Supporting Online Material for

RSY-1 Is a Local Inhibitor of Presynaptic Assembly in *C. elegans*

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Materials & Methods

Strains. Wildtype animals were of Bristol variety *N2* strain. Strains were maintained at 20°C on NGM agar plates with OP50 *E. coli*(*1*).

Mutants. LGI, *rsy-1(wy94)*, *rsy-1(wy95)*; LGII, *syd-1(ju82), unc-104(e1265)*; LGIV, *elks-1(tm1233)*; LGX, *syd-2(ju37)*.

Transgenic lines. *kyIs235* [*Punc-86::snb-1::yfp; Punc-4::lin-10::dsred; Podr-1::dsred*], *wyIs12* [*Punc-86::gfp::syd-2; Podr-1::gfp*], *wyEx146* [*Punc-86::git-1::yfp; Podr-1::dsred*], *wyEx196* [*Punc-86::elks-1::yfp; Podr-1::dsred*], *wyEx431* [*Punc-86::mcherry::rab-3; Podr-1::gfp*], *wyEx1583* [*Punc-86::rsy-1::cfp; Podr-1::gfp*], *wyEx1603* [*Punc-86::gfp::rsy-1; Podr-1::dsred*], *wyEx1616* [*Punc-86::rsy-1(1-502aa); Podr-1::gfp*], *wyEx1682* [*Punc-86::gfp::rsy-1(1-502aa); Punc-86::mcherry::rab-3; Podr-1::gfp*], *wyEx1686* [*Prsy-1::rsy-1::sl2::gfp; Podr-1::dsred*], *wyEx2498* [*Punc-86::mCherry::syd-2; Punc-86::gfp::rsy-1(1-502aa); Podr-1::gfp*], *wyEx2534* [*Punc-86::mCherry::rsy-1(1-502aa); Podr-1::dsred*], *wyEx2539* [*Punc-86::elks-1::yfp; Punc-86::mcherry::rsy-1(1-502aa); Podr-1::gfp*], *wyEx2541* [*Punc-86::gfp::rsy-1; Punc-86::mcherry; Podr-1::gfp*], *wyEx2543* [*Punc-86::gfp::rsy-1(1-502aa); Punc-86::mcherry; Podr-1::gfp*], *wyEx2566* [*Punc-86::git-1::yfp; Punc-86::mcherry::rsy-1(1-502aa); Podr-1::gfp*], *wyEx2590* [*Prab-3::gfp::rsy-1(1-502aa); Prab-3::3Xflag::syd-2; Podr-1::dsred*], *wyEx2613* [*Prab-3::gfp::rsy-1(1-502aa); Prab-3::3Xflag::syd-1; Podr-1::dsred*], *wyEx2649* [*Prab-3::3Xflag::syd-1; Podr-1::dsred*], *wyEx2656* [*Prab-3::3Xflag::syd-2; Podr-1::dsred*].

Molecular biology. Plasmids to make transgenic animals were made using pSM (with fluorophore) or ΔpSM (without fluorophore), derivatives of pPD49.26 (A. Fire) with extra cloning sites. For expression in the HSNL neuron, *unc-86* promoter was cloned between Sph I and Xma I. For pan-neuronal expression, *rab-3* promoter was utilized. Plasmids containing the *unc-86* promoter were injected at 0.5 – 1 ng/µl concentration and plasmids with the *rab-3* promoter were used at 10 ng/µl. Plasmids for expression in the Hek293 cells were made using pFLAG-CMV-2 or pEYFP-C3 (for prey), and pcDNA3.1/*myc*-His(-)A or mCerulean-C1 (for bait). The following plasmids were utilized:

pMP70 – *rsy-1* cDNA, isoform A (*C. elegans* ORFeome library, Open Biosystems) cloned into pSM between Nhe I and Kpn I using recombination (Gateway technology, Invitrogen) and in frame with *cfp* at the C-terminus. Used to make *wyEx1583*.

pMP68 – *rsy-1* cDNA, isoform A (*C. elegans* ORFeome library, Open Biosystems) cloned in frame with N-terminal *gfp* into pSM between Nhe I and Kpn I using recombination (Gateway technology, Invitrogen). Used to make *wyEx1603*.

pMP72 – *rsy-1(1-502aa)* cDNA, isoform A cloned into ΔpSM between Nhe I and Kpn I with primers:
5’ GAAAGGGCTAGCATGGAAGATTGGACCAACTATCATCAG
3’ GAAAGGGGTACCGAATCTGGAACGTGTAAATCATCAAC
Used to make *wyEx1616.*

pMP71 – *rsy-1(1-502aa)* cDNA, isoform A cloned in frame with *gfp* into pSM between Nhe I and Kpn I with the same primers as those used to make pMP72-2. Used to make *wyEx1682.*

pMP78 – Genomic *rsy-1* including the promoter and the coding region cloned into ΔpSM (without the *unc-86* promoter) between Not I and Kpn I with primers:
5’ GAAAGGGCGGCCGCGAAAAGCATGTAGTTTGGACAGG
3’ GAAAGGGGTACCTCAACGTCGACTATGCCGATCC
Used to make *wyEx1686.*

pMP156 – *rsy-1(1-502aa)* cDNA, isoform A cut from pMP71 with Nhe I and Kpn I and cloned into pSM downstream of and in frame with *mcherry*. Used to make *wyEx2534, wyEx2539, wyEx2566.*

pMP160 – cytoplasmic *mcherry* cloned between Kpn I and EcoR I downstream of *unc-86* promoter. Used to make *wyEx2541* and *wyEx2543*.

pMP167 – *git-1* cDNA (*C. elegans* ORFeome library, Open Biosystems) cloned in frame with C-terminal *gfp* into pSM between Nhe I and Kpn I using recombination (Gateway technology, Invitrogen). Used to make *wyEx2566.*

pMP161 – *gfp* cloned between Xma I and Nhe I of pSM with *rab-3* promoter.

pMP162 – *3Xflag* cloned between Xma I and Nhe I of pSM with *rab-3* promoter with primers:
5’
CCGGGATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATC
GATTACAAGGATGACGATGACAAGG
3’
CTAGCCTTGTCATCGTCATCCTTGTAATCGATGTCATGATCTTTATAATCA
CCGTCATGGTCTTTGTAGTCCATC

pMP165 – *rsy-1(1-502aa)* cDNA, isoform A cut from pMP71 with Nhe I and Kpn I and cloned into pMP161 in frame with N-terminal *gfp*. Used to make *wyEx2590* and *wyEx2613.*

pMP169 – *syd-1* cDNA cloned into pMP162 downstream of and in frame with *3Xflag*

with primers:
5’ GAAAGGGCTAGCATGCTCATATCCACAAGTTTAAAGTTCTTGAC
3’ GAAAGGGGTACCTCAGCACTGCGATTCCGACACATAG
Used to make *wyEx2613* and *wyEx2649.*

pMP170 – *syd-2* cDNA cloned into pMP162 downstream of and in frame with *3Xflag.* Used to make *wyEx2590* and *wyEx2656.*

pMP2 – 3X*myc* cloned into BamH I (primers kindly provided by B. Tasic, Stanford University) of pcDNA3.1/*myc*-His(-)A in frame with downstream *myc* and 6X*His*. *egfp* cDNA (kindly provided by H. Zhong, University of Oregon) cloned between Nhe I and Xho I with primers:
5’ GAAAGGGCTAGCATGGTGAGCAAGGGCGAGGAGCTG
3’ GAAAGGCTCGAGTCTTGTAAAGCTCGTCCATGCCGAG

pMP91 – *rsy-1*(1-502aa) cDNA cloned into pMP2 between Not I and BstX I in frame with *egfp* and 3X*myc* with primers:
5’ GAAAGGGCGGCCGCCATGGAAGATTGGACCAACTATCATCAG
3’ GAAAGGCCAGTGTGGTGGTGAATCTGGAACGTGTAAATCATCAAC

pMP6 – *syd-1* cDNA cloned into pMP2 between Not I and BstX I in frame with *egfp* and 3X*myc* with primers:
5’ GAAAGGGCGGCCGCCATGCTCATATCCACAAGTTTAAAGTTCTTG
3’ GAAAGGCCAGTGTGGTGGGCACTGCGATTCCGAGACATAGGCG

pMP101 – membrane targeting sequence, a modified N-terminus of MARCKS (KRAS)(*2*) cloned in frame with *egfp* cDNA in pMP2 between Not I and BstX I with primers:
5’
GGCCGCCAAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGACAAAGTGT
GTAATTATGTAACCACCACA
3’
GTGGTTACATAATTACACACTTTGTCTTTGACTTCTTTTTCTTCTTTTTACC
ATCTTTGGC

pMP99 – *kras* cloned into pMP91 between BstX I and in frame with *rsy-1*(1-502aa) with primers:
5’
GTGGAAAGATGGTAAAAAGAAGAAAAAGAAGTCAAAGACAAAGTGTGTA
ATTATGTAACCACCACA
3’
GTGGTTACATAATTACACACTTTGTCTTTGACTTCTTTTTCTTCTTTTTACC
ATCTTTCCACTGTG

pMP95 – *kras* cloned into pMP2 with same primers as those used to add *kras* into

pMP99. *elks-1* cDNA cloned into pMP2 between Not I and BstX I and in frame with *egfp* and *kras* with primers:
5’ GAAAGGGCGGCCGCGATGGCACCTGGTCCCGCACCATAC
3’ GAAAGGCCAGTGTGGTGGGGCCCAAATTCCGTCAGCATCGTC

pMP4 – vertebrate *mcherry* cloned into pFLAG-CMV-2 downstream of and in frame with *flag* using primers:
5’ GAAAGGAAGCTTATGGTGAGCAAGGGCGAGGAGGATAAC
3’ GAAAGGGAATTCATCTTGTACAGCTCGTCCATGCCGCC

pMP13 – *syd-2* cDNA cloned into pMP4 between Bgl II and Kpn I and in frame with *mcherry* with primers:
5’ GAAAGGAGATCTGATGAGCTACAGCAATGGAAACATAAATTG
3’ GAAAGGGGTACCCTAGGTATATAAATGAAACTCGTAGG

pMP30 – *syd-1* cDNA cloned into pMP4 between Bgl II and BamH I and in frame with *mcherry* with primers:
5’ GAAAGGAGATCTGATGCTCATATCCACAAGTTTAAAGTTCTTG
3’ GAAAGGGGATCCTCAGCACTGCGATTCCGAGACATAG

pMP82 – *rsy-1(1-502aa)* cDNA, isoform A cloned into pMP4 between EcoR I and Kpn I and in frame with *mcherry* with primers:
5’ GAAAGGGAATTCAATGGAAGATTGGACCAACTATCATC
3’ GAAAGGGGTACCTTATGAATCTGGAACGTGTAAATCATCAAC

pMP102 – *syd-2* (853-1035 aa) cDNA cloned into pMP4 between Bgl II and Kpn I and in frame with *mcherry* with primers:
5’ GAAAGGAGATCTGATGCTATTAGAGGAAGCCATGAAAGCAC
3’ GAAAGGGGTACCTCATGTGTTGATATTATCACACGCC

pMP178 – *syd-2(R184C)* cDNA cloned into pEYFP-C3 (kindly provided by T. Meyer, Stanford University) between Bgl II and Acc65 I downstream of and in frame with *eyfp*. R184C mutation introduced by PCR using primers:
5’ GAAAGGGCTAGCATGAGCTACAGCAATGGAAACATAAATTG
3’
CTTTTGTGCTCAGCTCTTCTTCAAGTGTCGCCACACGCTCCATTGCTACTCG
AAGACATTC

pMP181 – *elks-1* cDNA with *kras* at C-terminus cut from pMP95 with Xho I and Hind III and cloned into mCerulean-C1 (kindly provided by M. Park, Stanford University)

downstream of and in frame with mCerulean.

pMP182 – *kras* cut from pMP101 with Xho I and Hind III and cloned into mCerulean-C1 downstream of and in frame with mCerulean.

Genetic screen. Chemical mutagenesis with EMS was performed on *syd-1(ju82)* mutants with the *kyIs235* marker. Approximately 2500 haploid genomes were visually screened at the F2 generation for animals with significantly more SNB-1::YFP accumulation in the HSNL presynaptic region than in *syd-1(ju82)* single mutants.

Mapping. *rsy-1(wy94);syd-1(ju82)* mutants were outcrossed 3 times and *rsy-1(wy94)* was determined to be a single genetic event. *rsy-1(wy94);syd-1(ju82)* mutants were crossed into Hawaiian strain CB4856 to generate over 250 recombinants. We used snip-SNP mapping as described previously(*3*) to map rsy-1(wy94) to an approximately 2 map-unit region on the right arm of chromosome I. We sequenced genetic locus *y53h1a.1* (I:7.91) within the mapped region and found a C -> T base change in the coding region. We were able to confirm that the synaptic phenotype in *rsy-1(wy94)* mutants is caused by the mutation in *y53h1a.1* by rescuing the synaptic phenotype in *rsy-1(wy94)* mutants by transgenically expressing PCR product that contained the promoter and the coding regions of *y53h1a.1* and *y53h1a.2*.

Fluorescence quantification. Fluorescence images of synapses in young adults were captured using the same parameters across groups with a Zeiss AxioCam MRm camera on a Zeiss Axioplan 2 Imaging System with a 63X objective. The total fluorescence intensity of SNB-1::YFP was determined using Image J and Excel by integrating pixel intensity across the synaptic region in the HSNL from each animal. More than 20 animals were used to calculate the average fluorescence intensity for each group.

Egg-laying assay. About 50 L4 stage animals were allowed to develop overnight at 20° C into adults. Animals were transferred onto a fresh plate and the stage of each egg laid was determined after 30 minutes. The eggs were classified into one of three developmental stages: 1-8 cell, 9-32 cell, or >32 cell stage. These experiments were performed double blind.

Protein interaction assay. Hek293T cells were co-transfected with constructs expressing bait and prey under the CMV promoter. Fluorescent images of individual cells were obtained on the next day. To obtain the fluorescence intensity of prey at the cell membrane versus the cytoplasm, we performed line scans that went across the cell membrane and into cytoplasm. The pixel intensity at the membrane and the fluorescence intensity in the cytoplasm were used for quantification. Note: For obtaining fluorescence intensity of prey eYFP::SYD-2R184C, we took line scans across the region of the cell membrane that included eYFP::SYD-2^{R184C} puncta. eYFP::SYD-2^{R184C} cotransfected with control membrane targeted mCerulean was punctate in nature but the puncta were

mostly in the cell cytoplasm and not enriched on the cell membrane.

Coimmunoprecipitation. Methods were adopted from Dai et al, 2006(*4*) and Grill et al, 2007(*5*). Briefly, about 250-500 µl of packed worms were lysed by sonication in 2X volume lysis buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1µg/ml pepstatin, 40µg/ml PMSF, Complete Mini protease inhibitor cocktail (Roche), PhosSTOP phosphatase inhibitor cocktail (Roche)). After spinning in microcentrifuge for 10 minutes at 4 °C at maximum speed, supernatant was collected (total protein concentration ranged from 11 to 17 mg/ml). 450 µl of lysate was used for immunoprecipitation. Lysate was precleared for 2 hours at 4 °C with 40 µl of Protein-G Agarose beads. Cleared lysate was mixed with 1 µg 3E6 anti-GFP mouse monoclonal antibody (Invitrogen) for 15 minutes followed by 2 hour incubation of this mixture with 20 µl of Protein-G Agarose beads at 4 °C. Beads were then washed with lysis buffer 4 times before they were boiled in SDS sample buffer with DTT. SDS PAGE gel and transfer were performed according to standard protocol. Mouse monoclonal Anti-FLAG M2 antibody (Sigma) or anti-GFP mouse monoclonal antibody (Roche) were used at 1:1000 dilution as primary antibodies. HRP-conjugated goat anti-mouse antibody (Roche) was used at 1:15,000 to 1:25,000 dilution as the secondary anti-body. SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) was used for detecting HRP.

References

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2. M. D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, *Genesis* **45**, 593 (Sep, 2007).
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4. Y. Dai *et al.*, *Nat Neurosci* **9**, 1479 (Dec, 2006).
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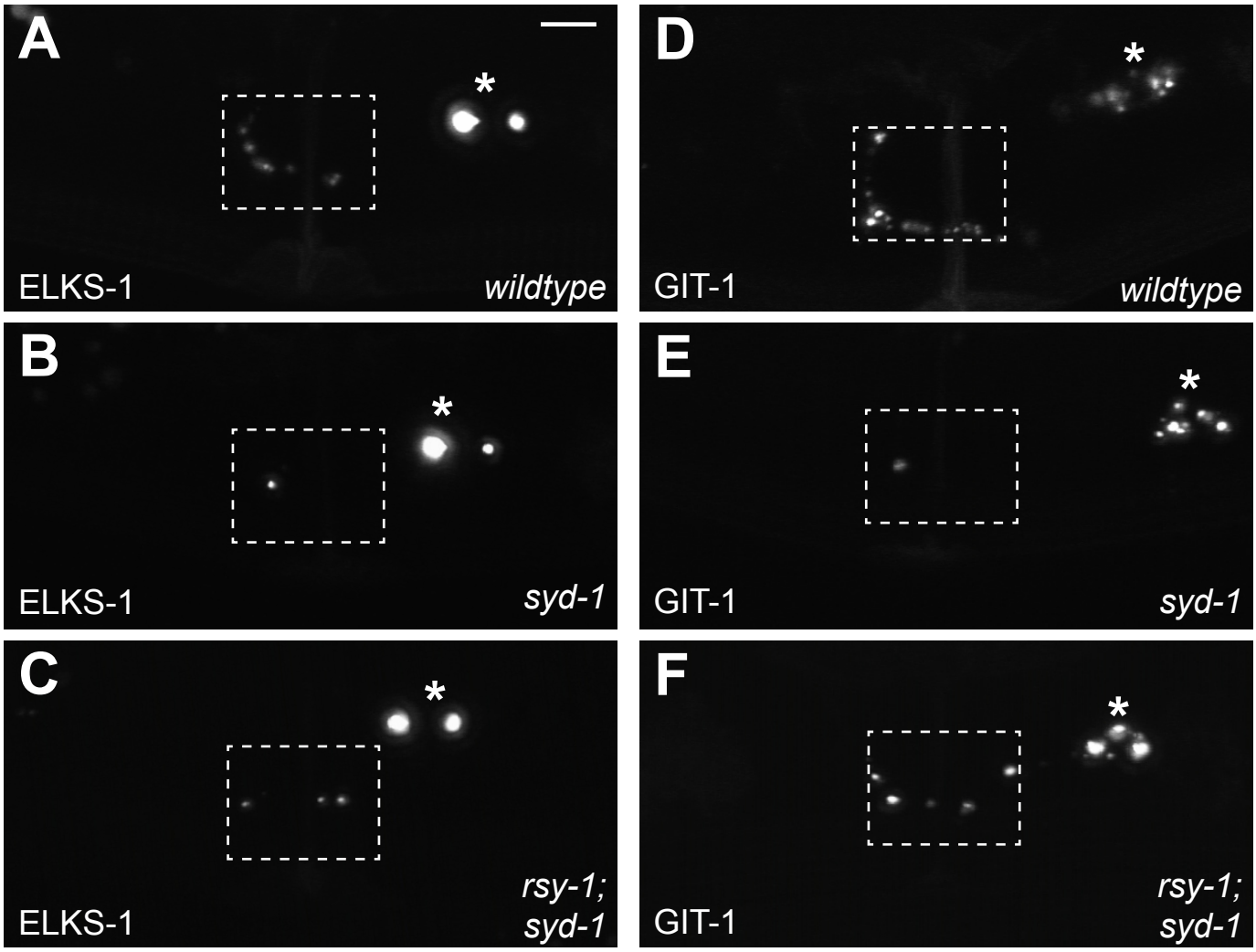
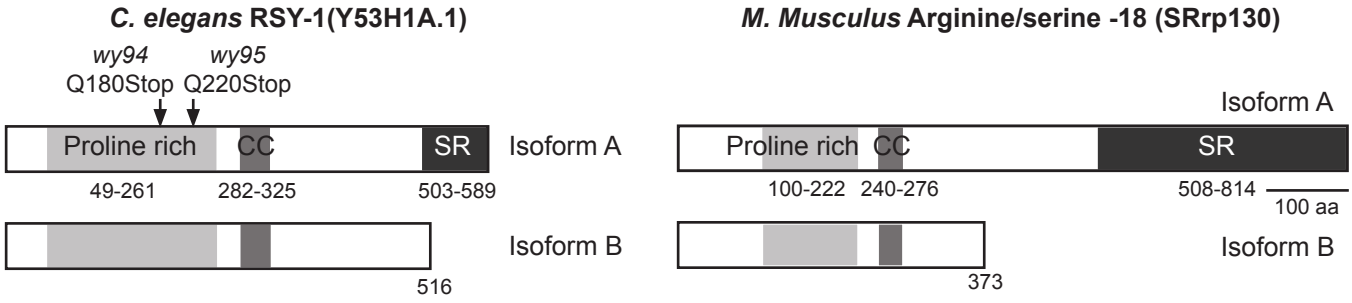
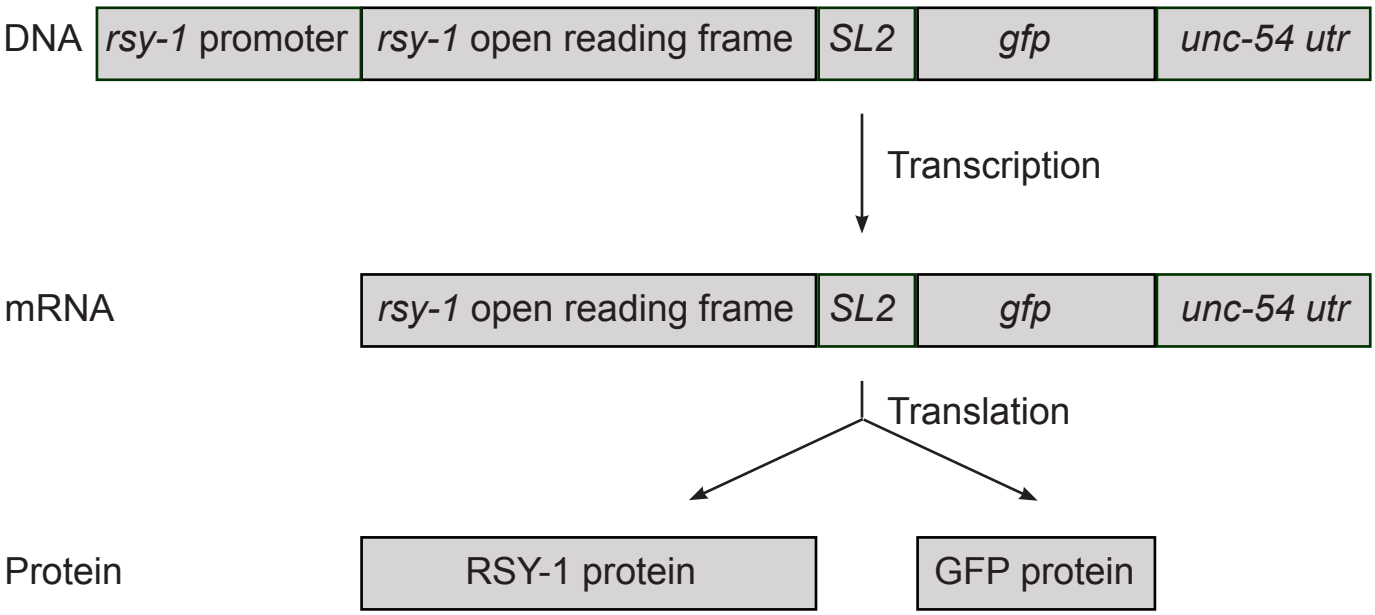


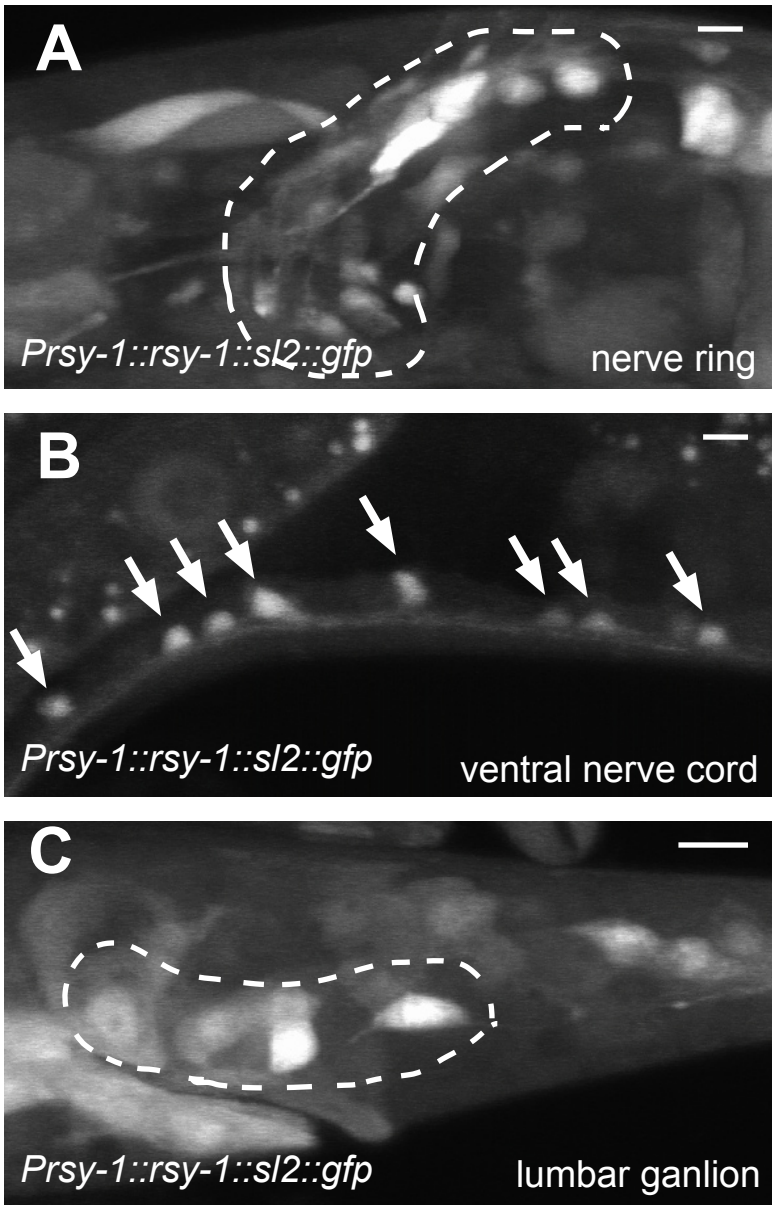
Supplementary Figure 1. Localization to presynaptic sites of multiple active zone proteins in *rsy-1;syd-1* mutants. **(A)** ELKS-1::YFP in *wildtype(N2)*, **(B)** *syd-1(ju82)*, and **(C)** *rsy-1(wy94);syd-1(ju82)* mutants. **(D)** GIT-1::YFP in *wildtype(N2)*, **(E)** *syd-1(ju82)*, and **(F)** *rsy-1(wy94);syd-1(ju82)* mutants. All images of adults. Dotted rectangle indicates synaptic region. Asterisk marks the HSNL cell body. Scale bar, 5 μm.



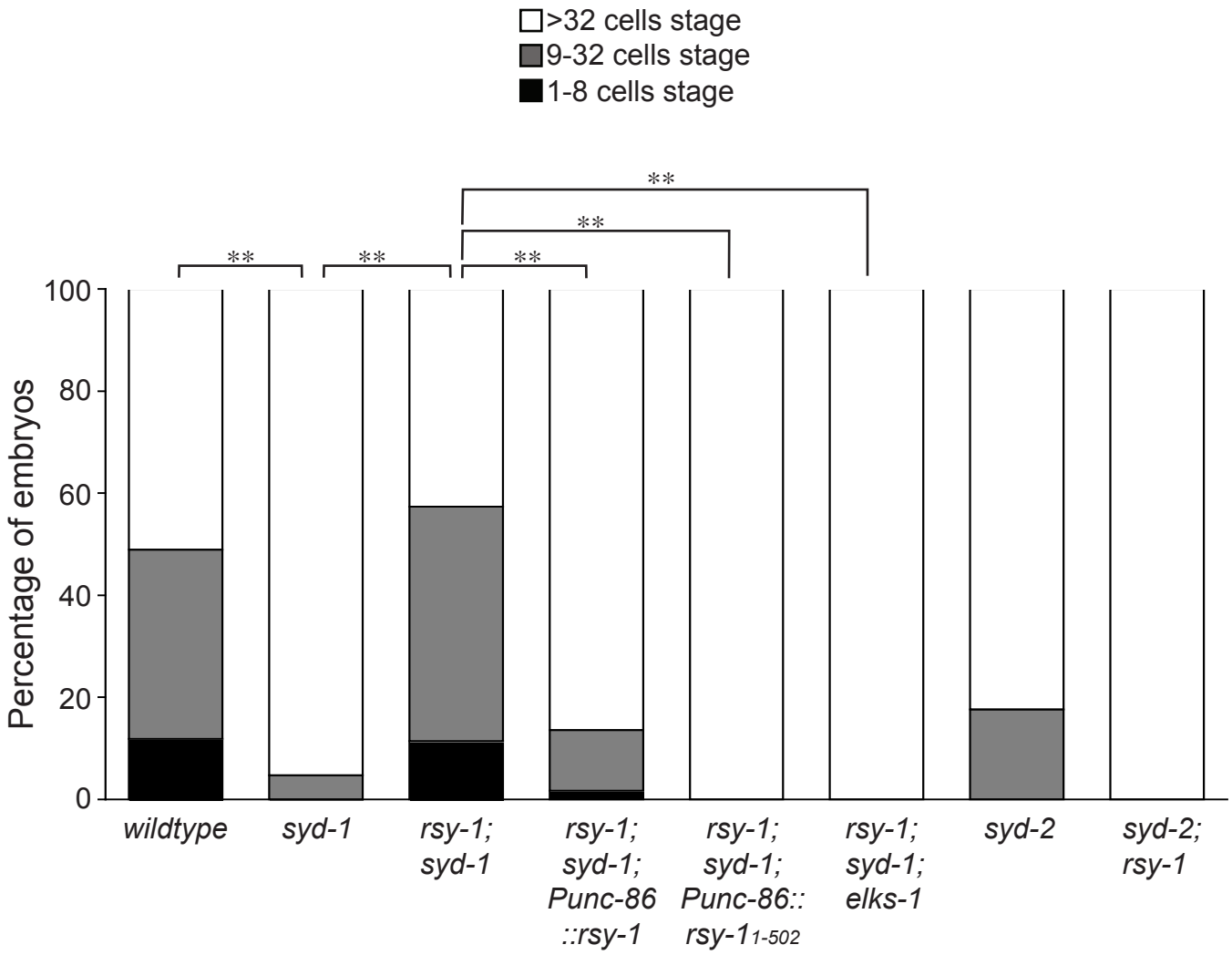
Supplementary Figure 2. Domain structure of two RSY-1 isoforms in *C. elegans* and their mouse homologs. Coiled-coil (CC), Serine/Arginine domain (SR). Amino acids that comprise each domain are shown below the structure. Arrows refer to the location of the point mutations in two alleles of *rsy-1* (*wy94* and *wy95*). Scale bar, 100 amino acids.



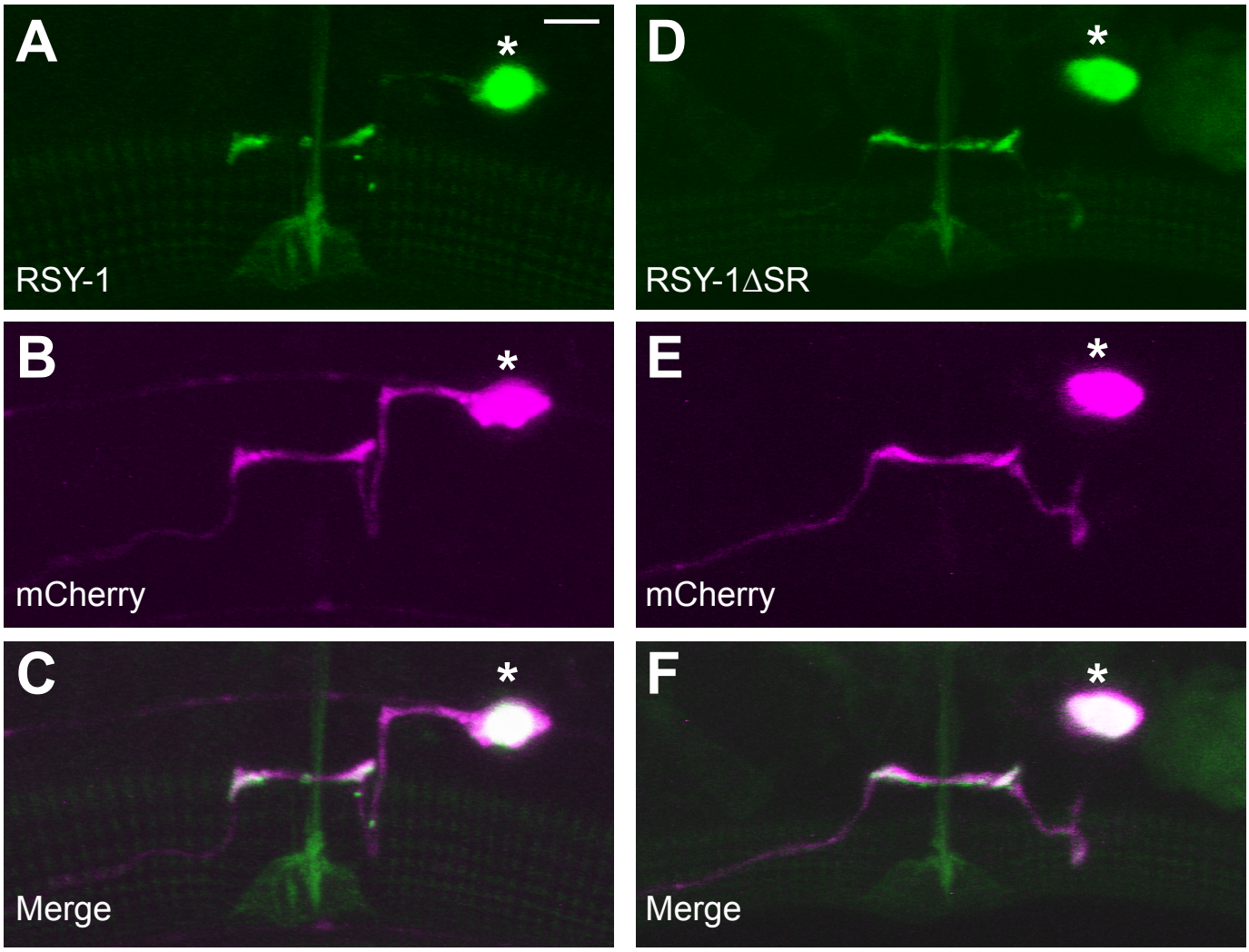
Supplementary Figure 3. Schematic of *rsy-1* expression construct. The *rsy-1* genomic locus with the 580bp promoter region upstream of the start codon was cloned into a plasmid with SL2 trans-splicing site and *gfp* at the C-terminus. Both *rsy-1* and *gfp* are expressed under the *rsy-1* promoter and transcribed as one mRNA. However, RSY-1 and GFP are translated as separate proteins, thus resulting in expression of cytoplasmic GFP under the *rsy-1* promoter.



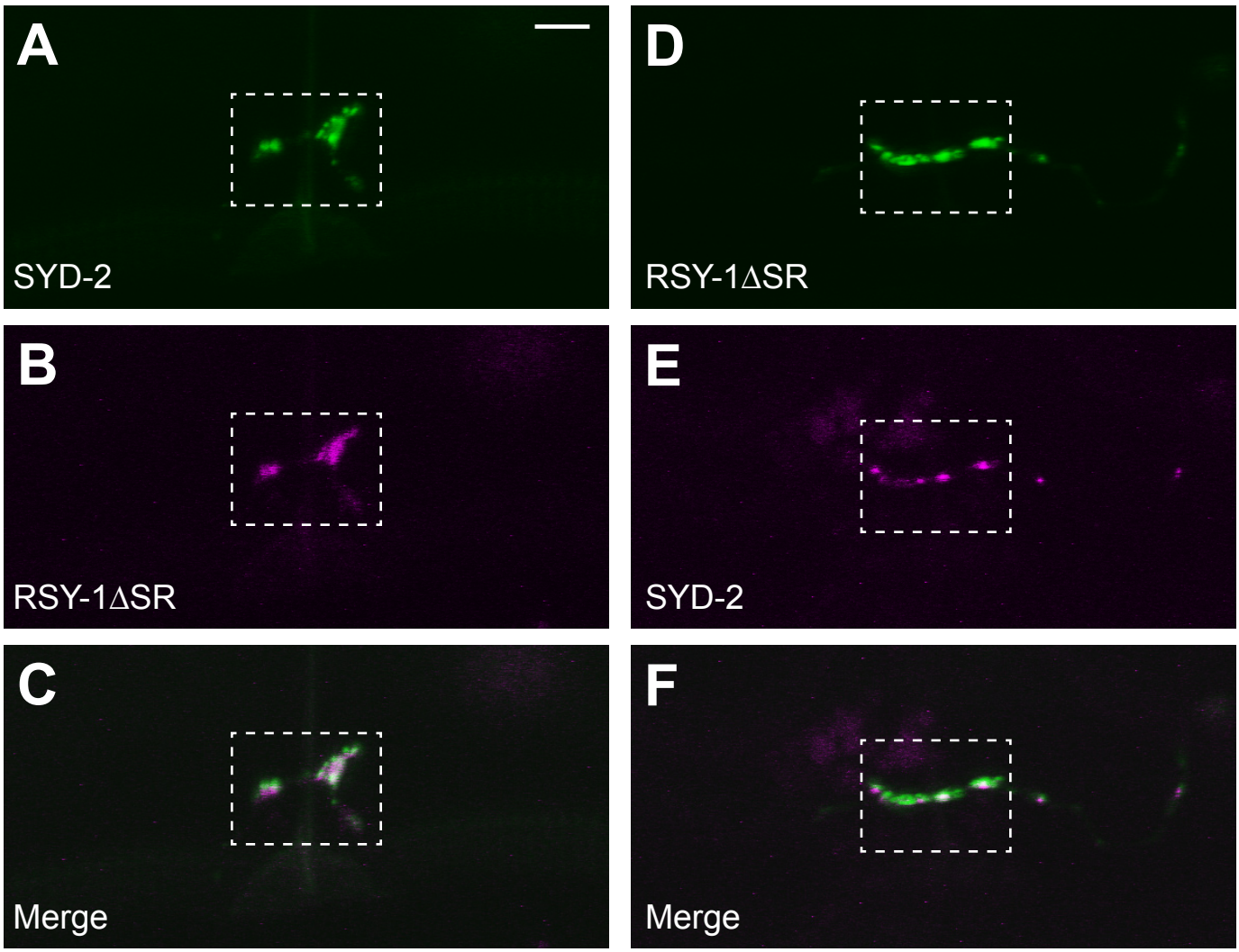
Supplementary Figure 4. Expression of *rsy-1* in neurons. **(A)** *rsy-1::sl2::gfp* expressed under the *rsy-1* promoter, which consists of 580 base pairs upstream of the *rsy-1* start site. Expression of *rsy-1* by neurons in the nerve ring (in the dashed region), **(B)** ventral nerve cord (neuron cell bodies marked by arrows), and **(C)** lumbar ganglion in the tail (in the dashed region). All images of adults. Scale bars, 5μm.



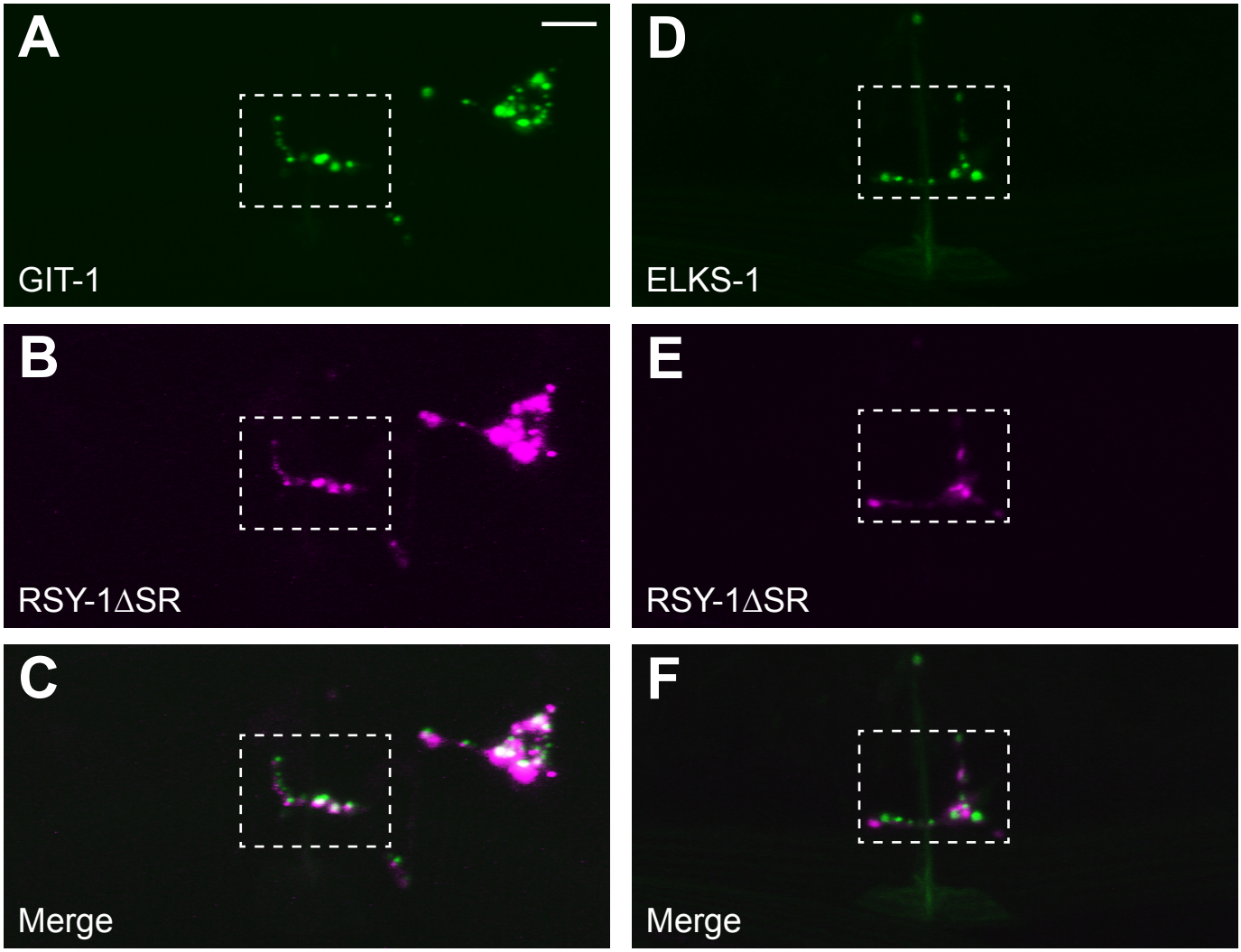
Supplementary Figure 5. Quantification of egg-laying, a behavioral output of synapse assembly in the HSNL. Proportion of eggs at a particular stage when laid. Scored double blind. **p<0.01, Fisher's exact test, n>20 per group.



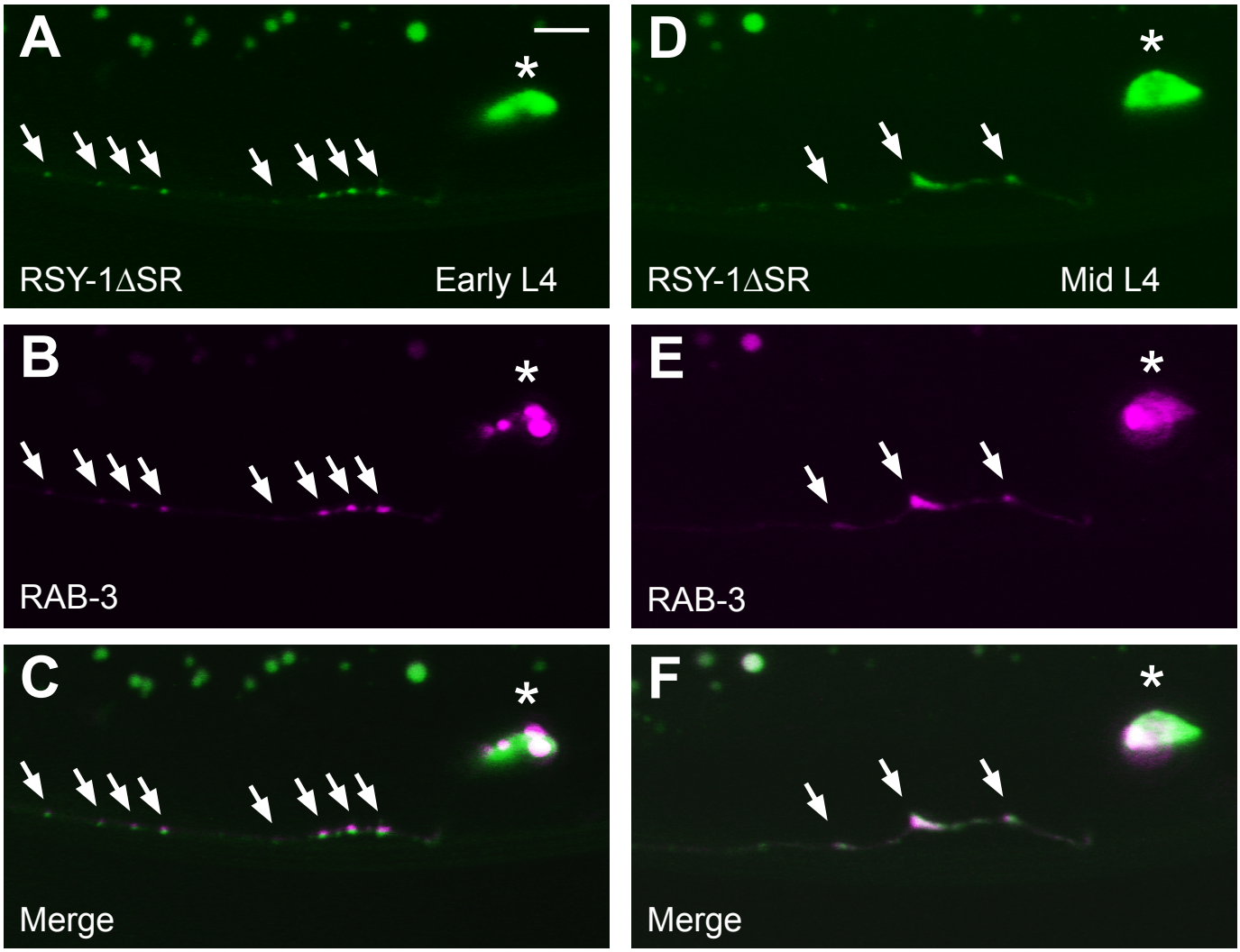
Supplementary Figure 6. Colocalization of RSY-1 with cytoplasmic mCherry. **(A)** GFP::RSY-1 (isoform A) coexpressed with **(B)** cytoplasmic mCherry. **(C)** Merged image of **A** and **B**. **(D)** GFP::RSY-1ΔSR coexpressed with **(E)** cytoplasmic mCherry. **(F)** Merged image of **D** and **E**. Note that GFP::RSY-1 and GFP::RSY-1ΔSR are enriched in the cell body and at the presynaptic sites while cytoplasmic mCherry is present throughout the whole neuron. All images of adults. Asterisk marks the HSNL cell body. Scale bar, 5 μm.



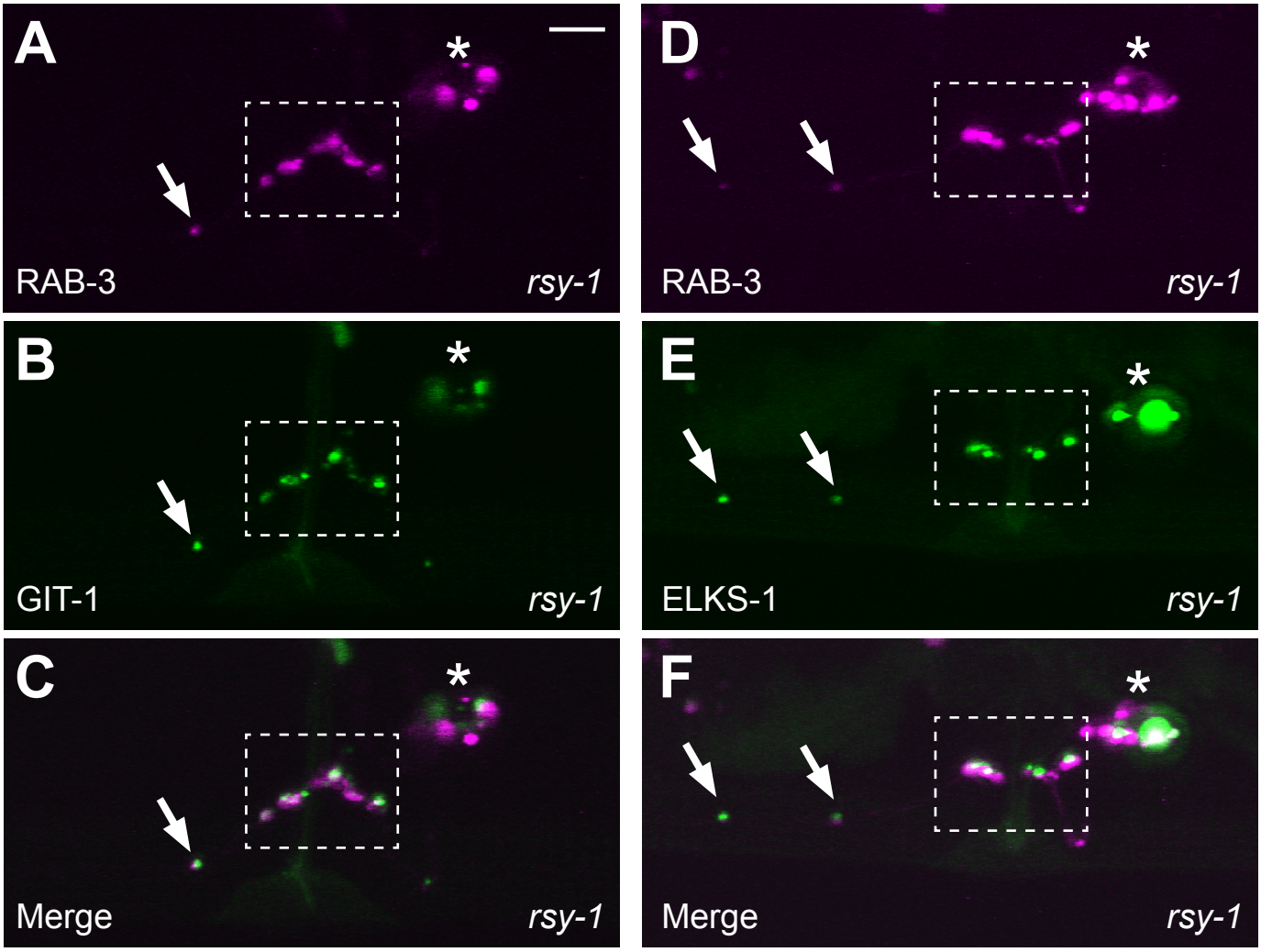
Supplementary Figure 7. Colocalization of RSY-1 with SYD-2. **(A)** GFP::SYD-2 coexpressed with **(B)** mCherry::RSY-1ΔSR. **(C)** Merged image of **A** and **B**. **(D)** GFP::RSY-1ΔSR coexpressed with **(E)** mCherry::SYD-2. **(F)** Merged image of **D** and **E**. Note that SYD-2 colocalizes with and is surrounded by RSY-1ΔSR regardless of the fluorophores they are tagged with. All images of adults. Dotted rectangle indicates synaptic region. Scale bar, 5 μm.



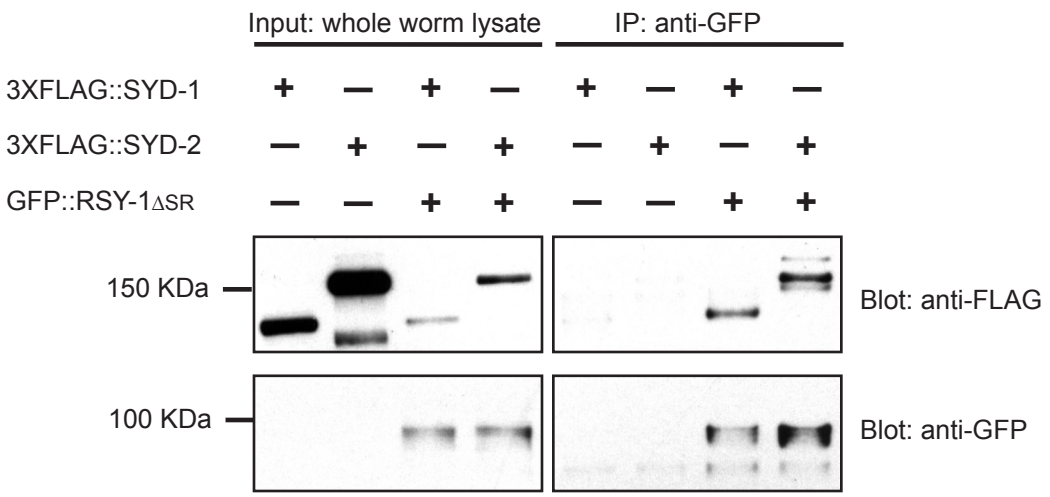
Supplementary Figure 8. Colocalization of RSY-1 with GIT-1 and ELKS-1. **(A)** GIT-1::YFP coexpressed with **(B)** mCherry::RSY-1ΔSR. **(C)** merged image of **A** and **B**. Note the tight colocalization of GIT-1 and RSY-1ΔSR puncta. **(D)** ELKS-1::YFP coexpressed with **(E)** mCherry::RSY-1ΔSR. **(F)** Merged image of **D** and **E**. Note that while ELKS-1 and RSY-1ΔSR occupy the same region of the HSNL, they do not necessarily occupy the same sub-domain of the synaptic region. All images of adults. Dotted rectangle indicates synaptic region. Scale bar, 5 μm.



Supplementary Figure 9. Time course of RSY-1 localization to presynaptic sites. **(A)** GFP::RSY-1ΔSR coexpressed with **(B)** mcherry::RAB-3 in early L4 stage. **(C)** Merged image of **A** and **B**. Arrows indicate colocalized RSY-1ΔSR and RAB-3 puncta. Note that even at this early point in synapse development, every single RAB-3 puncta colocalizes with RSY-1ΔSR. **(D)** GFP::RSY-1ΔSR coexpressed with **(E)** mcherry::RAB-3 in mid L4 stage. **(F)** Merged image of **D** and **E**. Note the continuing colocalization of RAB-3 with RSY-1ΔSR as synapses mature. Asterisk marks the HSNL cell body. Scale bar, 5 μm.



Supplementary Figure 10. Colocalization of multiple synaptic components to ectopic sites in *rsy-1(wy94)* mutants. **(A)** mcherry::RAB-3 and **(B)** GIT-1::YFP expressed in the HSNL in *rsy-1(wy94)* mutants. **(C)** Merged image of **A** and **B**. **(D)** mcherry::RAB-3 and **(E)** ELKS-1::YFP expressed in the HSNL in *rsy-1(wy94)* mutants. **(F)** Merged image of **D** and **E**. Arrows point to ectopic accumulation of synaptic material. All images of adults. Dotted rectangle indicates synaptic region. Asterisk marks the HSNL cell body. Scale bar, 5 μm.



Supplementary Figure 11. Coimmunoprecipitation of RSY-1 with SYD-1 and SYD-2 from worm lysate. Plus sign (+) indicates transgenic expression in animals of the corresponding protein under the pan-neuronal rab-3 promoter. Minus sign (–) indicates absence of the corresponding protein. Upper blot in the input column shows 3XFLAG::SYD-1 and 3XFLAG::SYD-2 while the lower blot shows GFP::RSY-1ΔSR. Lower of the two bands (input column, upper blot, lane with only 3XFLAG::SYD-2) is likely a degradation product. GFP::RSY-1ΔSR was immunoprecipitated with an anti-GFP antibody and membrane was blotted with anti-FLAG antibody to detect coimmunoprecipitation of 3XFLAG::SYD-1 or 3XFLAGSYD-2 (upper blot in the IP column). Membrane blotted with anti-FLAG antibody was stripped and blotted again with anti-GFP antibody to detect immunoprecipitation of GFP::RSY-1ΔSR (lower blot in the IP column). Results were verified by repeating the experiment three independent times.