

Drosophila Syd-1 Has RhoGAP Activity That Is Required for Presynaptic Clustering of Bruchpilot/ELKS but Not Neurexin-1

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ABSTRACT Syd-1 proteins are required for presynaptic development in worm, fly, and mouse. Syd-1 proteins in all three species contain a Rho GTPase activating protein (GAP)-like domain of unclear significance: invertebrate Syd-1s are thought to lack GAP activity, and mouse mSYD1A has GAP activity that is thought to be dispensable for its function. Here, we show that *Drosophila melanogaster* Syd-1 can interact with all six fly Rhos and has GAP activity toward Rac1 and Cdc42. During development, fly Syd-1 clusters multiple presynaptic proteins at the neuromuscular junction (NMJ), including the cell adhesion molecule Neurexin (Nrx-1) and the active zone (AZ) component Bruchpilot (Brp), both of which Syd-1 binds directly. We show that a mutant form of Syd-1 that specifically lacks GAP activity localizes normally to presynaptic sites and is sufficient to recruit Nrx-1 but fails to cluster Brp normally. We provide evidence that Syd-1 participates with Rac1 in two separate functions: (1) together with the Rac guanine exchange factor (RacGEF) Trio, GAP-active Syd-1 is required to regulate the nucleotide-bound state of Rac1, thereby promoting Brp clustering; and (2) Syd-1, independent of its GAP activity, is required for the recruitment of Nrx-1 to boutons, including the recruitment of Nrx-1 that is promoted by GTP-bound Rac1. We conclude that, contrary to current models, the GAP domain of fly Syd-1 is active and required for presynaptic development; we suggest that the same may be true of vertebrate Syd-1 proteins. In addition, our data provide new molecular insight into the ability of Rac1 to promote presynaptic development.

KEYWORDS Syd-1; Rac; Bruchpilot; Neurexin; Trio

THE assembly of synaptic connections between neurons and their targets is initiated by cell–cell adhesion and culminates in the clustering of presynaptic and postsynaptic components at apposing sites (Melom and Littleton 2011; Chia *et al.* 2013). Forward genetic screens in worm and fly have identified the presynaptic cytosolic protein Syd-1 as a critical regulator of presynaptic assembly (Hallam *et al.* 2002; Oswald *et al.* 2010; Holbrook *et al.* 2012). In both species, Syd-1 is among the first proteins to arrive at nascent presynaptic sites and is required for the subsequent recruitment of multiple presynaptic components, including scaffolding proteins, active zone (AZ) proteins, synaptic vesicles (SVs), and

mitochondria (Hallam *et al.* 2002; Dai *et al.* 2006; Patel *et al.* 2006; Oswald *et al.* 2010, 2012; Holbrook *et al.* 2012). Fly Syd-1 has been shown to directly bind and cluster the AZ ELKS family protein Bruchpilot (Brp; Oswald *et al.* 2010) and the presynaptic adhesion molecule Neurexin (Nrx-1; Oswald *et al.* 2012); presynaptic loss of fly Syd-1 disrupts Brp and Nrx-1 localization within neuromuscular junction (NMJ) boutons, decreasing the number of boutons that form (Oswald *et al.* 2010, 2012). More recently, a mouse Syd-1 ortholog, mSYD1A, was identified and shown to be required for the localization of SVs to presynaptic sites (Wentzel *et al.* 2013), suggesting that at least some aspects of Syd-1 function are conserved between invertebrates and vertebrates.

Syd-1 proteins in both invertebrates and vertebrates contain a Rho GTPase activating protein (GAP)-like domain; however, the importance of this domain to the ability of Syd-1 to promote presynaptic development is unclear. Classically, RhoGAPs increase the GTPase activity of one or more Rho family proteins—Rac, Rho, and Cdc42—accelerating their switch from an active, GTP-bound state to a conformationally distinct inactive, GDP-bound state

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(Scheffzek and Ahmadian 2005; Tcherkezian and Lamarche-Vane 2007). Rho GTPases are also regulated by guanine exchange factors (GEFs) that accelerate their switch back from the GDP- to the GTP-bound state (Rossman *et al.* 2005). Rho GTPases and their regulators have been implicated in multiple aspects of neural development, including presynaptic assembly, but the molecular mechanism(s) by which they regulate the latter are unknown (Ball *et al.* 2010; Nahm *et al.* 2010; Tolia *et al.* 2011). The RhoGAP domains of the worm and fly Syd-1 proteins lack key conserved amino acids and so have been thought to lack GAP activity (Hallam *et al.* 2002; Wentzel *et al.* 2013); indeed, while worm Syd-1 was recently found to bind GTP-bound MIG-2/Rac, no GAP activity has ever been detected (Hallam *et al.* 2002; Xu *et al.* 2015). By contrast, mouse mSYD1A does have GAP activity toward RhoA (Wentzel *et al.* 2013), but whether this activity is required for mSYD1A function is not clear. Mutant forms of mSYD1A that lack the RhoGAP domain are sufficient to cluster SVs at presynaptic sites in cultured neurons, but their ability to rescue the SV docking defect in *mSYD1A^{KO}* mutant mice has not been tested, and the mouse genome contains a second Syd-1 homolog (Wentzel *et al.* 2013), whose presence complicates the interpretation of these experiments (see *Discussion*).

Here, we show that, contrary to previous evidence (Wentzel *et al.* 2013), fly Syd-1 does have RhoGAP activity. We use a mutant form of Syd-1 that specifically lacks GAP activity to show that Syd-1 has both GAP-dependent and GAP-independent functions in presynaptic assembly. And, finally, we identify Rac1 as a Rho GTPase that is regulated by Syd-1 and provide evidence that Rac1 promotes presynaptic development by promoting clustering of both Nr_x-1 and Brp.

Materials and Methods

DNA constructs

New *syd-1* transgenes were created using a *UAS-syd-1-1xFLAG* DNA construct previously shown to rescue loss of *syd-1* from R7 photoreceptor neurons (Holbrook *et al.* 2012). Two new *UAS* full-length *syd-1* transgenes were created: (1) *UAS-syd-1^{wt}-3xFLAG* in which two additional FLAG tags were added to facilitate detection in pull-down experiments; and (2) *UAS-syd-1^{RA}-3xFLAG*, which is identical to *UAS-syd-1^{wt}-3xFLAG* but has a substitution at codon 979 (numbered according to the fly Syd-1 isoform C; GenBank accession: AAF57207.2) causing it to specify alanine instead of arginine (R979A). DNA encoding the region from Q643 to M1164 (again numbered according to the isoform C sequence), which includes the predicted RhoGAP domain but lacks the C2 domain, was subcloned from each of the two new constructs into the pGEX-4T1 vector in such a way as to be in-frame with the N-terminal glutathione S-transferase (GST) open reading frame and an added C-terminal HA epitope tag, resulting in two new GST transgenes: *GST-syd1^{wt} GAP* and *GST-syd-1^{RA} GAP*.

Biochemistry

Wild-type and constitutively-active mutant GST-Rac1, Rac2, Mtl, RhoA, RhoL, and Cdc42 fusion constructs were obtained

from the *Drosophila* Genomics Resource Center. GST-fusion proteins, including GST-Syd-1^{wt} GAP and GST-Syd-1^{RA} GAP, were expressed, in and purified from, *Escherichia coli* by standard methods (Amersham Pharmacia Biotech; Garcia-Mata *et al.* 2006). Total protein concentrations were determined by the Bradford protein assay, and protein purity by Coomassie Blue staining of SDS-polyacrylamide gels. Flies containing *actin (act)-Gal4* [from the Bloomington *Drosophila* Stock Center (BDSC)] were crossed to flies containing the *UAS-syd-1^{wt}-3xFLAG* or *UAS-syd-1^{RA}-3xFLAG* transgene. Lysates from the heads of adult progeny of both sexes were prepared as described in Emery (2007) and incubated with GST alone or a constitutively-active mutant GST-Rho GTPase bound to glutathione-Sepharose 4B resin (Amersham Pharmacia Biotech). After 1 hr on ice, samples were washed and analyzed by western blots probed with anti-FLAG antibody. GTPase activity assays were performed with EnzChek Phosphate Assay Kit (E-6646; Life Technologies).

Genetics and fly husbandry

Flies were grown on standard food at 25°. *BG380-Gal4* (Budnik *et al.* 1996) was used to drive expression of *UAS-Rac1^{wt}* (Luo *et al.* 1994), *UAS-Rac1^{V12}* (Luo *et al.* 1994), *UAS-syd-1^{wt}-1xFLAG* (Holbrook *et al.* 2012), or *UAS-syd-1^{RA}-3xFLAG* (see above) in motoneurons; wild-type animals contained *BG380-Gal4* alone, *syd-1* mutant animals were *syd-1^{w46}/syd-1^{CD}* transheterozygotes that contained *BG380-Gal4* alone (Holbrook *et al.* 2012), and *trio* mutant animals were *trio^{S6A}/trio^{S137203}* transheterozygotes (Ball *et al.* 2010). In all cases, we analyzed females only.

NMJ dissections and staining

Late third-instar larval NMJs were dissected in Schneider's insect medium (Sigma-Aldrich) and fixed with Bouin's solution (Sigma-Aldrich) for 15 min. All samples, except those stained with anti-Nr_x-1 (see below), were then stained by standard methods: washed 3× in PBS containing 0.3% Triton-X 100 (PBT), blocked for 30 min in 5% normal goat serum, incubated with primary antibodies at 4° overnight, washed 3× with PBT, incubated overnight with secondary antibodies at 4° overnight, washed 3× with PBT, and mounted in Vectashield (Vector Laboratories). We used the following antibodies: fluorescence-conjugated anti-HRP (1:250; Jackson Immuno Labs); mouse anti-Brp (nc82, 1:100; Developmental Studies Hybridoma Bank); rabbit anti-FLAG (1:250; Sigma-Aldrich); mouse anti-Dlg (4F3, 1:250; Developmental Studies Hybridoma Bank); guinea pig anti-Nr_x-1 (1:500; Li *et al.* 2007); rabbit anti-GluRIIC (1:2500; Marrus *et al.* 2004); all secondary antibodies (goat IgG coupled to Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 633; 1:500) were from Life Technologies.

Quantifying NMJ bouton number

Each bar graph with this quantification (Figure 2, G and H, Figure 3F, and Figure 4, E and F) contains the results of a single experiment in which all genotypes were grown,

dissected, and stained in parallel. Confocal images were collected on a Leica SP2 microscope (with a 63× 1.4 NA oil immersion objective) and analyzed with ImageJ software (Schindelin *et al.* 2012; <http://fiji.sc/>) ($n =$ animals). Within each experiment, the images were randomized, and quantifications were performed blind to genotype. As is standard in the field, for each animal we counted the number of boutons (identified based on shape, as visualized by anti-HRP and anti-Dlg staining) on muscle 4 or on muscles 6/7 on both sides of segment A3 (e.g., Ball *et al.* 2010). As is also standard, we divided bouton number by muscle area (measured in ImageJ by tracing the muscle perimeter visualized by anti-Dlg) to compensate for potential variations in animal size (Schuster *et al.* 1996; Ball *et al.* 2010). Note that we found no significant difference in muscle size among the genotypes tested (Supplemental Material, Figure S1).

Quantifying *Nrx-1* levels

Each bar graph with this quantification (Figure 3J, Figure 5E, and Figure 7A) contains the results of a single experiment in which all genotypes were grown and dissected in parallel. Dissected pelts were notched in a genotype-specific pattern, and then all pelts were stained and washed in a single tube.

Staining for *Nrx-1* was conducted as previously described (Li *et al.* 2007) using 0.05% Triton-X 100 and the Vectastain ABC system (Vector Laboratories) followed by Tyramide Signal Amplification (TSA; Life Technologies); TSA-treated NMJs were then incubated with fluorescence-conjugated anti-HRP (1:250; Jackson Immuno Labs) for 30 min at room temperature followed by washes with PBS.

Confocal images were collected on a Leica SP2 microscope and analyzed with ImageJ software (Schindelin *et al.* 2012; <http://fiji.sc/>) ($n =$ animals). For each bar graph, all samples were imaged on the same day using the same laser settings and exposure, the images were randomized, and quantifications were performed blind to genotype ($n =$ animals). For each animal, *Nrx-1* levels were quantified within boutons on muscle 4 in segment A3. To do so, we used the anti-HRP channel to trace 4–10 presynaptic boutons while remaining blind to the anti-*Nrx-1* channel; we duplicated this trace and moved it to a region within the sample that did not contain anti-HRP staining; we then measured the average fluorescence intensities in the anti-*Nrx-1* channel within each of the traces and subtracted the measurement outside the boutons from the measurement within boutons to obtain a final fluorescence value.

Quantifying *Brp* puncta size

The bar graph with this quantification (Figure 6F) contains the results of two experiments: one with *syd-1* genotypes and the other with *trio* mutants; wild-type animals were analyzed in each experiment (hence the two separate measurements of wild type shown). In each of the two experiments, all genotypes were grown and dissected in parallel. Dissected pelts were notched in a genotype-specific pattern, and then all pelts were stained and washed in a single tube. Images were collected

using three-dimensional (3D) structured illumination microscopy (SIM) with three grid rotations and five phases on a Zeiss Elyra S.1 inverted microscope with a Plan-Apochromat 63×/1.4 oil immersion lens. All samples were imaged on the same day using the same laser settings and exposure. Raw images were then batch-processed under identical settings using Zen software (Carl Zeiss). The reconstructed images were then analyzed by taking the maximum z-projections, and puncta sizes were quantified automatically by the “analyze particle” feature in ImageJ (<http://fiji.sc/>) ($n =$ hemisegments).

Data availability and statistical analysis

Fly strains and DNA constructs are available upon request. For each experiment, genotypes and sample sizes are described in the legend of the corresponding figure. Because some transgenes were on the X chromosome, we analyzed females only for the sake of consistency. As described above, all experiments were scored blind to genotype. For each experiment a one-way ANOVA was conducted to determine if there was significant variation among genotypes. For experiments that showed a significant difference, individual *post hoc* pairwise comparisons were conducted between genotypes of interest by using either one- or two-tailed *t*-tests (see individual figure legends). For all figures: ns, not significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Exact *P* values are provided within the figure legends. Statistical values are presented as mean value \pm SEM.

Results

Drosophila Syd-1 interacts with all six Rho GTPases, and its predicted GAP domain enhances the GTPase activities of *Rac1* and *Cdc42*, but not *RhoA*

Because the sequence of the RhoGAP-like domain fly *Syd-1* suggested that it might be inactive (Hallam *et al.* 2002; Wentzel *et al.* 2013), we originally hypothesized that fly *Syd-1* might simply bind Rho family GTPases without increasing their GTPase activity. To test this, we used GST pull-down assays to assess the abilities of each of the six fly Rho GTPases—three *Racs*, two *Rhos*, and one *Cdc42*—to interact with fly *Syd-1*. RhoGAPs preferentially bind the GTP-bound form of Rho GTPases, a form that can be transitory. To maximize the chance of observing this binding, we therefore used a mutant form of each fly Rho GTPase that stably mimics the GTP-bound form (Garcia-Mata *et al.* 2006). We expressed and purified each of the six mutant fly Rho:GST fusions from *E. coli* and tested the ability of each one to pull down full-length FLAG-tagged fly *Syd-1* from extracts of adult fly heads expressing FLAG-*Syd-1* under the control of the ubiquitous *actin-Gal4* driver. We found that each of the six fly Rho GTPases is able to pull down the tagged fly *Syd-1* in this assay (Figure 1A), consistent with our hypothesis that fly *Syd-1* can bind Rho GTPases.

We next assayed whether fly *Syd-1* has RhoGAP activity. Because full-length fly *Syd-1* could not be expressed in *E. coli* cells, we GST-tagged and expressed a smaller fragment that

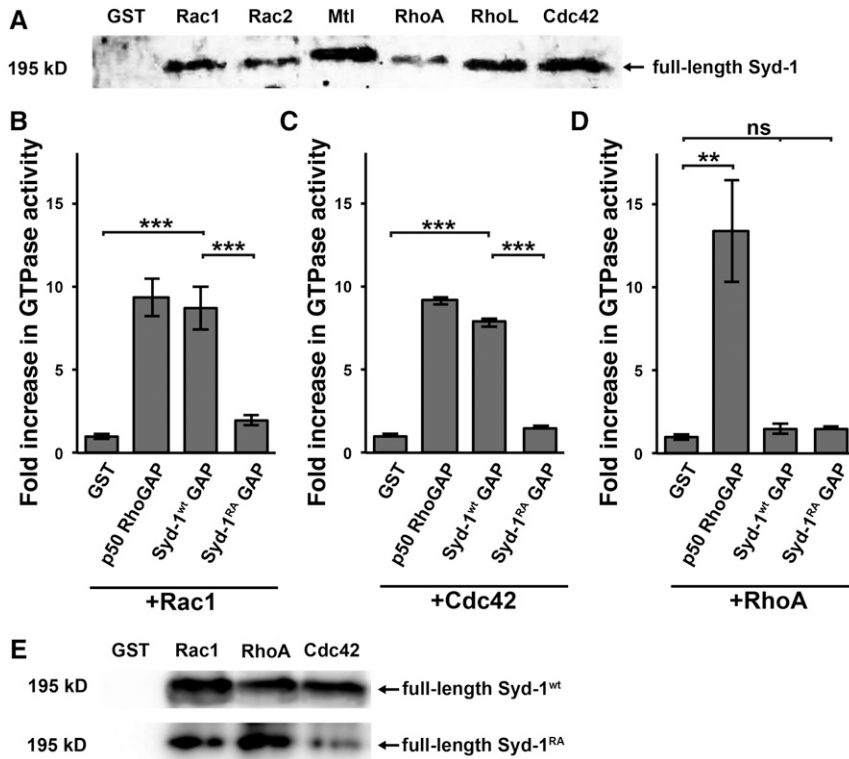


Figure 1 *Drosophila* Syd-1 interacts with all six Rho GTPases. Its predicted GAP domain enhances the GTPase activities of Rac1 and Cdc42 but not RhoA. (A) Western blot (probed with anti-FLAG antibodies) of protein pulled down by purified GST-Rho proteins. FLAG-tagged full-length wild-type fly Syd-1 expressed ubiquitously in adult head is pulled down by GST-tagged constitutively-active mutant forms of fly Rac1, Rac2, Mtl, RhoA, RhoL, or Cdc42, but not by GST alone. (B–D) Endpoint GTPase activity assays of wild-type fly Rac1 (B), Cdc42 (C), and RhoA (D). Each protein was assayed alone and in the presence of the negative control GST, the positive control p50 RhoGAP, the GAP domain of wild-type fly Syd-1 (Syd-1^{wt} GAP), or the GAP domain of fly Syd-1 in which the conserved arginine of the arginine finger is replaced by alanine (Syd-1^{RA} GAP). Activity is presented as fold-increase over the activity of each protein alone. Error bars represent SEM. *n*, Independent assays. As expected, GST does not increase the GTPase activity of Rac1 (1.00 ± 0.16 , $n = 7$), Cdc42 (1.00 ± 0.09 , $n = 3$), or RhoA (1.00 ± 0.22 , $n = 3$), whereas p50 RhoGAP significantly increases the GTPase activity of all three (Rac1: 9.36 ± 1.16 , $n = 7$, $P = 5.9 \times 10^{-6}$; Cdc42: 9.12 ± 0.22 , $n = 3$, $P = 2.2 \times 10^{-6}$; RhoA: 13.32 ± 3.04 , $n = 3$, $P = 0.0077$). We found that the wild-type GAP domain of Syd-1 significantly increases the GTPase activity of Rac1 (8.69 ± 1.29 , $n = 7$, $P = 3.5 \times 10^{-5}$) and Cdc42 (7.83 ± 0.27 ,

$n = 3$, $P = 9.0 \times 10^{-6}$) but not RhoA (1.48 ± 0.30 , $n = 3$, $P = 0.13$). By contrast, the Syd-1^{RA} mutant GAP domain has a severely reduced ability to increase the GTPase activity of Rac1 (1.98 ± 0.31 , $n = 7$, $P = 1.4 \times 10^{-4}$), and Cdc42 (1.41 ± 0.08 , $n = 3$, $P = 1.1 \times 10^{-5}$) and has no effect on RhoA (1.43 ± 0.18 , $n = 3$, $P = 0.10$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, based on one-tailed *t*-tests. (E) Like wild-type fly Syd-1 (Syd-1^{wt}), FLAG-tagged full-length R979A mutant fly Syd-1 (Syd-1^{RA}) expressed ubiquitously in adult head is pulled down by GST-tagged constitutively active mutant forms of Rac1, RhoA, and Cdc42 but not by GST alone.

included the RhoGAP but not the PDZ or C2 domains (“GST-Syd-1^{wt} GAP”). We purified this Syd-1 fragment and assayed its ability to increase the GTPase activities of each of the three major Rho family members in fly: Rac1, RhoA, and Cdc42. We found that the RhoGAP domain of fly Syd-1 significantly increases the GTPase activity of Rac1 and Cdc42 but not that of RhoA (Figure 1, B–D). The degree of increase is similar to that caused by the positive control p50 RhoGAP, which has previously been shown to increase the GTPase activity of all three Rho family members (Zhang *et al.* 1998). We conclude that, despite its noncanonical amino acid sequence, the RhoGAP-like domain of fly Syd-1 does have significant RhoGAP activity toward Rac1 and Cdc42.

Decreasing *syd-1* dosage impairs the ability of wild-type but not constitutively-active Rac1 to increase NMJ bouton number

We next wanted to test whether the GAP activity of fly Syd-1 might be required to regulate Rac or Cdc42 *in vivo*. Like Syd-1, both Rac1 and Cdc42 have previously been shown to act within motorneurons to regulate synaptic bouton number at fly NMJ (Rodal *et al.* 2008; Ball *et al.* 2010; Nahm *et al.* 2010). However, Cdc42 does so by attenuating retrograde BMP signaling, which is not known to be affected by Syd-1 loss. We therefore focused instead on testing whether Syd-1 might regulate Rac1. To do so, we took advantage of a

genetic interaction test that was previously used to implicate the RacGEF Trio as a regulator of Rac1 at NMJ (Ball *et al.* 2010). Overexpression of Rac1 in motorneurons of wild-type animals significantly increases NMJ bouton number (Ball *et al.* 2010). While loss of one copy of *trio* has no effect on NMJ development in otherwise wild-type animals, it significantly impairs the ability of overexpressed Rac1 to increase bouton number (Ball *et al.* 2010). By contrast, Rac1^{V12}, which stably mimics the GTP-bound state of Rac1 (Luo *et al.* 1994), increases NMJ bouton number unimpaired by loss of *trio*. Together, these results suggest that Trio is required to potentiate Rac1, and does so by regulating the nucleotide-bound state of Rac1.

We used the same genetic interaction test to ask whether Syd-1 might also regulate Rac1. We found that overexpression of wild-type Rac1 or the mutant Rac1^{V12} in motorneurons of wild-type animals significantly increases NMJ bouton number, as previously reported (Figure 2, A, D, G, and H). Loss of one copy of *syd-1* from otherwise wild-type animals has no effect on NMJ development in otherwise wild-type animals (Figure 2, A, B, G, and H). However, loss of one copy of *syd-1* does prevent wild-type Rac1 from increasing NMJ bouton number (Figure 2, D, E, G, and H), indicating that, like Trio, Syd-1 is required to potentiate the ability of Rac1 to promote NMJ growth. Furthermore, loss of one copy of *syd-1* does not affect the ability of Rac1^{V12} to increase NMJ bouton number

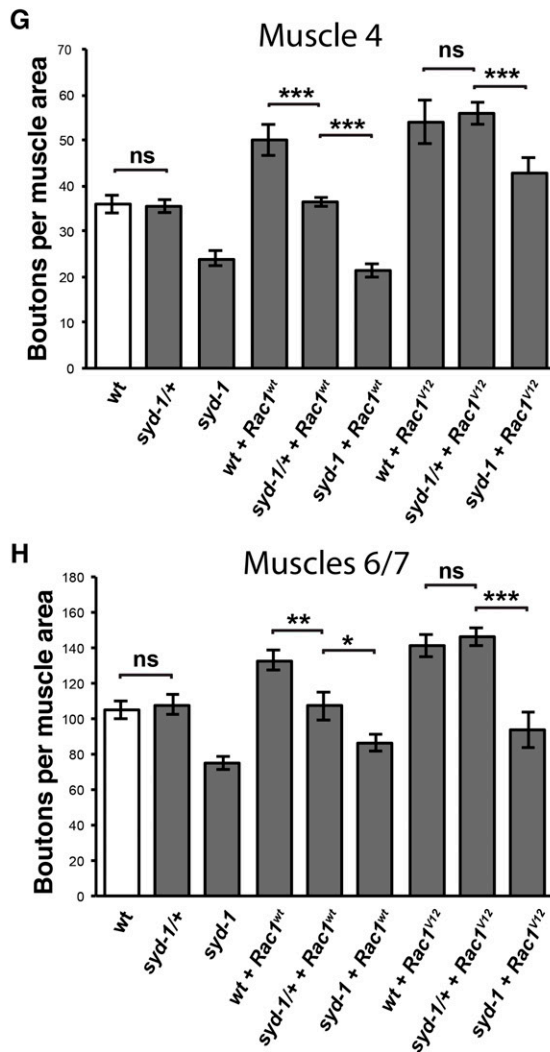
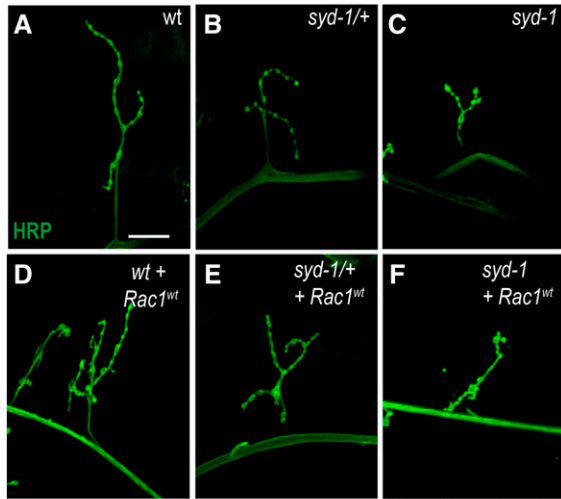


Figure 2 Wild-type Rac1, unlike Rac1^{V12}, requires wild-type Syd-1 levels in order to promote NMJ growth. (A–F) Third-instar larval NMJs at muscle 4 in abdominal segment 3 stained with anti-HRP (green). Bar, 25 μ m. All genotypes also contain the motorneuron driver *BG380-Gal4* and were costained with anti-Dlg. (A) Wild type. (B) *syd-1^{CD/+}* heterozygote. (C) *syd-1^{CD/syd-1^{w46}}* mutant. (D) Wild-type animal in which motorneurons express wild-

(Figure 2, G and H), indicating that, like Trio, Syd-1 is only required to potentiate Rac1 that can change its nucleotide-bound state. We note that Rac1^{wt} and Rac1^{V12} increase synaptic bouton number to similar degrees at muscle 6/7 ($P = 0.38$), suggesting that the differential effect of *syd-1* dosage on the two proteins is unlikely to be caused by a difference in the strengths of their effects. [There may be a slight difference in the strengths of their effects at muscle 4, although it is not statistically significant in the data we have ($P = 0.057$).] These results are consistent with a model in which the GAP activity of Syd-1 directly regulates Rac1 by altering its nucleotide-bound state, but they do not rule out the possibility that the effect of Syd-1 on Rac1 is indirect.

We did note one difference between the effects of *trio* and *syd-1* loss on Rac1. Completely eliminating *trio* has no effect on the ability of Rac1^{V12} to increase NMJ bouton number, indicating that the only role for Trio in this process is to promote the GTP-bound state of Rac1 (Ball *et al.* 2010). By contrast, we found that both Rac1^{wt} and Rac1^{V12} are significantly less able to increase NMJ bouton number in the complete absence of *syd-1* (Figure 2, A, F, G, and H). Since the GAP activity of Syd-1 would not be predicted to affect Rac1^{V12}, this result suggested that Syd-1 might have GAP-independent roles in promoting Rac1-induced NMJ growth, in addition to any GAP-dependent role it might have. We note

type Rac1 (Rac1^{wt}). (E) *syd-1^{CD/+}* heterozygote in which motorneurons express Rac1^{wt}. (F) *syd-1^{CD/syd-1^{w46}}* mutant in which motorneurons express Rac1^{wt}. (G) Quantification of the number of boutons on muscle 4 in abdominal segment 3 per muscle area. Loss of one copy of *syd-1* does not cause a decrease in bouton number (36.3 ± 1.3 , $n = 14$) compared to wild type (*BG380-Gal4* alone: 35.7 ± 1.9 , $n = 12$). By contrast, while overexpressing wild-type Rac1 (49.5 ± 3.4 , $n = 12$) significantly increases bouton number compared to the wild-type control ($P = 0.0011$), loss of one copy of *syd-1* prevents this increase (36.8 ± 1.0 , $n = 17$, $P = 1.9 \times 10^{-8}$ compared to wild-type Rac1 in wild type). By contrast, loss of one copy of *syd-1* has no effect on the increase in bouton number caused by overexpression of a constitutively-active form of Rac1, Rac1^{V12} (Rac1^{V12} alone: 58.5 ± 3.5 , $n = 8$ ($P = 4.8 \times 10^{-5}$ compared to wild type); Rac1^{V12} in *syd-1/+*: 56.0 ± 2.5 , $n = 12$). Complete loss of *syd-1* impairs the ability of wild-type Rac1 (21.4 ± 1.6 , $n = 14$, $P = 1.9 \times 10^{-8}$) or Rac1^{V12} (36.6 ± 4.2 , $n = 11$, $P = 1.4 \times 10^{-4}$) to increase bouton number. Error bars represent SEM. $n =$ animals. ns, not significant; *** $P < 0.001$, based on two-tailed *t*-tests. (H) Quantification of the number of boutons on muscles 6/7 in abdominal segment 3 per muscle area. We observed the same effects on bouton number as at both muscle 4. Wild type (105.4 ± 3.1 , $n = 14$) and *syd-1/+* heterozygotes (108.0 ± 5.7 , $n = 14$) have similar numbers of boutons. Overexpressing either wild-type Rac1 (133.0 ± 5.7 , $n = 14$, $P = 1.3 \times 10^{-4}$) or Rac1^{V12} (141.0 ± 6.5 , $n = 8$, $P = 2.6 \times 10^{-5}$) causes an increase in bouton number. Removing one copy of *syd-1* prevents wild-type Rac1 from increasing bouton number (107.4 ± 8.1 , $n = 14$, $P = 0.0015$ compared to wild-type Rac1 in wild type) but has no effect on Rac1^{V12} (146.0 ± 5.2 , $n = 17$). Complete loss of *syd-1* impairs the ability of wild-type Rac1 (86.8 ± 5.0 , $n = 15$, $P = 1.5 \times 10^{-6}$) or Rac1^{V12} (93.5 ± 7.8 , $n = 14$, $P = 5.3 \times 10^{-4}$) to increase bouton number. Error bars represent SEM. $n =$ animals. ns, not significant; * $P < 0.05$, * $P < 0.01$, *** $P < 0.001$, based on two-tailed *t*-tests.

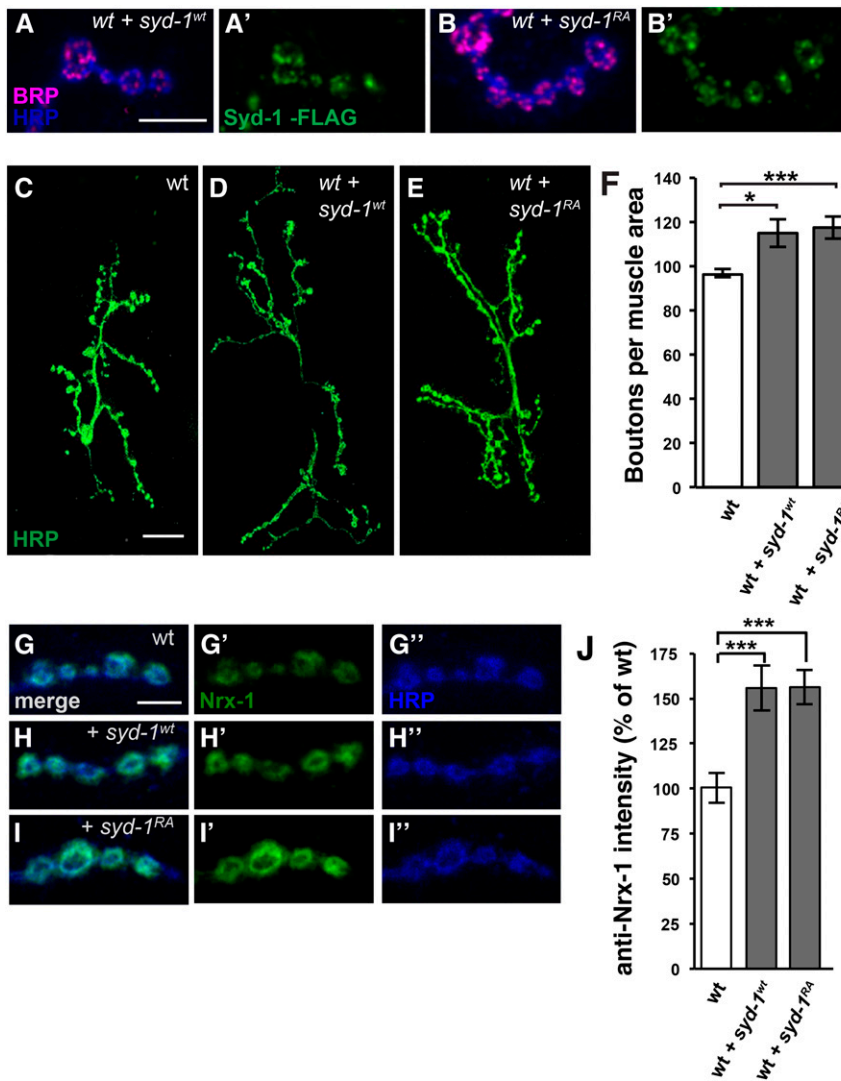


Figure 3 Wild-type and R979A mutant Syd-1 colocalize with Brp and cause similar increases in NMJ bouton number and Nr-x-1 levels when expressed in wild-type motoneurons. (A–B') Third-instar larval NMJ boutons on muscle 4 stained with anti-HRP (blue), anti-Brp (magenta), and anti-FLAG (green). Bar, 5 μ m. (A and A') A wild-type animal in which motoneurons express wild-type full-length FLAG-tagged Syd-1 driven by *BG380-Gal4*. Like endogenous Syd-1, tagged wild-type Syd-1 forms puncta that are adjacent to, and partly overlapping with, the AZ protein Brp. (B and B') A wild-type animal in which motoneurons express R979A mutant full-length FLAG-tagged Syd-1 (Syd-1^{RA}) driven by *BG380-Gal4*. The punctate pattern and levels of Syd-1^{RA} resemble those of Syd-1^{wt}. (C–E) Third-instar larval NMJs at muscles 6/7 in abdominal segment 3 stained with anti-HRP (green). Bar, 25 μ m. All genotypes also contain the motoneuron driver *BG380-Gal4*, and were costained with anti-Dlg. (C) Wild type. (D) Wild type in which motoneurons express wild-type full-length Syd-1. (E) Wild type in which motoneurons express R979A mutant full-length Syd-1. (F) Quantification of the number of boutons on muscles 6/7 in abdominal segment 3 per muscle area. Overexpressing wild-type (114.9 ± 6.4 , $n = 10$) or R979A mutant Syd-1 (117.8 ± 5.3 , $n = 7$) causes similar, significant increases in bouton number ($P = 0.02$ and 5.9×10^{-4} , respectively) compared to the wild-type control (*BG380-Gal4* alone: 96.9 ± 1.8 , $n = 9$). Error bars represent SEM. $n =$ animals. * $P < 0.05$, *** $P < 0.001$, based on two-tailed *t*-tests. (G–I'') Third-instar larval NMJ boutons on muscle 4 stained with anti-HRP (blue) and anti-Nrx-1 (green). Bar, 5 μ m. All genotypes also contain the motoneuron driver *BG380-Gal4*. (G–G'') Wild type. (H–H'') Wild type in which motoneurons express wild-type Syd-1. (I–I'') Wild type in which motoneurons express R979A mutant Syd-1. (J) Quantification of anti-Nrx-1 staining. Overexpressing wild-type ($155 \pm 12.4\%$, $n = 14$) or R979A mutant Syd-1 ($156 \pm 9.4\%$, $n = 13$) significantly increases Nr-x-1

1 levels in NMJ boutons ($P = p = 9.2 \times 10^{-4}$ and 3.1×10^{-4} , respectively) compared to the wild-type control (*BG380-Gal4* alone: $100 \pm 8.4\%$, $n = 15$). Error bars represent SEM. $n =$ animals. ns, not significant; * $P < 0.05$, ** $P < 0.01$, based on one-tailed *t*-tests.

that Rac1^{V12} can increase bouton number somewhat even in the absence of *syd-1* ($P = 2.8 \times 10^{-4}$ at muscle 4; but $P = 0.12$ at muscles 6/7), indicating that Rac1^{V12} can act partly independently of Syd-1. To tease apart Syd-1's potential GAP-dependent and independent functions, we decided to create a mutant version of Syd-1 that specifically lacks GAP activity.

Overexpression of wild-type or R979A mutant Syd-1 in wild-type motoneurons causes similar increases in NMJ bouton number and Nr-x-1 levels

Canonical RhoGAP proteins contain a conserved arginine that is required for their GAP activity (Scheffzek and Ahmadian 2005; Tcherkezian and Lamarche-Vane 2007). To test whether the same might be true of Syd-1, we constructed a GST-tagged Syd-1 RhoGAP domain in which we replaced the codon encoding this conserved arginine (at position 979, see *Materials and Methods*) with a codon encoding alanine (R979A; "GST-Syd-1^{RA} GAP"). We found that, indeed, the

R979A substitution severely decreases the RhoGAP activity of Syd-1's RhoGAP domain (Figure 1, B–D). We next used the transgene encoding full-length wild-type Syd-1 (*UAS-syd-1^{wt}*; Holbrook *et al.* 2012) to create an equivalent transgene encoding R979A mutant Syd-1 (*UAS-syd-1^{RA}*). We tested whether, like other RhoGAP proteins with this R to A substitution, Syd-1^{RA} retains the ability to interact with Rho GTPases despite losing its GAP activity (Graham *et al.* 1999; Scheffzek and Ahmadian 2005). Indeed, we found that FLAG-tagged R979A mutant Syd-1 is pulled-down from adult fly extracts by GST-tagged constitutively-active forms of all three major Rho family members: Rac1, RhoA, and Cdc42 (Figure 1E). We conclude that the R979A substitution specifically disrupts Syd-1's GAP activity, providing us with a tool to analyze the latter's importance for Syd-1 function.

As a first test of the abilities of Syd-1^{wt} and Syd-1^{RA} to promote synapse development, we overexpressed each in motoneurons of wild-type animals. We observed that, like endogenous Syd-1 (Owald *et al.* 2010), both Syd-1^{wt} and

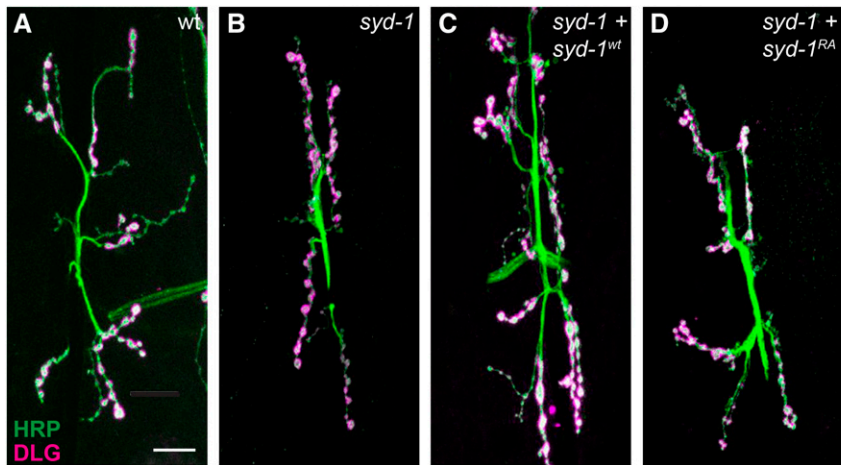
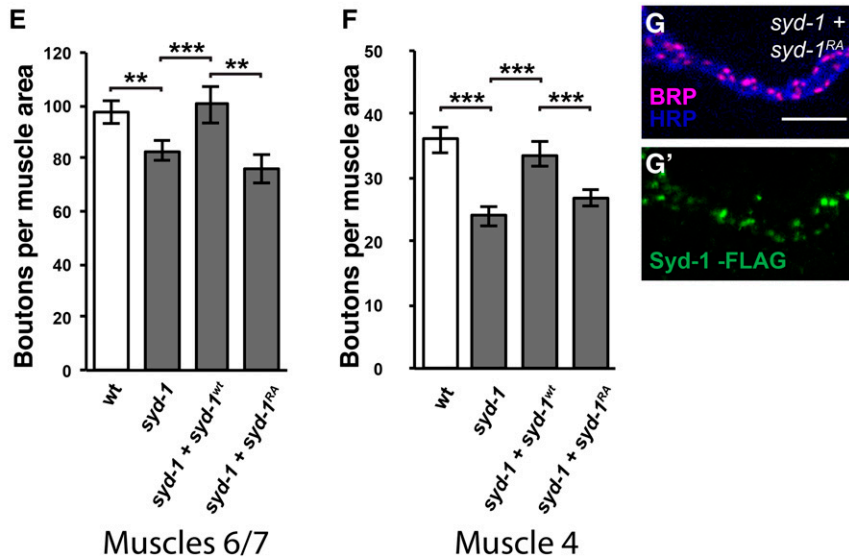


Figure 4 The decrease in NMJ bouton number in *syd-1* mutants is fully rescued by wild-type Syd-1 but unaltered by R979A mutant Syd-1. (A–D) Third-instar larval NMJs at muscles 6/7 in abdominal segment 3 stained with anti-HRP (green) and anti-Dlg (magenta). Bar, 25 μ m. All genotypes also contain the motorneuron driver *BG380-Gal4*. (A) Wild type. (B) *syd-1^{CD}/syd-1^{w46}* mutant. (C) *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express wild-type Syd-1. (D) *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express R979A mutant Syd-1. (E) Quantification of the number of boutons on muscles 6/7 in abdominal segment 3 per muscle area. As previously reported, loss of *syd-1* causes a modest but significant decrease in NMJ bouton number (*BG380-Gal4* alone: 97.6 ± 4.5 , $n = 14$; *syd-1* mutant: 82.6 ± 3.7 , $n = 10$, $P = 3 \times 10^{-3}$). Overexpressing Syd-1^{wt} in the motorneurons of *syd-1* mutants rescues this defect (100.4 ± 7.0 , $n = 10$, $P = 0.0018$). Overexpressing Syd-1^{RA} does not (76.0 ± 5.2 , $n = 16$, $P = 0.0044$ compared to *syd-1* mutant expressing Syd-1^{wt}). Error bars represent SEM. $n =$ animals. ns, not significant; * $P < 0.05$, ** $P < 0.01$, based on one-tailed t -tests. (F) Quantification of the number of boutons on muscle 4 in abdominal segment 3 per muscle area. Again, loss of *syd-1* decreases NMJ bouton number (*BG380-Gal4* alone: 36.0 ± 2.1 , $n = 12$; *syd-1* mutant: 24.0 ± 1.6 , $n = 10$, $P = 5.5 \times 10^{-5}$), and presynaptically expressed Syd-1^{wt} rescues this defect (33.7 ± 1.9 , $n = 12$, $P = 9.9 \times 10^{-5}$), but Syd-1^{RA} does not (26.8 ± 1.2 , $n = 13$, $P = 7.9 \times 10^{-4}$ compared to *syd-1* mutant expressing Syd-1^{wt}). Error bars represent SEM. $n =$ animals. ns, not significant; *** $P < 0.001$, based on two-tailed t -tests. (G and G') Third-instar larval NMJ boutons on muscle 4 of *syd-1^{CD}/syd-1^{w46}* mutant animals in which motorneurons express R979A mutant full-length Syd-1 driven by *BG380-Gal4*, stained with anti-HRP (blue), anti-Brp (magenta), and anti-FLAG (green) as in Figure 3, A–B'. Bar, 5 μ m. As in wild type, tagged R979A mutant Syd-1 expressed in *syd-1* mutant motorneurons forms puncta that are adjacent to and partly overlapping with Brp. Note, however, that the endogenous Brp puncta appear somewhat abnormal (see Figure 6).



and anti-FLAG (green) as in Figure 3, A–B'. Bar, 5 μ m. As in wild type, tagged R979A mutant Syd-1 expressed in *syd-1* mutant motorneurons forms puncta that are adjacent to and partly overlapping with Brp. Note, however, that the endogenous Brp puncta appear somewhat abnormal (see Figure 6).

Syd-1^{RA} localize to presynaptic puncta that are adjacent to and partly overlapping with the AZ protein Brp (Figure 3, A–B'), indicating that Syd-1's GAP activity is not required for its gross subcellular localization. Presynaptic Syd-1 is necessary and sufficient to recruit Nr1 to AZs (Owald *et al.* 2012), and overexpression of Nr1 is sufficient to increase NMJ bouton number (Li *et al.* 2007). We therefore predicted that presynaptic Syd-1^{wt} overexpression would increase both Nr1 levels and NMJ bouton number. Indeed, we found that overexpression of either Syd-1^{wt} or Syd-1^{RA} significantly increases the number of boutons in wild-type animals; the effects of the two proteins are not significantly different (Figure 3, C–F). Using anti-Nrx-1 antibodies to quantify endogenous Nr1 levels, we found that overexpression of either Syd-1^{wt} or Syd-1^{RA} also significantly increases Nr1 levels within NMJ boutons to similar degrees (Figure 3, G–J). We conclude that Syd-1's RhoGAP activity is not required for its ability to increase bouton number and promote Nr1 localization in wild-type animals.

Unlike wild-type Syd-1, R979A mutant Syd-1 does not rescue the decrease in NMJ bouton number in *syd-1* mutants, despite restoring Nr1 levels

Loss of *syd-1* causes a decrease in NMJ bouton number and a loss of Nr1 staining. We next wanted to compare the abilities of Syd-1^{wt} and Syd-1^{RA} to rescue these defects in *syd-1* mutant animals. We expressed the two proteins in motorneurons of *syd-1^{w46}/syd-1^{CD}* transheterozygotes and found that Syd-1^{wt} fully rescues the decrease in NMJ bouton number (Figure 4, A–C, E, and F) but that Syd-1^{RA} has no effect (Figure 4, A–F). We conclude that Syd-1^{RA}'s ability to increase NMJ bouton number in wild-type animals requires the presence of endogenous wild-type Syd-1 and that Syd-1's RhoGAP activity is required for normal NMJ growth. We confirmed that, as in wild-type animals, both Syd-1^{wt} and Syd-1^{RA} form Brp-associated puncta in *syd-1* mutant animals (compare Figure 4, G and G' with Figure 3, A–B'), indicating that Syd-1^{RA}'s inability to rescue *syd-1* loss is not caused by a

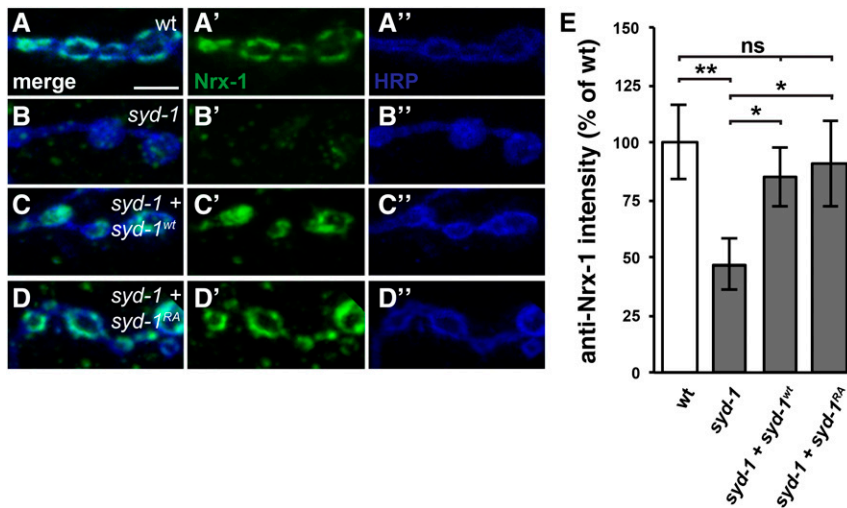


Figure 5 Both wild-type and R979A mutant Syd-1 fully restore NMJ Nrx-1 levels in *syd-1* mutants. (A–D'') Third-instar larval NMJ boutons on muscle 4 stained with anti-HRP (blue) and anti-Nrx-1 (green). Bar, 5 μ m. All genotypes also contain the motorneuron driver *BG380-Gal4*. (A–A'') Wild type. (B–B'') *syd-1^{CD}/syd-1^{w46}* mutant. (C–C'') *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express wild-type Syd-1. (D–D'') *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express R979A mutant Syd-1. (E) Quantification of anti-Nrx-1 staining. As previously reported, loss of *syd-1* decreases Nrx-1 levels in presynaptic boutons (*BG380-Gal4* alone: 100 \pm 16%, n = 13; *syd-1* mutant: 47 \pm 11%, n = 12, P = 0.0043). Overexpressing either wild-type or R979A mutant Syd-1 in the motorneurons of *syd-1* mutants rescues this defect (85 \pm 13% (n = 12, P = 0.013) and 91 \pm 19% (n = 9, P = 0.021), respectively). Error bars represent SEM. n = animals. ns, not significant; * P < 0.05, ** P < 0.01, based on one-tailed t -tests.

defect in its gross subcellular localization. This is the first evidence in any organism that Syd-1's GAP activity is required for presynaptic development.

We next wanted to pinpoint which of Syd-1's molecular functions depends upon its GAP activity. *syd-1* loss significantly decreases presynaptic Nrx-1 levels (Owald *et al.* 2012), and loss of *nrx-1* reduces NMJ bouton number to the same degree as loss of *syd-1* (Li *et al.* 2007; Oswald *et al.* 2012). We therefore first tested whether the inability of Syd-1^{RA} to rescue NMJ bouton number might be caused by an inability to recruit Nrx-1 in the absence of endogenous wild-type Syd-1. We again expressed Syd-1^{wt} or Syd-1^{RA} in motorneurons of *syd-1* mutants and quantified anti-Nrx-1 staining within NMJ boutons. We found that, like Syd-1^{wt}, Syd-1^{RA} is sufficient to fully restore Nrx-1 to wild-type levels in *syd-1* mutant boutons (Figure 5). We conclude that Syd-1's RhoGAP activity is not required for its ability to localize Nrx-1.

Syd-1's RhoGAP activity is required for normal Brp clustering, as is the RacGEF Trio

In addition to binding and localizing Nrx-1, Syd-1 also directly binds and clusters Brp, a major structural component of AZs that is required for normal NMJ size (Kittel *et al.* 2006; Oswald *et al.* 2010). Loss of Syd-1 results in disorganized Brp puncta that are enlarged and irregularly spaced (Owald *et al.* 2010). We therefore next compared the abilities of Syd-1^{wt} and Syd-1^{RA} to promote Brp clustering. We found that expressing Syd-1^{wt} in motorneurons fully restores the size of Brp puncta in *syd-1* mutants (Figure 6, A–C' and F), whereas expressing Syd-1^{RA} has no effect on the Brp defect (Figure 6, D, D', and F). We conclude that Syd-1's RhoGAP activity is required for normal Brp clustering.

The obvious implication of this result is that Syd-1 regulates Brp, at least in part, by regulating the GTPase activity of one or more Rho proteins. Having already found biochemical and genetic evidence that Syd-1 regulates Rac1, we wanted to test whether Brp localization is disrupted by Rac1 loss. However, Rac1 is partly redundant with the other two fly *Rac* genes, and

triple *Rac* mutant flies die early (Hakeda-Suzuki *et al.* 2002). We therefore chose instead to analyze Brp localization in animals lacking the Rac1 GEF Trio. Rac1 has been shown to be unable to promote NMJ bouton development in *trio^{S6A}/trio^{S137203}* null mutants, which nonetheless survive until the end of larval development (Ball *et al.* 2010). We analyzed Brp puncta within the NMJ boutons of *trio^{S6A}/trio^{S137203}* mutants and found them to be abnormally enlarged, like Brp puncta in *syd-1* mutants (Figure 6, E and F). We conclude that Trio, like Syd-1, is required for proper Brp clustering, consistent with a model in which their coregulation of Rac1 and/or other Rho GTPases is required for this process.

Syd-1 is required for Rac1^{V12} to increase Nrx-1 levels

Finally, we wanted to follow up on our finding that complete loss of *syd-1* prevents Rac1^{V12} from increasing NMJ bouton number. This result suggested that Syd-1 has one or more activities that are required for Rac1-induced NMJ growth but separate from Syd-1's effect on the nucleotide-bound state of Rac1. An obvious candidate for such an activity is Syd-1's recruitment of Nrx-1, since this occurs independently of Syd-1 GAP activity. If so, we would expect that: (1) Rac1^{V12} overexpression promotes NMJ growth, at least in part, by increasing Nrx-1 levels; and (2) eliminating Syd-1 prevents Rac1^{V12} from causing this Nrx-1 increase. We tested these two predictions and, indeed, found that expressing Rac1^{V12} in the motorneurons of wild-type animals causes a significant increase in Nrx-1 levels at NMJ (Figure 7A) and that complete loss of Syd-1 prevents this increase (Figure 7A). We conclude that Syd-1 participates with Rac1 in two separate functions that promote NMJ bouton development: (1) together with Trio, the RacGAP activity of Syd-1 is required to regulate the nucleotide-bound state of Rac1, which is required for normal Brp clustering; and (2) Syd-1, independent of its RacGAP activity, is required for the recruitment of Nrx-1 to boutons, including the recruitment of Nrx-1 that is promoted by Rac1 (Figure 7B).

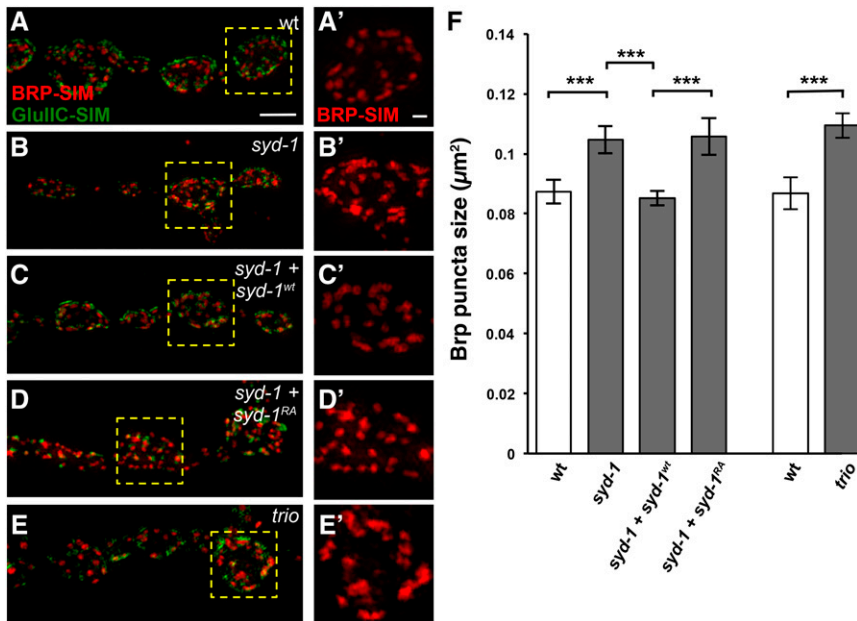


Figure 6 The defect in Brp clustering in *syd-1* mutants is fully rescued by wild-type Syd-1, unaltered by R979A mutant Syd-1, and phenocopied by loss of the RacGEF Trio. (A–E') SIM images of third-instar larval NMJ boutons on muscle 4 stained with anti-Brp (red) and anti-GluRIIC (green). Bar, 1 μm. Genotypes in (A–D') also contain the motorneuron driver *BG380-Gal4*. (A and A') Wild type. (B and B') *syd-1^{CD}/syd-1^{w46}* mutant. (C and C') *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express wild-type Syd-1. (D and D') *syd-1^{CD}/syd-1^{w46}* mutant in which motorneurons express R979A mutant Syd-1. (E and E') *trio^{6A}/trio^{S137203}* mutant. (F) Quantification of the average size of anti-Brp puncta. As previously reported (Owald *et al.* 2010), loss of *syd-1* increases the average size of anti-Brp puncta ($0.105 \pm 0.004 \mu\text{m}^2$, $n = 17$) compared to wild type ($0.087 \pm 0.004 \mu\text{m}^2$, $n = 21$, $P = 3.0 \times 10^{-4}$). Overexpressing Syd-1^{wt} in the motorneurons of *syd-1* mutants rescues this defect ($0.085 \pm 0.002 \mu\text{m}^2$, $n = 14$, $P = 6.3 \times 10^{-4}$). By contrast, overexpressing Syd-1^{RA} in the motorneurons of *syd-1* mutants does not ($0.106 \pm 0.006 \mu\text{m}^2$, $n = 12$, $P = 9.7 \times 10^{-4}$ compared to *syd-1* mutant expressing Syd-1^{wt}). In a separate experiment, we

found that loss of Trio increases the average size of anti-Brp puncta ($0.109 \pm 0.004 \mu\text{m}^2$, $n = 14$, $P = 1.2 \times 10^{-4}$) to a degree similar to that caused by loss of Syd-1. Error bars represent SEM. $n =$ hemisegments. ns, not significant; ** $P < 0.01$, *** $P < 0.001$, based on one-tailed *t*-tests.

Discussion

The role of Syd-1's GAP activity in clustering Brp

In this paper, we have shown that Syd-1^{wt} and Syd-1^{RA} promote NMJ growth to similar degrees in wild-type animals and recruit similar levels of Nr x -1 to presynaptic boutons in both wild type and *syd-1* mutants. However, unlike Syd-1^{wt}, Syd-1^{RA} is unable to restore Brp clustering in *syd-1* mutants, indicating that Syd-1's GAP activity is required for this process. Our results further suggest that Syd-1's GAP activity promotes Brp clustering by working together with the RacGEF Trio to potentiate Rac1. However, while loss of a GEF for Rac1 would be predicted to increase the proportion of GDP-bound, and therefore inactive, Rac1, we note that loss of a GAP for Rac1 should instead increase the proportion of GTP-bound, and therefore active, Rac1 (Scheffzek and Ahmadian 2005; Tcherkezian and Lamarche-Vane 2007). Why, instead, might loss of Syd-1 GAP activity impair Rac1 function at NMJ?

Given our finding that Syd-1's GAP domain also increases the GTPase activity of Cdc42, we considered the possibility that Syd-1 might potentiate Rac1 only indirectly, by acting upon Cdc42. The phenotype caused by Cdc42 loss from motorneurons—an increase in NMJ bouton number—is the same as that caused by Rac1 gain, suggesting that Cdc42 and Rac1 might antagonize one another during NMJ development. Syd-1 loss might then impair Rac1 by increasing the proportion of GTP-bound, active Cdc42. However, we do not think this model is likely for three reasons. First, presynaptic loss of *cdc42* significantly enhances the formation of abnormally positioned “satellite” boutons, a hallmark of increased BMP signaling (Nahm *et al.* 2010), and we do not observe an increase in satellite boutons in animals overexpressing Syd-1^{wt} (or in *syd-1* mutants; Figure S2), suggesting that Syd-1

does not normally regulate Cdc42 at NMJ. Second, we directly tested whether coexpressing Cdc42 with Rac1 would impair the latter's ability to increase NMJ bouton number and found that it does not (Figure S2). Third, a model in which decreasing *syd-1* dosage potentiates a Rho GTPase with antagonistic effects on Rac1 does not explain the specific sensitivity of Rac1^{wt} and not Rac1^{V12} to this manipulation. Finally, we note that Syd-1 could also theoretically potentiate Rac1 indirectly by acting upon one of the other two fly Racs, if either of the latter were antagonistic to Rac1. However, there is no evidence of such antagonism: reducing the levels of one, two, or all three fly Racs has previously been shown either to have no effect or to decrease NMJ bouton number (Ball *et al.* 2010), and our third point (above) applies to this model too.

Our data are therefore more consistent with Syd-1 directly regulating the nucleotide-bound state of Rac1. In previous cases in which loss of the RhoGAP or RhoGEF for a single Rho GTPase causes the same defect, the explanation has been that the ability of the GTPase to cycle between GDP- and GTP-bound states is critical (Symons and Settleman 2000; Parrini and Camonis 2011; Sharif and Maddox 2012; Fritz and Pertz 2016); loss of the regulatory RhoGAP or RhoGEF then causes the same defect because either manipulation reduces the rate of Rho cycling. While it is theoretically possible that, instead, both GDP-bound and GTP-bound forms of Rac1 are separately required to promote Brp clustering at NMJ, there is no precedent for such a model: GDP-bound Rac1 is thought to be inactive (Symons and Settleman 2000; Parrini and Camonis 2011; Sharif and Maddox 2012; Fritz and Pertz 2016). We therefore favor a model in which Rac1 cycling is important for Brp clustering at NMJ.

How might Rac1 cycling affect Brp recruitment? Brp has previously been shown to directly bind *in vitro* to N- and

C-terminal fragments of Syd-1 that lack the RhoGAP domain (Owald *et al.* 2010). One possibility is that the ability of Syd-1 to bind Brp *in vivo* is somehow regulated intramolecularly by the process of transforming a GTP-bound Rho GTPase into a GDP-bound form (simply binding the GTP-bound form would not be sufficient, since R979A Syd-1 binds all six Rhos yet cannot cluster Brp). Alternatively, Syd-1's RhoGAP activity may have an indirect effect on Brp by, in parallel, promoting a Rho GTPase-dependent change in presynaptic structure that facilitates the ability of Syd-1 to cluster Brp properly. Rho GTPases are classically involved in regulating actin assembly (Stankiewicz and Linseman 2014), and presynaptic development is characterized by the early appearance of actin-rich structures to which other molecules, including Syd-1, are recruited (Chia *et al.* 2012, 2013; Nelson *et al.* 2013). Perhaps Rac1, regulated by Syd-1 and Trio, sculpts the local actin environment at presynaptic sites, creating a permissive environment for Syd-1 to recruit additional presynaptic components, including Brp.

The roles of Rac1 and Syd-1 in recruiting Nrxx-1

In contrast to our evidence that Rac1 cycling may be important for Brp clustering, we found that Rac1^{V12}, which stably mimics the GTP-bound state, increases Nrxx-1 levels in wild type, and that Syd-1 lacking GAP activity is sufficient to increase Nrxx-1 levels, even in the absence of endogenous Syd-1. These results suggest that Rac1 does not need to enter the GDP-bound state in order to promote Nrxx-1 recruitment and that Rac1 cycling is therefore not required for this process. Nonetheless, complete loss of *syd-1* prevents Nrxx-1 recruitment, even by Rac1^{V12}. Together, these results indicate that Syd-1 is required downstream of or in parallel to GTP-bound Rac1 to recruit Nrxx-1 to boutons. Syd-1 and Nrxx-1 have previously been shown to bind via an interaction between the former's PDZ domain and the latter's PDZ-binding domain; each protein depends on the other for its localization (Owald *et al.* 2012). One possibility is that Syd-1 localization or its ability to recruit Nrxx-1 is potentiated by direct binding between Syd-1 and GTP-bound Rac1, an interaction of which Syd-1^{RA} remains capable.

Fly Syd-1 compared with worm Syd-1 and mouse mSYD1A

We have shown that fly Syd-1 requires its GAP activity to cluster the ELKS protein Brp. However, worm Syd-1 apparently lacks GAP activity and yet is able to cluster ELKS (Xu *et al.* 2015). How might this work? Both worm and fly Syd-1 also recruit the scaffolding protein Liprin-alpha (Syd-2 in worm), which binds ELKS. Perhaps in worm this parallel mechanism of ELKS recruitment is sufficient for normal ELKS localization, alleviating the need for Rac cycling. Alternatively, some other RacGAP in worm might promote Rac cycling during synaptogenesis, creating a permissive environment for the GAP-dead worm Syd-1 to recruit ELKS.

By contrast, mouse mSYD1A does have GAP activity toward RhoA, but this activity is not required for its ability to rescue the presynaptic defects caused by mSYD1A loss (in cell culture at least; Wentzel *et al.* 2013). An obvious possibility is that

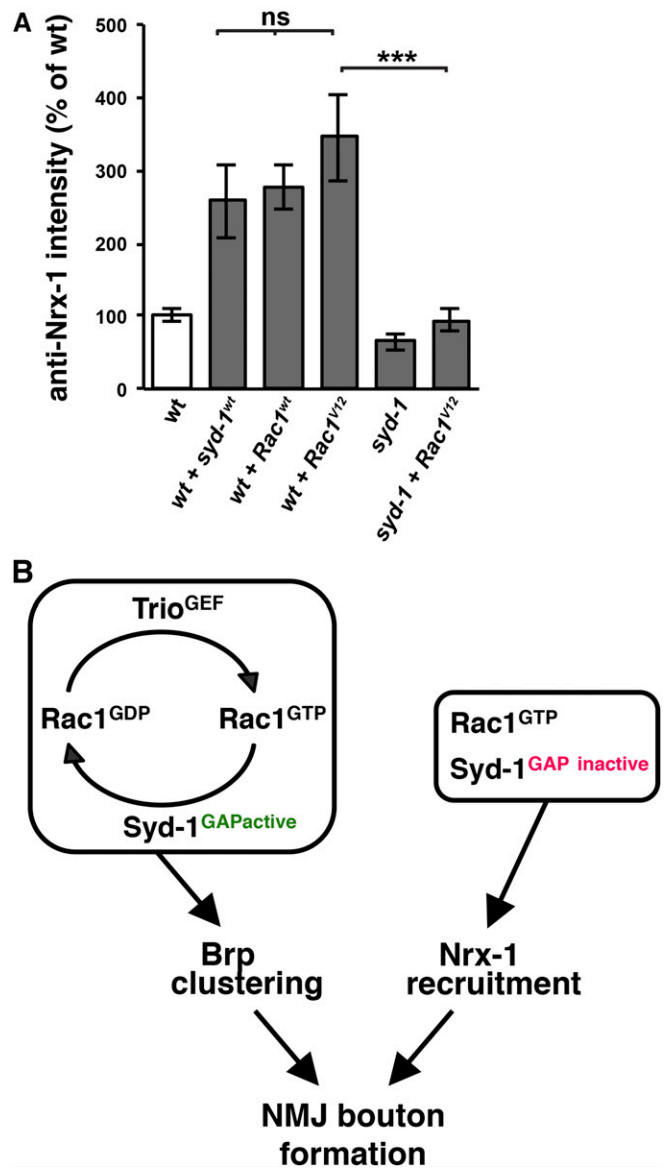


Figure 7 Rac1^{V12} requires Syd-1 in order to increase Nrxx-1 levels at NMJ. (A) Quantification of anti-Nrxx-1 staining. Overexpressing either Rac1^{wt} or Rac1^{V12} in the motoneurons of wild-type animals increases Nrxx-1 levels in presynaptic boutons [to 278 ± 31%, $n = 14$, $P = 4.3 \times 10^{-6}$, or 345 ± 60%, $n = 16$, $P = 4.7 \times 10^{-4}$, of wild-type levels, respectively (*BG380-Gal4* alone: 100 ± 9%, $n = 15$)]. Loss of *syd-1* significantly impairs the ability of Rac1^{V12} to increase Nrxx-1 levels (94 ± 16%, $n = 8$, $P = 1.2 \times 10^{-4}$ compared to Rac1^{V12} in wild type). Error bars represent SEM. $n =$ animals. ns, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, based on one-tailed *t*-tests. (B) Model. Previous work has shown that Syd-1 is required presynaptically for the recruitment of Nrxx-1 and clustering of Brp at NMJ, and that each of these processes is required for the formation of a wild-type number of NMJ boutons. In this paper, we provide evidence that Syd-1's GAP activity is required together with the RacGEF Trio to promote proper Brp puncta size by regulating the nucleotide-bound state of Rac1. The similarity between the Brp defects caused by *syd-1* or *trio* loss suggests that Rac1 cycling between GTP- and GDP-bound states is required for normal Brp clustering (see Discussion). By contrast, we find that Syd-1 does not require RacGAP activity to recruit Nrxx-1 to boutons and that GTP-bound Rac1 is sufficient for this process but that Rac1 cannot increase Nrxx-1 levels in the absence of Syd-1. See Discussion for more details.

the molecular mechanisms that promote presynaptic assembly differ substantially between vertebrates and invertebrates. However, we note that mice have a second Syd-1 homolog, mSYD1B, which has not yet been analyzed but which may have assumed some of the functions that depend on the single Syd-1 in invertebrates (Wentzel *et al.* 2013). Consistent with this possibility, the presynaptic defects caused by mSYD1A loss are far milder than those of the invertebrate *syd-1* mutants (Wentzel *et al.* 2013). Because fly Syd-1^{RA} can promote increased synaptogenesis in the presence of wild-type endogenous Syd-1, one possibility is that mouse mSYD1A lacking the RhoGAP domain can promote SV clustering in *mSYD1A*^{KO} knockout cells because wild-type mSYD1B is present. It will be interesting to examine the effects of deleting both mouse Syd-1 proteins and to test the functionality of mutant versions of those proteins in the double mutant animals. Fly Syd-1 was previously thought to lack RhoGAP activity in part because mouse mSYD1A GAP activity toward RhoA is eliminated by changing a single one of its residues into the corresponding fly residue (Wentzel *et al.* 2013). Our results confirm that fly Syd-1's RhoGAP domain does not have GAP activity toward RhoA, but instead we found that it can regulate Rac1 and Cdc42. It will be interesting to see whether the GAP domain of mouse mSYD1B might share fly Syd-1's specificity.

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Literature Cited

- Ball, R. W., M. Warren-Paquin, K. Tsurudome, E. H. Liao, F. Elazzouzi *et al.*, 2010 Retrograde BMP signaling controls synaptic growth at the NMJ by regulating Trio expression in motor neurons. *Neuron* 66: 536–549.
- Budnik, V., Y. H. Koh, B. Guan, B. Hartmann, C. Hough *et al.*, 1996 Regulation of synapse structure and function by the *Drosophila* tumor suppressor gene Dlg. *Neuron* 17: 627–640.
- Chia, P. H., M. R. Patel, and K. Shen, 2012 NAB-1 instructs synapse assembly by linking adhesion molecules and F-actin to active zone proteins. *Nat. Neurosci.* 15: 234–242.
- Chia, P. H., P. Li, and K. Shen, 2013 Cellular and molecular mechanisms underlying presynapse formation. *J. Cell Biol.* 203: 11–22.
- Dai, Y., H. Taru, S. L. Deken, B. Grill, B. Ackley *et al.*, 2006 SYD-2 Liprin-alpha organizes presynaptic active zone formation through ELKS. *Nat. Neurosci.* 9: 1479–1487.
- Emery, P., 2007 Protein extraction from *Drosophila* heads. *Methods Mol. Biol.* 362: 375–377.
- Fritz, R. D., and O. Pertz, 2016 The dynamics of spatio-temporal Rho GTPase signaling: formation of signaling patterns. *F1000Res* 5: F1000 Faculty Rev–749.
- Garcia-Mata, R., K. Wennerberg, W. T. Arthur, N. K. Noren, S. M. Ellerbroek *et al.*, 2006 Analysis of activated GAPs and GEFs in cell lysates. *Methods Enzymol.* 406: 425–437.
- Graham, D. L., J. F. Eccleston, and P. N. Lowe, 1999 The conserved arginine in Rho-GTPase-activating protein is essential for efficient catalysis but not for complex formation with Rho.GDP and aluminum fluoride. *Biochemistry* 38: 985–991.
- Hakeda-Suzuki, S., J. Ng, J. Tzu, G. Dietzl, Y. Sun *et al.*, 2002 Rac function and regulation during *Drosophila* development. *Nature* 416: 438–442.
- Hallam, S. J., A. Goncharov, J. McEwen, R. Baran, and Y. Jin, 2002 SYD-1, a presynaptic protein with PDZ, C2, and RhoGAP-like domains, specifies axon identity in *C. elegans*. *Nat. Neurosci.* 5: 1137–1146.
- Holbrook, S., J. K. Finley, E. L. Lyons, and T. G. Herman, 2012 Loss of *syd-1* from R7 neurons disrupts two distinct phases of presynaptic development. *J. Neurosci.* 32: 18101–18111.
- Kittel, R. J., C. Wichmann, T. M. Rasse, W. Fouquet, M. Schmidt *et al.*, 2006 Bruchpilot promotes active zone assembly, Ca²⁺ channel clustering, and vesicle release. *Science* 312: 1051–1054.
- Li, J., J. Ashley, V. Budnik, and M. A. Bhat, 2007 Crucial role of *Drosophila* *neurexin* in proper active zone apposition to postsynaptic densities, synaptic growth, and synaptic transmission. *Neuron* 55: 741–755.
- Luo, L., Y. J. Liao, L. Y. Jan, and Y. N. Jan, 1994 Distinct morphogenetic functions of similar small GTPases: *Drosophila* Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* 8: 1787–1802.
- Marrus, S. B., S. L. Portman, M. J. Allen, K. G. Moffat, and A. DiAntonio, 2004 Differential localization of glutamate receptor subunits at the *Drosophila* neuromuscular junction. *J. Neurosci.* 24: 1406–1415.
- Melom, J. E., and J. T. Littleton, 2011 Synapse development in health and disease. *Curr. Opin. Genet. Dev.* 21: 256–261.
- Nahm, M., S. Kim, S. K. Paik, M. Lee, S. Lee *et al.*, 2010 dCIP4 (*Drosophila* Cdc42-interacting protein 4) restrains synaptic growth by inhibiting the secretion of the retrograde Glass bottom boat signal. *J. Neurosci.* 30: 8138–8150.
- Nelson, J. N., A. K. Stavoe, and D. A. Colon-Ramos, 2013 The actin cytoskeleton in presynaptic assembly. *Cell Adhes. Migr.* 7: 379–387.
- Owald, D., W. Fouquet, M. Schmidt, C. Wichmann, S. Mertel *et al.*, 2010 A Syd-1 homologue regulates pre- and postsynaptic maturation in *Drosophila*. *J. Cell Biol.* 188: 563–579.
- Owald, D., O. Khorramshahi, V. K. Gupta, D. Banovic, H. Depner *et al.*, 2012 Cooperation of Syd-1 with Neurexin synchronizes pre- with postsynaptic assembly. *Nat. Neurosci.* 15: 1219–1226.
- Parrini, M. C., and J. Camonis, 2011 Cell motility: the necessity of Rac1 GDP/GTP flux. *Commun. Integr. Biol.* 4: 772–774.
- Patel, M. R., E. K. Lehrman, V. Y. Poon, J. G. Crump, M. Zhen *et al.*, 2006 Hierarchical assembly of presynaptic components in defined *C. elegans* synapses. *Nat. Neurosci.* 9: 1488–1498.
- Rodal, A. A., R. N. Motola-Barnes, and J. T. Littleton, 2008 Nervous wreck and Cdc42 cooperate to regulate endocytic actin assembly during synaptic growth. *J. Neurosci.* 28: 8316–8325.
- Rossmann, K. L., C. J. Der, and J. Sondek, 2005 GEF means go: turning on Rho GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* 6: 167–180.

- Scheffzek, K., and M. R. Ahmadian, 2005 GTPase activating proteins: structural and functional insights 18 years after discovery. *Cell. Mol. Life Sci.* 62: 3014–3038.
- Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair *et al.*, 2012 Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9: 676–682.
- Schuster, C. M., G. W. Davis, R. D. Fetter, and C. S. Goodman, 1996 Genetic dissection of structural and functional components of synaptic plasticity. I. Fasciclin II controls synaptic stabilization and growth. *Neuron* 17: 641–654.
- Sharif, B., and A. S. Maddox, 2012 Wound healing: GTPases flux their muscles. *Dev. Cell* 23: 236–238.
- Stankiewicz, T. R., and D. A. Linseman, 2014 Rho family GTPases: key players in neuronal development, neuronal survival, and neurodegeneration. *Front. Cell. Neurosci.* 8: 314.
- Symons, M., and J. Settleman, 2000 Rho family GTPases: more than simple switches. *Trends Cell Biol.* 10: 415–419.
- Tcherkezian, J., and N. Lamarche-Vane, 2007 Current knowledge of the large RhoGAP family of proteins. *Biol. Cell* 99: 67–86.
- Tolias, K. F., J. G. Duman, and K. Um, 2011 Control of synapse development and plasticity by Rho GTPase regulatory proteins. *Prog. Neurobiol.* 94: 133–148.
- Wentzel, C., J. E. Sommer, R. Nair, A. Stiefvater, J. B. Sibarita *et al.*, 2013 mSYD1A, a mammalian synapse-defective-1 protein, regulates synaptogenic signaling and vesicle docking. *Neuron* 78: 1012–1023.
- Xu, Y., H. Taru, Y. Jin, and C. C. Quinn, 2015 SYD-1C, UNC-40 (DCC) and SAX-3 (Robo) function interdependently to promote axon guidance by regulating the MIG-2 GTPase. *PLoS Genet.* 11: e1005185.
- Zhang, B., J. Chernoff, and Y. Zheng, 1998 Interaction of Rac1 with GTPase-activating proteins and putative effectors: a comparison with Cdc42 and RhoA. *J. Biol. Chem.* 273: 8776–8782.

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