E), demonstrating a cell-autonomous function of the exocyst in dendrite growth. Furthermore, consistent with Sec5 and Sec6 functioning together in dendrite development, Sholl analysis revealed a remarkable phenotypic similarity between C4da neurons deficient for Sec5 or Sec6 (Fig. 3F). Of note, a comparable reduction in the number of dendritic intersections was observed in Rop\textsuperscript{G27} C4da MARCM clones (data not shown). Outgrowth of the major dendritic branches was only moderately affected in the exocyst mutants as the primary dendrites of Sec5\textsuperscript{E10} and Sec6\textsuperscript{Ex15} C4da MARCM clones extended to the dorsal midline and segment borders (Fig. 3B,C). However, varicosities along portions of major dendrites and thinning of terminal dendrites were apparent in Sec5 and Sec6 mutant clones (Fig. 3B,C), a phenotype reminiscent of Rop\textsuperscript{G27} C4da MARCM clones.

Based on the phenotypic similarity of Rop and Sec5/6 mutants, we investigated whether Rop and the exocyst function together in dendrite development. First, we determined whether Rop interacts genetically with Sec5 and/or Sec6 in the control of dendrite morphogenesis. Dosage-dependent genetic interactions are often indicative of a shared function in C4da neuron dendrite development (Parrish et al., 2007), therefore we assayed for dendrite growth defects in larvae with trans-heterozygous combinations of Rop\textsuperscript{G27} with the Sec5\textsuperscript{E13} or Sec6\textsuperscript{Ex15} allele (Fig. 3G-L). Indeed, trans-heterozygous combination of Rop\textsuperscript{G27} with Sec5\textsuperscript{E13} resulted in reduced numbers of terminal dendritic branches compared to C4da neurons in Rop\textsuperscript{G27}/+ and Sec5\textsuperscript{E13}/+ heterozygotes (p = 0.06; Sec5\textsuperscript{E13}/+; Rop\textsuperscript{G27}/+ trans-heterozygotes vs. Sec5\textsuperscript{E13} heterozygotes). We also found that C4da neurons in Sec6\textsuperscript{Ex15}/+; Rop\textsuperscript{G27}/+ trans-heterozygotes exhibited significant reductions in terminal dendritic branching compared to C4da neurons in Rop\textsuperscript{G27}/+ and Sec6\textsuperscript{Ex15}/+ heterozygotes alone (p < 0.05) (Fig. 3M). Thus, Rop genetically interacts with Sec5 and Sec6 to regulate dendrite development.

Since Rop genetically interacts with genes encoding exocyst subunits to regulate dendritic branching, we next tested whether Rop could physically associate with individual exocyst subunits in vivo. We performed co-immunoprecipitation experiments using a monoclonal antibody against Rop and found that Rop co-immunoprecipitated with endogenous Sec6 from adult Drosophila whole brain extracts as revealed by Western blot with anti-Sec6 antibody (Fig. 3N). We further found that Rop co-immunoprecipitated with endogenous Sec15 from whole brain extracts (Fig. 3N). Thus, Rop forms complexes with exocyst subunit proteins in vivo. Taken together, these data suggest that Rop can associate with multiple subunits of the
exocyst complex and that *Rop* and exocyst genes likely function in the same pathway to control dendritic morphogenesis.

**Exocytosis preferentially occurs along primary dendrites**

During larval development, terminal dendrite growth/addition accounts for the majority of C4da dendrite growth (Fig. 4A,B). Our finding that mutations in *Rop* and exocyst subunits preferentially affected growth and maintenance of terminal dendrites during larval development suggested that growing terminal dendrites may be the primary target for exocytosis during neuronal growth. If this is the case, we reasoned that the exocytic machinery would be preferentially localized to terminal dendrites. To address this possibility, we monitored endogenous distribution of Rop in larval C4da neurons using a monoclonal antibody to Rop (Harrison et al., 1994). Although we observed Rop immunoreactivity in major dendritic branches, Rop signal was not detectable or much weaker at terminal dendrites of C4da neurons (Fig. 4C-E). However, Rop immunoreactivity was present throughout the body wall, precluding high-resolution analysis of Rop distribution in terminal dendrites. To circumvent this problem, we expressed an epitope tagged version of Rop (Myc-Rop) in C4da neurons. As with endogenous Rop, epitope tagged Rop was enriched in major dendrites; weak signal was present in some higher order dendrites, but rarely extended the entire length of terminal dendrites (Fig. 4F-H). We conclude that the distribution of Rop is not uniform in C4da dendrite arbors and appears to be predominantly localized to major dendrites.

It is possible that the exocyst spatially restricts the localization of assembled SNARE complexes (Carr et al., 1999; Grote et al., 2000; Wiederkehr et al., 2004), and in this manner, tethers Rop to specific sites of exocytosis. We reasoned that if the exocyst accumulated at terminal dendrites, then it might efficiently recruit Rop to terminal dendrites, even though the majority of Rop is restricted to major dendrites. To test this possibility, we monitored exocyst distribution in C4da neurons by co-expressing a GFP-tagged version of Sec15, which has been previously used to visualize the exocyst (Jafar-Nejad et al., 2005), in C4da neurons also expressing the CD4-tdTomato membrane marker. Interestingly, we observed that Sec15-GFP is enriched in the soma, where it accumulates in large punctate structures, and is also distributed along major dendrites with notable accumulations at dendritic branch points (Fig. 4I-K). As with
Rop, Sec15-GFP was present at much lower levels in terminal dendrites. Therefore, our data suggest that the exocytic machinery is enriched in major dendrites.

The distribution of Rop and exocyst components in C4da dendrites suggested that dendritic exocytosis occurs primarily along the major dendritic branches rather than at terminal dendrites. To test this possibility, we used two imaging-based approaches. First, we reasoned that visualization of Rop-exocyst complex formation \textit{in vivo} would allow us to identify sites of vesicle-plasma membrane attachment. To this end, we used Bimolecular Fluorescence Complementation (BiFC) in which two interacting proteins are fused to two non-fluorescent fragments of yellow fluorescent protein (YFP), the N-terminal (NYFP) and C-terminal (CYFP) fragments (Gohl et al., 2010; Hu et al., 2002). In this assay, binding of the interacting proteins facilitates YFP reconstitution, thus YFP fluorescence serves as a proxy for tracking protein-protein interactions \textit{in vivo}. Reassembly of split-YFP is irreversible \textit{in vitro} (Hu et al., 2002), thus BiFC should provide a cumulative record of Rop-exocyst complex formation and hence facilitate visualization of complexes throughout the cell.

Yeast Sec1 and Sec6 can directly interact \textit{in vitro} (Morgera et al., 2012), and we found that Rop and Sec6 co-immunoprecipitate in \textit{Drosophila} neurons (Fig. 3N), therefore we examined whether Rop and Sec6 directly interact in \textit{Drosophila} S2 cells. First, as a negative control, we co-expressed the two halves of split YFP (CYFP and NYFP) in S2 cells and observed no detectable YFP fluorescence (supplementary material Fig. S4), demonstrating that interacting proteins are required for efficient YFP reassembly. Next, we generated both N- and C-terminal fusions of Rop with NYFP and N- and C-terminal fusions of Sec6 with CYFP and tested for YFP reconstruction in S2 cells. Strong BiFC signal was apparent only when NYFP-Rop was transfected with Sec6-CYFP, demonstrating the specificity of the interaction (supplementary material Fig. S4). Thus, we conclude that Rop and Sec6 can directly interact in \textit{Drosophila} S2 cells.

Using NYFP-Rop and Sec6-CYFP, we next monitored complex formation between Rop and Sec6 in the \textit{Drosophila} larval body wall. First, we assayed \textit{for in vivo} function of NYFP-Rop and Sec6-CYFP. We expressed \textit{UAS-NYFP-Rop} in \textit{Rop} mutant C4da MARCM clones and \textit{UAS-Sec6-CYFP} in \textit{Sec6} mutant C4da MARCM clones, and found that both transgenes rescued the respective mutant phenotypes (Fig. 2D,E and supplementary material Fig. S4). These results indicate that NYFP-Rop and Sec6-CYFP are functional proteins and suggest that BiFC signal
would likely reflect the normal localization of Rop-Sec6 complexes in vivo. Sec6 complexes localize to basolateral junctional domains in polarized epithelial cells where they appear to mediate polarized delivery of cargo (Grindstaff et al., 1998), therefore we next examined whether Rop-Sec6 BiFC signal accumulates at junctional domains in the larval epidermis. Indeed, co-expression of NYFP-Rop with Sec6-CYFP using the epidermal A58-Gal4 driver yielded robust YFP reconstitution in body wall epithelial cells, with Rop-Sec6 complexes enriched at junctional domains (supplementary material Fig. S4). Finally, we assayed for Rop-Sec6 complex formation in C4da neurons. When we co-expressed NYFP-Rop and Sec6-CYFP in C4da neurons, we observed strong YFP fluorescence in major dendrites but little or no signal in terminal dendrites (Fig. 5A,B), suggesting that Rop-exocyst interaction, and hence vesicle tethering to the plasma membrane, primarily occurs in major but not terminal dendrites. In contrast to the dendritic distribution of Rop-Sec6 complexes, co-expressing NYFP-Rop and Sec6-CYFP yielded robust YFP reconstitution in C4da axon terminals (Fig. 5C), consistent with prior reports that the exocyst is required for exocytosis of Golgi-derived vesicles in axon terminals of Drosophila motor neurons (Murthy et al., 2003; Murthy et al., 2005). Thus, the Rop-Sec6 complexes are differentially distributed in different compartments within C4da neurons: they are enriched in axon terminals and major dendrites, but present only at low levels in terminal dendrites. These results suggest that the capacity for exocytosis within C4da neurons is not uniform.

To more directly visualize sites of exocytosis and function, we utilized a pH-sensitive reporter (pHluorin-CD4-tdTomato; pH-Tom) comprised of the pH-sensitive green fluorescent protein (ecliptic pHluorin) fused to the amino-terminus of a red fluorescent membrane marker (CD4-tdTomato) (Kanamori et al., 2015) (Fig. 6A). The chromophore of ecliptic pHluorin is protonated at acidic pH (Miesenbock et al., 1998), hence pHluorin fluorescence is quenched within acidified intracellular compartments. As a result, the pH-Tom reporter is both green and red fluorescent at the cell surface but loses green fluorescence in acidified intracellular compartments such as exocytic vesicles. In cells expressing this reporter, regions with low levels of exocytosis should have reduced levels of green fluorescence compared to regions with high levels of exocytosis.

When we constitutively expressed pH-Tom in C4da neurons beginning during late embryogenesis, we observed comparable ratios of green/red fluorescence throughout dendrite
arbors in third instar larvae (Fig. 6B-D). However, over extended timescales, endocytosis and lateral diffusion might obscure regional differences in exocytosis as reported by pH-Tom. We therefore used a Flp recombinase-inducible ppk-Gal4 driver (Kanamori et al., 2013) to inducibly and selectively express pH-Tom in C4da neurons, reasoning that by tracking newly synthesized pH-Tom we could more accurately monitor patterns of exocytic vesicle delivery. Using this approach, we found that the pH-Tom reporter accumulates to detectable levels ~24 h after induction of Flp expression. We therefore induced Flp expression at 48 h AEL and monitored pH-Tom fluorescence in C4da dendrites at 72 h AEL, the time at which dendrite defects were first apparent in Rop mutant C4da clones (Fig. 6E). Similar to what we observed following constitutive expression of the reporter, green (pHluorin) and red (Tomato) fluorescent signals were readily detectable in major dendrite branches (Fig. 6F-H). However, compared to major dendrites, the ratio of green/red fluorescence was significantly reduced in terminal dendrites, suggesting that exocytosis preferentially occurs along major dendrites (Fig. 6H). Alternatively, these results could reflect heightened rates of endocytosis in terminal dendrites. However, we observed a similar distribution of pH-Tom fluorescence when we concomitantly induced pH-Tom expression and blocked endocytosis using a temperature-sensitive dominant-negative allele of shibire (shi^{ts1}) (Kitamoto, 2001) (Fig. 6H), suggesting that the absence of pHluorin fluorescence in terminal dendrites is not a consequence of endocytosis. Thus, although the pH-Tom reporter readily accumulates in terminal dendrites, pH-Tom is more efficiently delivered to the plasma membrane in major dendrites.

Our results thus far indicated that exocytosis occurs primarily along major dendrites, whereas the majority of dendrite growth occurs in terminal dendrites. We therefore investigated the possibility that material delivered to major dendrites might be supplied to terminal dendrites by diffusion to facilitate growth. If terminal dendrites relied on diffusion for materials to support membrane expansion, then diffusion of materials into terminals would need to occur at a faster rate than diffusion out of terminals. To examine this possibility, we monitored the dynamics of a membrane-associated protein in dendrites by measuring the lateral diffusion of a myristoylated photoactivatable fluorescent protein (myr-Eos) after photoconversion. Specifically, we photoconverted myr-Eos in major dendrites and monitored diffusion of the photoconverted protein in major dendrites and into terminal dendrites (Fig. 7A,B), or photoconverted myr-Eos in terminal dendrites and monitored diffusion within the terminals and into major dendrites (Fig.
Using this approach, we observed robust lateral diffusion of myr-Eos within major dendrites or within terminal dendrites, modest diffusion from major dendrites into terminals, and very little diffusion from terminals into main dendrites (Fig. 7B,C). Although the photoconverted myr-EOS likely comprises both cytosolic and membrane-bound forms of the fluorescent protein, a cytosolic version of Eos that is not membrane-bound freely diffused between the two compartments (Fig. 7B,C), suggesting the directional bias in myr-Eos diffusion is primarily due to its association with the membrane. We next tested the possibility that mutations in Rop affect the dynamics of membrane proteins by monitoring diffusion of a membrane-targeted GFP (mCD8-GFP) in both wild-type and Rop\textsuperscript{G27} C4da MARCM clones using fluorescence recovery after photobleaching (FRAP). Notably, when we photobleached mCD8-GFP in terminal dendrites and assayed recovery from mCD8-GFP in major dendrites, we observed no difference between control and Rop mutant clones (Fig. 7D). Likewise, the recovery halftime and the percentage of fluorescence recovery in major dendrites were unchanged in Rop mutant neurons compared to wild-type controls (Fig. 7D), suggesting that lateral diffusion of membrane-associated proteins is not impaired when exocytosis is attenuated. Taken together, these results are consistent with the hypothesis that material delivered to primary dendrites by exocytosis diffuses into terminals to support terminal dendrite growth.

**Blocking endocytosis mitigates dendrite growth defects of Rop mutants**

At first glance, our findings that terminal dendrites have a heightened requirement for exocytic machinery and that exocytic machinery/activity are concentrated in primary dendrites seemed at odds with one another. However, dendrites in many systems exhibit high rates of endocytic activity and the balance of exocytosis and endocytosis is likely a critical factor in ensuring the proper development of the dendritic arbor (Blanpied et al., 2002; Parton et al., 1992). We therefore hypothesized that an imbalance in exocytosis and endocytosis contributes to the growth defect of terminal branches in Rop mutants. If this was the case, we reasoned that blocking endocytosis might partially limit the terminal dendrite growth defects caused by depleted Rop function. To test this possibility, we selectively blocked endocytosis in C4da neurons by expressing shi\textsuperscript{ts1} in control and Rop\textsuperscript{G27} MARCM clones. Larvae with C4da MARCM clones were shifted to the non-permissive temperature for shi\textsuperscript{ts1} (30°C) at 48 h AEL, prior to the onset of dendrite growth defects in Rop mutants, and C4da neuron clones were visualized at 96 h AEL,
when dendrite growth defects are broadly manifest in Rop mutant C4da neurons. Thus, shi was selectively inactivated in these clones from 48 h to 96 h AEL.

Under this temperature regime, we observed a modest decrease in dendrite branching of control neurons (compare Fig. 8E to Fig. 2B), reflecting a mild temperature sensitivity of the branching program, but little effect on overall dendrite length. Rop mutant clones still exhibited characteristic defects including significant decreases in total dendrite length and an abundance of varicosities along major dendrites (Fig. 8B,E). Blocking shi function from 48 h to 96 h AEL similarly reduced overall dendrite growth (Fig. 8C,E), consistent with a prior report that constitutively blocking shi function compromises dendrite growth (Yang et al., 2011). In addition, blocking shi resulted in a significant increase in dendrite-dendrite crossing events, suggesting that endocytic signaling is required for dendrite-dendrite avoidance (Fig. 8C,G). Interestingly, we found that blocking shi function from 48 h to 96 h AEL significantly ameliorated the dendrite defects of RopG27 C4da MARCM clones, leading to an overall increase in the number of terminal dendrites and dramatically reducing the appearance of varicosities along the major dendrites (Fig. 8D-F). Thus, endocytosis in the absence of exocytosis is likely a major contributory cause to the terminal dendrite growth defect and degeneration of primary dendrites in Rop mutant neurons. We also noted a synergistic effect on dendrite-dendrite crossing defects (Fig. 8D,G), demonstrating that endocytic/exocytic signaling plays critical roles in dendrite-dendrite avoidance. Altogether, these results demonstrate that the balance of endocytosis and exocytosis is critically important in supporting dendrite growth and maintenance and that these types of membrane fusion events are differentially localized during neuronal maturation to ensure the correct construction of neuronal architecture.

**Discussion**

The plasma membrane of the developing neuron must continuously expand to keep pace with the rapid growth of the axon and dendrite. Although the addition of neuronal membrane is mediated by exocytosis, there is little in vivo evidence to support the requirement of SM proteins in this process. Our study not only reveals an autonomous function for the SM protein Rop and the exocyst in dendrite growth, but also reveals a differential requirement for the exocytosis machinery in the development of different compartments of the dendritic arbor. Depletion of Rop in C4da neurons causes an initial defect in terminal dendrites followed by profound degeneration.
of primary dendrites. Unexpectedly, we found that although Rop and the exocyst are required for terminal dendrite growth, Rop, the exocyst components Sec15 and Sec6, and Rop-Sec6 complexes are largely excluded from terminal branches. Likewise, we demonstrate that exocytosis predominantly occurs along major dendrites in these neurons, despite the fact that terminal dendrites account for the majority of dendrite growth in larval C4da neurons.

Our results show that terminal dendrites likely depend on the diffusion of material from primary dendrites to support their growth and in the process reveal key differences in how membrane is allocated in different compartments of the dendritic arbor. Primary and terminal dendrites of C4da neurons differ in their cytoskeletal composition and dynamics (Grueber et al., 2002; Jinushi-Nakao et al., 2007; Lee et al., 2015), which can create differences in membrane tension (Dai and Sheetz, 1999). As membrane tends to flow from a region of low tension to a region of higher tension (Dai and Sheetz, 1995), the resulting tension gradient could then explain how membrane proteins readily diffuse from major branches into terminals, but not in the opposite direction. Why then are terminal dendrites particularly sensitive to reduced Rop function? Growing dendrites exhibit high rates of endocytic activity compared to growing axons (Parton et al., 1992; Ye et al., 2007), and dynamic clathrin assembly/disassembly occurs throughout the dendritic arbor, consistent with a requirement for clathrin-dependent endocytosis in dendrite growth (Blanpied et al., 2002; Yang et al., 2011; Zheng et al., 2008). We found that blocking endocytosis significantly ameliorated the terminal dendrite loss observed in Rop mutant neurons, suggesting that unchecked endocytosis contributes to the rapid retraction of terminal dendrites in Rop mutants. Thus, even though terminal dendrites show low levels of exocytosis compared to primary dendrites, it is necessary to counterbalance the high levels of endocytic activity and replenish the plasma membrane, underscoring the delicate balance of exocytosis and endocytosis required for dendrite growth (Fig. 8H).

Although our data suggest that lateral diffusion of material contributes to terminal dendrite growth, we cannot exclude the possibility that satellite secretory pathways (or Golgi outposts) can act as a local source of membrane within some terminal branches (Horton et al., 2005; Ye et al., 2007). The presence of Golgi outposts within terminal branches of C4da neurons would seem to suggest that local exocytosis is an important mode of terminal dendrite growth, but recent studies support microtubule nucleation as a key role for Golgi outposts within these branches (Ori-McKenney et al., 2012). Therefore, we propose a model in which primary
dendrites are the major sites of exocytosis and membrane addition for expansion of the dendritic arbor and terminal dendrites primarily rely on diffusion of material to support their growth (Fig. 8H).

In mature dendrites, exocytosis primarily occurs in dendritic spines enriched with clusters of the t-SNARE Syntaxin 4, which mediates activity-dependent exocytosis during long-term potentiation (Kennedy et al., 2010). By contrast, how sites of exocytosis are defined in a growing dendrite remains largely unresolved. The restricted localization of Rop in primary dendrites of C4da neurons suggests that the exocytic machinery is likewise targeted to specific sites of membrane expansion during development. Consistent with this notion, Rop interacts with exocyst subunits Sec5 and Sec6 to regulate dendrite growth, demonstrating that the exocyst/Sec1 interaction is functionally relevant in higher eukaryotes and may be an important mechanism by which SNARE complexes are recruited to specific sites of membrane expansion. In our BiFC analysis, we found that Rop directly interacts with Sec6 and that these complexes accumulate at sites with high exocytic activity, reflecting the importance of this interaction in biologically relevant contexts. In epithelial cells, we found that Rop-Sec6 complexes accumulate at junctional domains, consistent with the localized recruitment of the exocyst to regions of the epithelial membrane undergoing active exocytosis (Grindstaff et al., 1998), and suggesting that BiFC between Rop and Sec6 can be used as a proxy for exocytic activity in neurons. Indeed, we found that Rop-Sec6 complexes accumulate along major dendritic branches in C4da neurons, which correlate with the primary sites of exocytosis as revealed by the \textit{in vivo} pH-Tom reporter. Thus, we propose that the exocyst actively recruits Rop to specific sites of membrane expansion in dendrites to promote growth.

We also observed the accumulation of Rop-Sec6 complexes in C4da axon terminals. This result, together with the axon phenotype that we observed in \textit{Rop} mutant neurons, points to a role for Rop in vesicular trafficking in axon growth and/or maintenance, in addition to its well-characterized role in synaptic vesicle release. The exocyst plays a key role in axon outgrowth and discriminates between vesicular trafficking mechanisms that regulate membrane insertion and cell growth and those that regulate neurotransmitter secretion (Murthy et al., 2003). The exocyst itself is dispensable for neurotransmitter release (Mehta et al., 2005; Murthy et al., 2003), suggesting that binding of Rop to the exocyst can differentially affect Rop-mediated vesicular trafficking events in the presynaptic terminal.
Our finding that \textit{Rop}^{G27} C4da MARCM clones show a progressive loss of terminal dendrites and blebbing/fragmentation of primary dendrites during later stages of larval development further suggests that Rop is not only important for growth, but is also broadly required for dendrite maintenance. The neurodegeneration in \textit{Rop} mutant neurons parallels observations in neurons from \textit{munc18-1} knockout mice, which show severe and widespread neurodegeneration (Heeroma et al., 2004; Verhage et al., 2000). Importantly, degeneration in \textit{munc18-1} null neurons cannot be fully explained by the loss of synaptic activity because mice deficient for both \textit{munc13-1} and \textit{munc13-2}, which are synaptically silent, show no apparent signs of neurodegeneration (Varoqueaux et al., 2002). The function of Munc18-1 in neuronal maintenance is therefore likely to entail a function distinct from its role in neurotransmitter secretion. The ability of a dominant-negative allele of \textit{shibire} to mitigate dendrite degeneration phenotypes of \textit{Rop} mutant neurons suggests that a failure in exocytosis of vesicles derived from dynamin-dependent pathways is part of the mechanism by which \textit{Rop} depletion causes degeneration of primary dendrites. Indeed, endocytic dysfunction is a common theme in neurodegeneration and is likely to underlie the pathology of many neurodegenerative disorders (Nixon, 2005). Given the strong association of Munc18-1 with Syntaxin-1 (Sudhof and Rothman, 2009), it is not surprising then that a recent study also showed that expression of a dominant-negative form of dynamin could delay neurodegeneration induced by botulinum toxin-mediated cleavage of Syntaxin-1 in cultured hippocampal neurons (Peng et al., 2013). Syntaxin-1 is therefore important for neuronal maintenance, but an early requirement for Syntaxin-1 in neurite outgrowth (Igarashi et al., 1996) is consistent with the differential requirement for the exocytic machinery in the developing neuron.

Our findings are particularly interesting in light of the fact that mutations in \textit{MUNC18-1} and exocyst subunit genes are associated with a spectrum of neurodevelopmental disorders. \textit{de novo} mutations in the gene encoding MUNC18-1, (also known as Syntaxin binding protein 1, STXBP1), have been identified in individuals diagnosed with Ohtahara syndrome (Saitsu et al., 2008). In addition to intractable seizures, patients with mutations in \textit{MUNC18-1} further exhibit profound intellectual disability, physical deficits, and varying degrees of cerebral atrophy (Saitsu et al., 2008). While disruption of \textit{MUNC18-1} is most closely associated with Ohtahara syndrome, \textit{de novo} mutations in \textit{MUNC18-1} have also been identified in individuals with severe intellectual disability and non-syndromic epilepsy, individuals with non-syndromic intellectual
disability, and patients with Rett syndrome (Hamdan et al., 2011; Hamdan et al., 2009; Olson et al., 2015; Romaniello et al., 2015). Likewise, recent studies revealed possible connections between mutations in exocyst genes and neurological disorders (Evers et al., 2014; Fruhmesser et al., 2013; Wen et al., 2013). Given that dendrite defects are the strongest pathological correlate of intellectual disability (Kaufmann and Moser, 2000), we speculate that dendritic abnormalities may contribute to intellectual disability associated with MUNC18-1 and exocyst dysfunction.

Materials and Methods

Fly stocks

The following lines were used: Gal4221 (Grueber et al., 2003), ppk-Gal4 (Grueber et al., 2007), A58-Gal4 (Galko and Krasnow, 2004), ppk-Frt-stop-Frt-Gal4 (Kanamori et al., 2013), UAS-phLucor-CD4-ttdTomato (Kanamori et al., 2015), E7-2-36 (Brewster and Bodmer, 1995), sensory organ precursor (SOP)-FLP (Shimono et al., 2014), UAS-myrEos (Pfeiffer et al., 2010), ppk-CD4-ttdTomato, UAS-CD4-tdGFP and UAS-CD4-ttdTomato (Han et al., 2011), UAS-shi.K44A (Moline et al., 1999), and UAS-Dicer-2 (Dietzl et al., 2007). RopG27 and RopA19 (Harrison et al., 1994), Sec5E10 and Sec5E13 (Murthy et al., 2003), Sec6Ex15 (Murthy et al., 2005), Sec620 (Beronja et al., 2005), and UAS-GFP::Sec15 (Jafar-Nejad et al., 2005) are previously described. RNAi lines were generated by the Transgenic RNAi Project at Harvard Medical School (Ni et al., 2009). UAS-NYFP-Rop and UAS-CYFP-Sec6 transgenic lines were generated by site-specific integration into attP40 and attP2 dockers lines, respectively.

MARCM labeling

RopG27, FRT2A/TM6B, RopA19, FRT2A/TM6B, or w; FRT2A were mated with 109(2)80-Gal4, UAS-mCD8::GFP, SOP-FLP; tub-Gal80, FRT2A females. Sec5E10, FRT40A/CyO or w; FRT40A males were mated with elav-Gal4, UAS-mCD8::GFP, hsFLP; tub-Gal80, FRT40A females, and FRT42D, Sec6Ex15/CyO or w; FRT42D males were mated with elav-Gal4, UAS-mCD8::GFP, hsFLP; FRT42D, tub-Gal80 females. For SOP-FLP-bearing crosses, embryos were collected for 2 h in standard cornmeal molasses agar and allowed to develop to 72, 96, or 144 h, at which time MARCM clones were imaged live using confocal microscopy. For hsFLP-bearing crosses, embryos were collected for 2 h on grape juice agar plates and allowed to develop for 3-5 h at
25°C before being heat-shocked for 1 h at 38°C. Embryos were then allowed to develop to late third instar larvae, at which point they were dissected and processed for immunocytochemistry.

**Immunocytochemistry**

Embryos and larvae were processed for immunocytochemistry as previously described (Kim et al., 2006) using the following antibodies: mouse anti-Rop 4F8 at 1:200 (Developmental Studies Hybridoma Bank) (Harrison et al., 1994), rabbit anti-Myc at 1:1000 (A-14, Santa Cruz Biotechnology), rabbit anti-β-galactosidase at 1:5,000 (Cortex Biochem), rat anti-mCD8 at 1:200 (Life Technologies), rabbit anti-Dcp-1 at 1:500 (Cell Signaling Technologies), Cy5-conjugated anti-Horseradish peroxidase at 1:250 (Jackson Laboratories), and appropriate fluorophore-conjugated secondary antibodies at 1:200 (Jackson Laboratories). Whole-mount embryos and image stacks of MARCM clones were acquired at 1024 x 1024 pixel resolution using either a Leica TCS SP2 or an Olympus FluoView FV1000 confocal microscope.

**Live Imaging**

At the appropriate time, a single embryo/larva was mounted in 90% glycerol and imaged on a Leica SP5 confocal microscope with a 40x 1.25NA lens. MARCM clones were imaged at 1024 x 1024 pixel resolution. For late time-points, image stacks from multiple fields of view were assembled into a montage. For pHluorin imaging, larvae of the genotype *ppk-gal4/UAS-pHluorin::CD4-tdTomato* were imaged at 96 h AEL and larvae of the genotype UAS hsFLP/+; *ppk-Frt-Stop-Frt-Gal4/UAS-pHluorin::CD4-tdTomato; UAS-pHluorin::CD4-tdTomato/+* were heat-shocked (37°C for 1 h) at 48 h AEL and imaged at 72 h AEL at 1024 x 512 pixel resolution. For BiFC imaging, larvae of the genotype *ppk-Gal4/UAS-NYFP::Rop; ppk-CD4tdTomato/UAS-Sec6::CYFP* were imaged at 96 h AEL at 1024 x 512 pixel resolution.

**FRAP analysis of membrane protein diffusion**

Photoconversion and FRAP assays were conducted on a Leica SP5 with a 40x/1.25 oil objective at 1024 x 256 pixel resolution. For photoconversion assays, 10 µm of either a terminal or a major dendrite of a *ppk-gal4/+; UAS-myr-tdEosFP/+* or *ppk-gal4/+; UAS-tdEosFP/+* larvae at 72 h AEL was selected as a region of interest (ROI). The ROI was illuminated for 5 s with a 50mW 405nm Diode laser (Leica) at 40% output to photoconvert tdEOS and imaged at 10 s and 300 s
post-conversion with a 500-520nm bandwidth. The fluorescence intensity of the converted signal was measured for the central 8 μm of the converted region and the adjacent 8 μm in both directions. Intensity was analyzed using line plots (Image J) with eight individual samples. For FRAP assays, ROIs in control and RapG27 mutant C4da MARCM clones were photobleached for 3s with a 405nm laser at 60% power such that 50% of the initial fluorescence intensity was bleached. Baseline fluorescence was measured from five images acquired prior to bleaching and fluorescence recovery was measured with 20 scans at 2 s intervals after bleaching. Background fluorescence values were subtracted from measurements of a nearby, unbleached dendritic region at each time point. To control for photobleaching and sample movement out of focus, fluorescence intensity was monitored in multiple regions of the arbor outside of the ROI; only samples for which intensities remained steady in these control regions were included in data analysis. Recovery halftime and recoverable pool were measured using Leica FRAP Wizard software.

**Immunoprecipitation**

Adult fly heads (50 per experiment) were isolated and homogenized in 500 ml of lysis buffer (15 mM Tris/HCl at pH 7.6, 0.25 mM sucrose, 15 mM NaCl, 15 mM MgCl₂, 25 mM EDTA, 1 mM EGTA, and protease inhibitor cocktail). After pre-clearing with Protein A agarose (Life Technologies), extracts were incubated with 15 ml anti-Rop antibody and 50 ml of Protein A agarose beads. The beads and input extract were eluted 1:1 in Laemmli buffer with 2-mercaptoethanol (Bio-Rad Laboratories) and boiled for 5 minutes. Samples were resolved on a 4–20% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in Blocking Buffer (Rockland Immunochemicals) and incubated overnight at 4°C with primary antibodies, anti-Sec6 at 1:250 (Beronja et al., 2005) or anti-Sec15 at 1:250 (Mehta et al., 2005). Membranes were then incubated with secondary antibodies (anti-guinea pig IRDye700 at 1:10,000; Rockland Immunochemicals) and blots were imaged and processed on an Odyssey Infrared Imaging System (LI-COR Biosciences).

**Bimolecular fluorescence complementation**

Rop and Sec6 coding sequences were PCR amplified from genomic DNA and inserted into pUAST-BiFC vectors (Gohl et al., 2010) using Gateway cloning (Life Technologies) to generate
expression vectors with *Rop* fused to coding sequences for the N-terminal portion of *YFP* and *Sec6* fused to the C-terminal portion of *YFP* (*pUAST-NYFP-Rop, pUAST-Rop-NYFP, pUAST-CYFP-Sec6, pUAST-Sec6-CYFP*). As negative controls, we generated *pUAST-NYFP* and *pUAST-CYFP* expression vectors that contained coding sequences for the N-terminal and C-terminal portion of *YFP*, respectively. To assay for *YFP* reconstitution we used Effectene (Qiagen) to transfect S2 cells with Actin-Gal4, UAS-RFP, and all possible combinations of the above *NYFP* and *CYFP* expression constructs; only *pUAST-NYFP-Rop + pUAST-Sec6-CYFP* yielded BiFC signal, therefore we used those expression constructs to monitor Rop/Sec6 complex formation *in vivo*.

**Quantitative and statistical analysis**

2D projections of image z-stacks were used for computer-assisted dendrite tracing and analysis with either the NeuronJ plugin in ImageJ (Meijering et al., 2004) or Neurolucida (Fig. 2, 4, and 8; MBF Bioscience). For Sholl analysis (Sholl, 1953), neuron traces were generated in NeuronJ and analysis was performed using the Sholl Analysis plugin in ImageJ. Statistical tests are indicated in the figure legends.

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**Author Contributions**

Competing Interests

The authors declare no competing financial interests.
References


Fig. 1. **Rop regulates dendrite growth.** (A) Dendritic morphologies of wild-type dorsal class I da (C1da) neurons ddaD and ddaE in a late stage embryo. (B) C1da neurons in a Rop\(^{G27}\) homozygous mutant exhibit reduced outgrowth of the primary dendrite and absence of lateral branching. (C) Dendritic morphology of wild-type dorsal class IV da (C4da) neuron ddaC in a late stage embryo. (D) ddaC neurons in a Rop\(^{G27}\) homozygous mutant exhibit reduced primary dendrite outgrowth and lateral branching defects. (E-G) Multidendritic (md) neurons in a late stage embryo labeled by the E7-2-36 lacZ enhancer trap line revealed by staining with anti-β-galactosidase (E) and anti-Rop antibody (F). Rop is expressed in all da neurons in the dorsal cluster (G). Anterior is to the left and dorsal is up in this and all subsequent figures.
Fig. 2. Differential requirement for Rop function in dendrite growth. (A) Dendritic morphologies of representative wild-type or Rop<sup>G27</sup> C4da MARCM clones at indicated time-points. Arrowheads mark varicosities on dendrite branches. Clones were imaged live and all images are shown at the same magnification. (B) Quantification of total number of terminal dendrite branches and total dendrite length in wild type or Rop<sup>G27</sup> mutant C4da-ddaC MARCM clones at indicated times. The number of clones analyzed for each time-point/genotype combination is indicated. *** p < 0.001 (one-way ANOVA with Tukey’s HSD post hoc analysis). (C) At 144 h AEL, 4/19 Rop<sup>G27</sup> mutant C4da-ddaC MARCM clones analyzed exhibit a total loss of terminal dendrites and extensive fragmentation of major dendrites. (D) Dendritic morphologies of representative wild-type, Rop<sup>A19</sup>, Rop<sup>G27</sup>, or Rop<sup>G27</sup> rescue (Rop<sup>G27</sup> + UAS-NYFP-Myc-Rop) C4da-ddaC MARCM clones at 144 h AEL. (E) Quantification of total number of terminal dendrite branches and total dendrite length, with the number of neurons analyzed for each genotype indicated. *** p < 0.001, ns, not significant, compared to wild-type controls (one-way ANOVA with Tukey’s HSD post hoc analysis).
Fig. 3. Rop interacts with the exocyst complex. (A-C) Dendritic morphology of a wild-type (A), Sec5<sup>E10</sup> (B), or Sec6<sup>Ex15</sup> (C) C4da-ddaC MARCM clone in a fixed preparation of late-stage third instar larva. (D, E) Quantification of total number of terminal dendrite branches (D) and total dendrite length (E) in wild-type, Sec5<sup>E10</sup>, and Sec6<sup>Ex15</sup> C4da-ddaC MARCM clones. *** p < 0.001 (one-way ANOVA with Tukey’s HSD post hoc analysis). (F) Sholl analysis of wild-type, Sec5<sup>E10</sup>, and Sec6<sup>Ex15</sup> C4da-ddaC MARCM clones. FRT40A and FRT42D control clones were pooled as wild type in (D-F) for purposes of statistical analysis. (G-L) Dendritic morphology of C4da-ddaC neuron in a wild-type (G), Rop<sup>G27/+</sup> heterozygous (H), Sec5<sup>E13/+</sup> heterozygous (I),
Sec6^{Ex15/+} heterozygous (J), Sec5^{E13/+}; Rop^{G27/+} trans-heterozygous (K), and Sec6^{Ex15/+}; Rop^{G27/+} trans-heterozygous third instar larva at 144 h AEL (L). All neurons were imaged live. (M) Quantification of total number of terminal dendritic branches per 10.1 x 10^4 μm^2 field. # denotes statistical comparison to wild type; * p < 0.05, ### p < 0.001 (one-way ANOVA with Tukey’s HSD post hoc analysis). (N) Rop forms a complex with exocyst components Sec6 and Sec15. Co-immunoprecipitation using Rop monoclonal antibody 4F8 precipitates Sec6 and Sec15 from adult whole brain lysates as detected by immunoblotting.
Fig. 4. Exocytic machinery preferentially localizes to major dendrites. (A, B) Time-lapse imaging of dendrite growth in larval C4da neurons. (A) A representative individual C4da neuron (positively labeled using MARCM) is shown at indicated time-points. (B) Mean values and standard deviation for total dendrite length, terminal dendrite length and primary dendrite length for 5 ddaC MARCM clones imaged at each time-point. (C-E) GFP (to mark C4da neurons) and
Rop immunostaining of a *ppk-EGFP* third instar fillet reveals prominent Rop immunoreactivity in major dendrites (arrowheads). (F-H) Images of a third instar larva expressing GFP and epitope-tagged Rop (NYFP-Myc-Rop) specifically in C4da neurons immunostained with anti-GFP and anti-Myc antibodies. (I-K) The exocyst is enriched in major dendrites and accumulates at dendritic branch points. Live images of a third instar larva expressing tdTomato and GFP-tagged Sec15 specifically in C4da neurons.
**Fig. 5. Visualization of Rop-Sec6 complexes in vivo.** (A) Schematic representation of C4da neurons in the larval PNS. C4da axons project into the ventral nerve cord (VNC) of the central nervous system. (B) Rop-Sec6 complexes preferentially localize to major branches in C4da dendrite arbors. Dendrite arbors of C4da neurons expressing UAS-NYFP-Rop together with UAS-Sec6-CYFP were labeled with ppk-CD4-tdTomato and Rop-Sec6 complex formation was assayed by monitoring YFP fluorescence as NYFP-Rop-Sec6-CYFP interactions promote YFP reassembly (BiFC signal). Dendrite arbors were segmented into 15 μm segments, and BiFC and CD4-tdTomato signal intensities were measured in 25 such segments of major dendrites and terminal dendrites. Ratios of BiFC/CD4-tdTomato signal are displayed according to a lookup table for one neuron (arrows mark terminal dendrites) and measurements are shown for 5 neurons (circles, major dendrites; squares, terminal dendrites). Bars mark median values and interquartile range. *** $p < 0.001$, paired $t$-test (comparing means of BiFC/Tomato ratios in major dendrites and terminal dendrites). (C) Rop-Sec6 BiFC signal is readily detectable in terminal arbors of C4da axons in the VNC.
Fig. 6. Exocytosis preferentially occurs on major dendrites. (A) Schematic of pHluorin-CD4-tdTomato (pH-Tom) reporter, which has different fluorescent properties at acidic and neutral pH. (B) CD4-tdTomato and (C) pHluorin fluorescence at 96 h AEL following constitutive pH-Tom reporter expression in C4da neurons. Signal intensity is depicted according to a lookup table. Note that pHluorin signal is detectable throughout the dendrite arbor. (D) Dendrite arbors were segmented into 15 μm segments, and ratios of pHluorin and CD4-tdTomato signal intensities are shown for 25 such segments of major dendrites (circles) and terminal dendrites (squares) for each neuron analyzed. n = 5 neurons. Bars mark median values and interquartile range. ns, p > 0.05, paired t-test (comparing means of pHluorin/Tomato ratios in major dendrites and terminal dendrites). (E) Schematic of pulse-chase experiment to monitor exocytosis during dendrite growth. (F) CD4-tdTomato, (G) pHluorin fluorescence at 72 h AEL, and (H) fluorescence intensity measurements 24 h after induction of pH-Tom reporter expression in wild-type C4da neurons (left) or C4da neurons in which endocytosis was blocked during induction of pH-Tom reporter expression (ppk>shi^ts; right). Larvae were raised at 29°C during the 24 h period following induction of Flp activity to block endocytosis. *** p < 0.001, paired t-test (comparing means of pHluorin/Tomato ratios in major dendrites and terminal dendrites).
Fig. 7. Directional diffusion of membrane proteins into terminal dendrites. (A) Dendrites of a C4da neuron expressing photo-convertible cytosolic EOS or myr-EOS imaged prior to photoconversion (t = 0), 10 sec after conversion in the region marked by the red hatched box (t = 10 s) and 300 sec after conversion (t = 300 s). Green fluorescent myr-EOS (488 nm excitation) and photo-converted red fluorescent myr-EOS (561 nm excitation) are shown. Arrows mark the
boundary of lateral diffusion within the dendrite branch and double carats mark diffusion into terminal dendrites. Mean intensity of red fluorescent EOS or myr-EOS within an 8 μm photo-converted region (B) on a major dendrite and 8 μm neighboring regions on the same dendrite branch and in an adjacent terminal dendrite or (C) in a terminal dendrite branch and 8 μm neighboring regions on the same dendrite branch and in an adjacent major dendrite at 10 s and 300 s after photo-conversion. n=8 regions of interest, each from a different neuron, no more than 2 neurons were sampled from the same larva; error bars represent standard deviation. (D) FRAP analysis of membrane protein diffusion in Rop$^{G27}$ mutant neurons. Plots shown mean values and standard deviation for $t_{\text{half}}$ (halftime of recovery) and % recovery for mCD8-GFP in 10 μm ROIs within major dendrites or entire terminal dendrites of wild type and Rop$^{G27}$ mutant C4da MARCM clones. n > 25 dendrites for each category/genotype pair.
Fig. 8. Blocking endocytosis attenuates dendrite defects in Rop mutant neurons. (A-D)
Representative images of C4da MARCM clones depicting effects of blocking endocytosis and exocytosis on dendrite growth. Larvae bearing MARCM clones of the following genotypes are shown: (A) wild type control; (B) Rop^{G27} mutant; (C) C4da neuron expressing UAS-shi^{ts}; (D) Rop^{G27} mutant C4da neuron expressing UAS-shi^{ts} were raised at 25°C for 48 h, shifted to 29°C for 48 h and imaged using live confocal microscopy. Arrows, dendrite-dendrite crossing points. Quantification of total number of terminal dendrite branches (E), total dendrite length (F), and number of crossing points normalized to dendrite length (G). * p < 0.05, ** p < 0.01 (Student’s t-
test). The number of neurons analyzed for each genotype is indicated. (H) Model of exocytic/endocytic trafficking in developing dendrites. In wild-type neurons (1), Rop (green rectangle) is recruited to the membrane by the exocyst (blue oval) and mediates exocytosis. In the absence of Rop (2), endocytosis proceeds unchecked causing terminal branch retraction, which can be mitigated by expression of dominant negative dynamin (3).
Figure S1. Axon and dendrite phenotypes of Rop mutant neurons are unlikely to be a consequence of cell death. (A) Axons of Rop^{G27} da neuron MARCM clones appear thin with varicosities along the terminals at 144 h AEL compared to wild-type controls. (B) Dorsal cluster of da neurons as revealed by staining with horseradish peroxidase (HRP). Wild-type C4da neurons and Rop^{G27} C4da-ddaC MARCM clones (labeled in green) show no detectable signal for Death caspase-1 (Dcp-1). By contrast, C4da neurons expressing the apoptosis activator Reaper (ppk>rpr) stain positive for Dcp-1. Scale bars, 25 μm.
Figure S2. Rop protein perdures through early larval stages in Rop^{G27} mutant C4da MARCM clones. (A) Rop^{G27} mutant C4da MARCM clones and Rop^{G27/+} heterozygous control C4da neurons (from the neighboring segment in the same larva) were stained with antibodies for HRP (to label sensory neurons) and Rop. Representative images and (B) quantification (mean and standard deviation, number of neurons indicated for each sample) of Rop levels (mean Rop immunoreactivity in the C4da soma, outlined with white hatched line) are shown for each genotype-timepoint combination. Scale bar, 25 μm.
Figure S3. SNARE proteins regulate terminal dendritic branching. Dendritic morphologies of representative C4da neurons in a UAS-Dicer-2 control (A) or expressing RNAi transgenes targeting Syx1A (B), Snap24 (C), Snap25 (D), and α-Snap (E). (F) Mean and standard deviation of terminal branch number with the number of neurons analyzed for each genotype indicated. *** p < 0.001, compared to control (one-way ANOVA with Tukey’s HSD post hoc analysis). Scale bar, 50 μm.
Figure S4. Monitoring Rop-Sec6 complex formation using BiFC. (A) S2 cells co-transfected with Actin-Gal4 + UAS-RFP (to mark transfected cells) and the indicated expression constructs (e.g. UAS-NYFP + UAS-NYFP) were DAPI stained and imaged using epifluorescence microscopy to assay for YFP reassembly (BiFC signal). (B) Co-expression of UAS-NYFP-Rop and UAS-Sec6-CYFP under control of the epidermal A58-Gal4 driver, but not expression of UAS-NYFP-Rop alone, results in YFP fluorescence in the body wall, indicative of Rop-Sec6 complex formation. YFP fluorescence is enriched at cell-cell junctions (dashed line). (C) Dendritic morphologies of representative wild-type, Sec6Δ20, or Sec6Δ20 + UAS-Sec6-CYFP C4da-ddaC MARCM clones. Scale bar, 100 μm. (D,E) Mean and standard deviation of total dendrite length (D) and terminal dendrite number (E) is shown, with the number of neurons analyzed for each genotype indicated. Bars represent mean values *** p < 0.001, ns, not significant, compared to wild type controls (one-way ANOVA with Tukey’s HSD post hoc analysis).