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Supplemental information

Semaphorin3A/PlexinA3 association with the Scribble scaffold for cGMP increase is required for apical dendrite development

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Supplementary Figures and Figure Legends

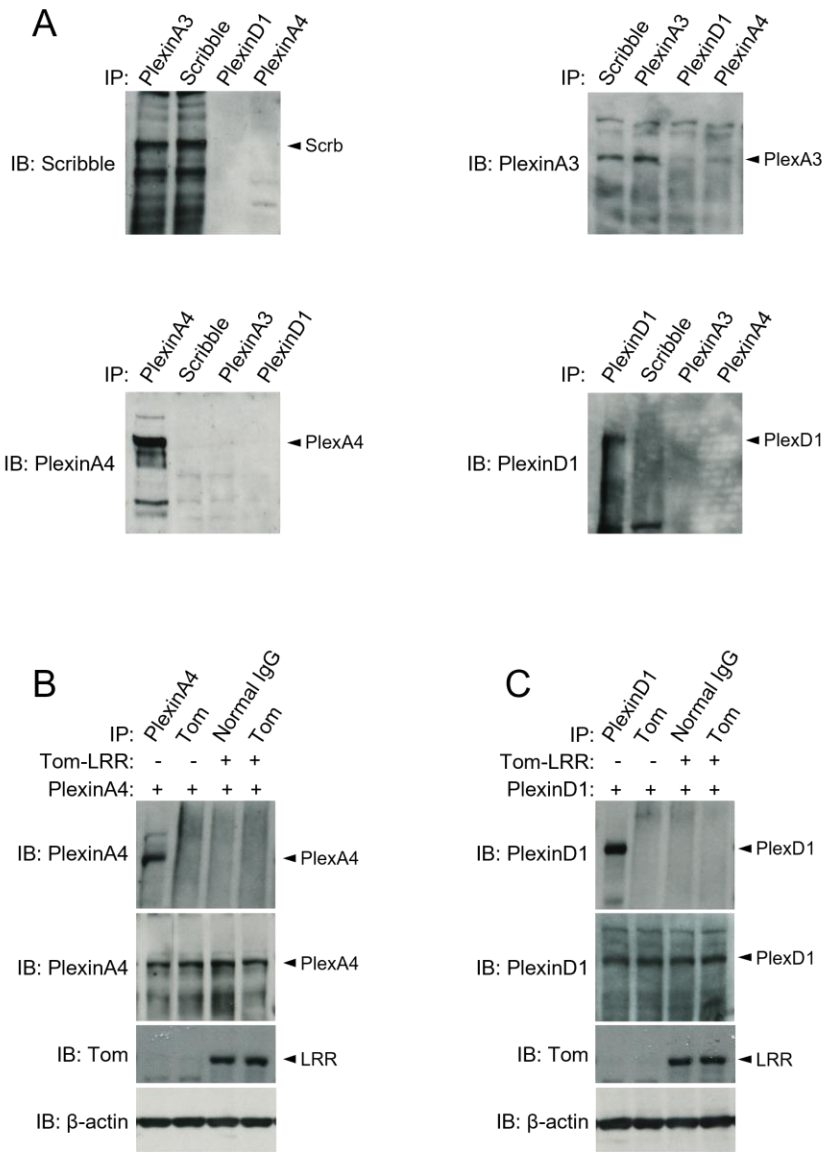

Figure S1. Co-IP of Scribble with Plexin co-receptors from the embryonic brain and co-IP of PlexinA4 and PlexinD1 with the Scribble LRR domain

(A) Reciprocal co-IP of PlexinA3, PlexinA4, or PlexinD1, with Scribble, from rat E18 embryonic brain lysates. Reciprocal IP was with Ab to PlexinA3, PlexinA4, or PlexinD1, or Scribble, and IB with Ab to these proteins (n = 3). There was reciprocal co-IP among PlexinA3 and Scribble, but not among PlexinA4 or PlexinD1 and Scribble. IP and IB with PlexinA4 and PlexinD1 confirmed their expression in the E18 embryonic brain lysates.

(B, C) Co-IP (top blot panel) of WT PlexinA4 (B) or PlexinD1 (C) with dTom-LRR, from HEK-293 cell lysates co-expressing WT PlexinA4 (B) or PlexinD1 (C) with dTom-LRR. IP was with Tom and IB for PlexinA4 (B) or PlexinD1 (C). Control IP was with Ab to the respective receptor or normal IgG. Total cell lysates were immunoblotted for PlexinA4 (B), PlexinD1 (C), or LRR, to check protein expression (n = 3). PlexinA4 or PlexinD1 did not associate with the Scribble LRR domain.

Related to Figure 1A and 1C.

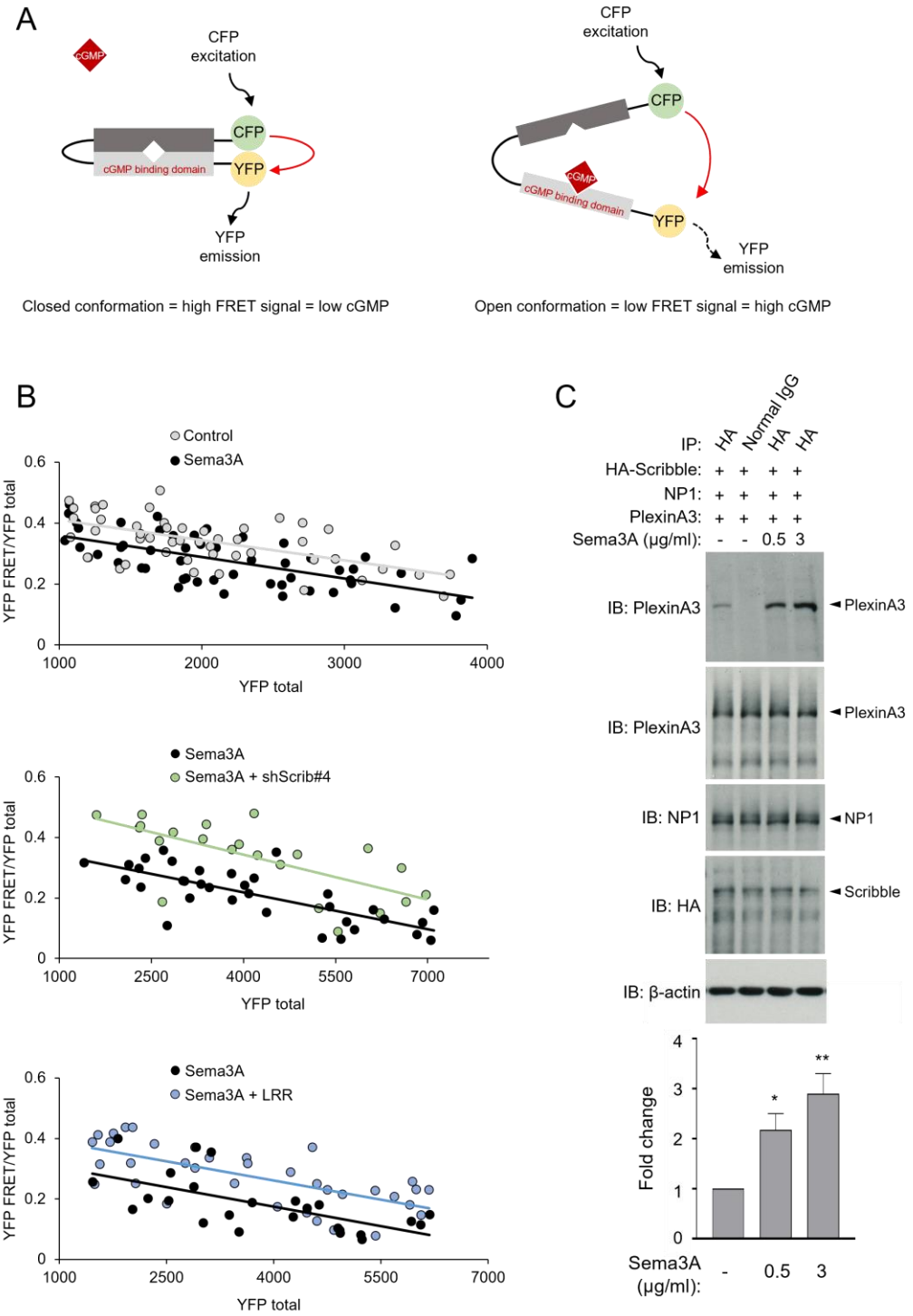

Figure S2. Sema3A mediates cGMP increase via PlexinA3 – Scribble association

(A) Schematic depiction of the FRET cGMP reporter cGi-500 (Russwurm et al., 2007). The FRET signal was determined by the YFP fluorescence measured following CFP excitation (YFP-FRET). Upon cGMP increase and cGMP binding, cGi-500 sensor relaxes its conformation resulting in YFP-FRET reduction, whereas cGMP decrease is reflected in YFP-FRET increase.

(B) cGMP measurements in cultured hippocampal neurons following treatment with recombinant Sema3A using FRET cGMP sensor cGi-500, and effects of LRR overexpression or Scribble knockdown on Sema3A-mediated cGMP increase. Neurons were co-transfected with cGi-500 together with LRR, Scribble-shRNA (shScrib#4), or

control vector. FRET measurements performed at 3-4DIV, following Sema3A treatment. Control, cells not treated with Sema3A. FRET signal was determined by YFP fluorescence measured following CFP excitation (YFP-FRET). Because the FRET signal (YFP-FRET) depends on the level of probe expression, YFP-FRET values were normalized to total YFP levels determined following YFP excitation (YFP-total), reflecting the level of probe expression. cGMP levels for control cells without Sema3A treatment was determined by FRET signal in each cell normalized to probe expression level in that cell (YFP-FRET / YFP-total), presented for all cells with varying probe concentration (YFP-total; arbitrary fluorescence units) (grey circles). A range in which FRET (YFP-FRET / YFP-total) demonstrated linear correlation with levels of probe expression (YFP-total), represents cGMP-levels in control cells (grey circles, linear fit). Normalized FRET signals (YFP-FRET / YFP-total) were determined following Sema3A treatment in cells transfected with control vectors (black circles), which expressed cGi-500 at a level for which control cells showed a linear response. YFP-FRET / YFP-total values for Sema3A treated cells were scattered below those for control, indicating cGMP increase upon Sema3A treatment. FRET signals (YFP-FRET / YFP-total) in cells expressing LRR (blue circles) or shScrib#4 (green circles) that were treated with Sema3A, were scattered above those for control cells treated with Sema3A, indicating cGMP decrease following LRR expression or Scribble knockdown compared to control Sema3A treated cells.

(C) Co-IP (top blot panel) of WT PlexinA3 with WT Scribble following treatment with different Sema3A concentrations (0.5 and 3 µg/ml), from HEK-293 cell lysates co-expressing WT PlexinA3 and WT Tom-NP-1 (constituting the receptor dimer for Sema3A binding) together with WT HA-Scribble. Cell lysates were subjected to IP with HA and IB with PlexinA3. Association of PlexinA3 with HA-Scribble following Sema3A treatment was quantified as fold change (n=4, One-way ANOVA, Dunnett's Multiple Comparison Test, *p=0.0297; **p=0.0014) relative to Sema3A untreated cells, normalized to total PlexinA3 protein levels. PlexinA3 – Scribble association was promoted by Sema3A.

*p≤0.05; **p≤0.01. Error bars represent SEM.

Related to Figure 1A, 1C and 1D and Figure 1F-1H.

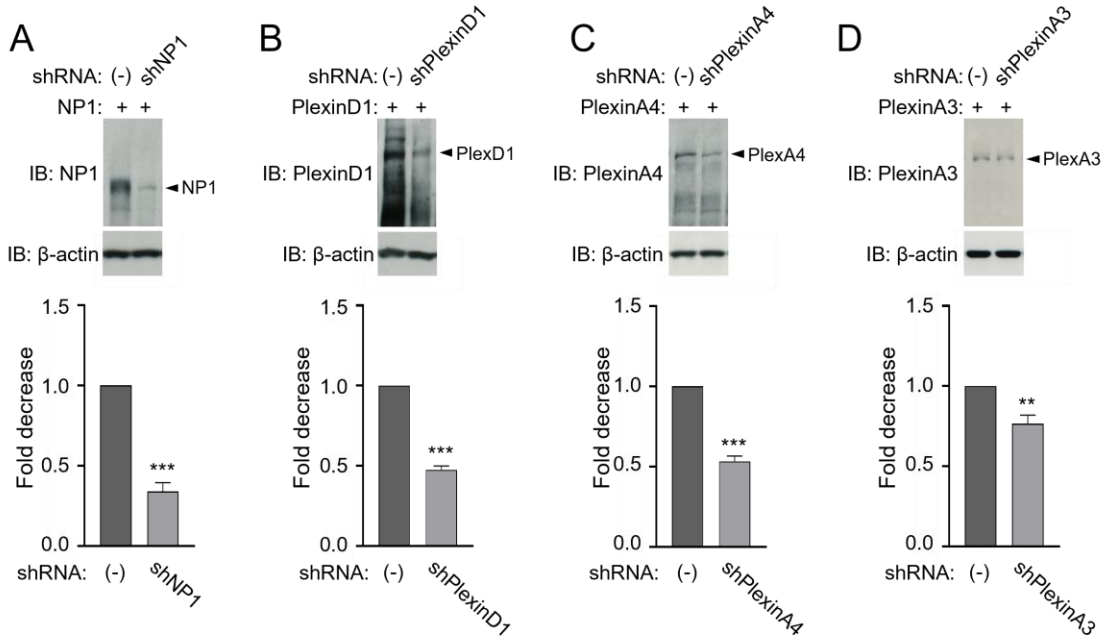

Figure S3. Knock-down efficiency of Plexin co-receptors and NP-1 with shRNAs

(A, B, C, D) Knock-down efficiency of NP-1 (A), PlexinD1 (B), PlexinA4 (C) or PlexinA3 (D) with specific shRNAs. shRNA sequences targeting rat NP-1 (A), PlexinD1 (B), PlexinA4 (C) or PlexinA3 (D) cloned in pRNAT-U6.3 vector that also drives EGFP or dTom expression, were co-transfected in HEK-293 cells together with targeted NP-1 (A), PlexinD1 (B), PlexinA4 (C) or PlexinA3 (D). Whole-cell extracts immunoblotted for NP-1 (A), PlexinD1 (B), PlexinA4 (C) or PlexinA3 (D). "(-)", control shRNA. Knockdown levels with shRNAs were quantified as fold reduction (n=3, Unpaired t-test, **p≤0.01; ***p≤0.001) relative to control shRNA, normalized to β-actin. For FRET experiments (Figure 1), shRNAs were cloned into pRNAT-U6.3 vector that expresses dTom instead of EGFP. **p≤0.01; ***p≤0.001. Error bars represent SEM.
Related to Figure 1 and 3.

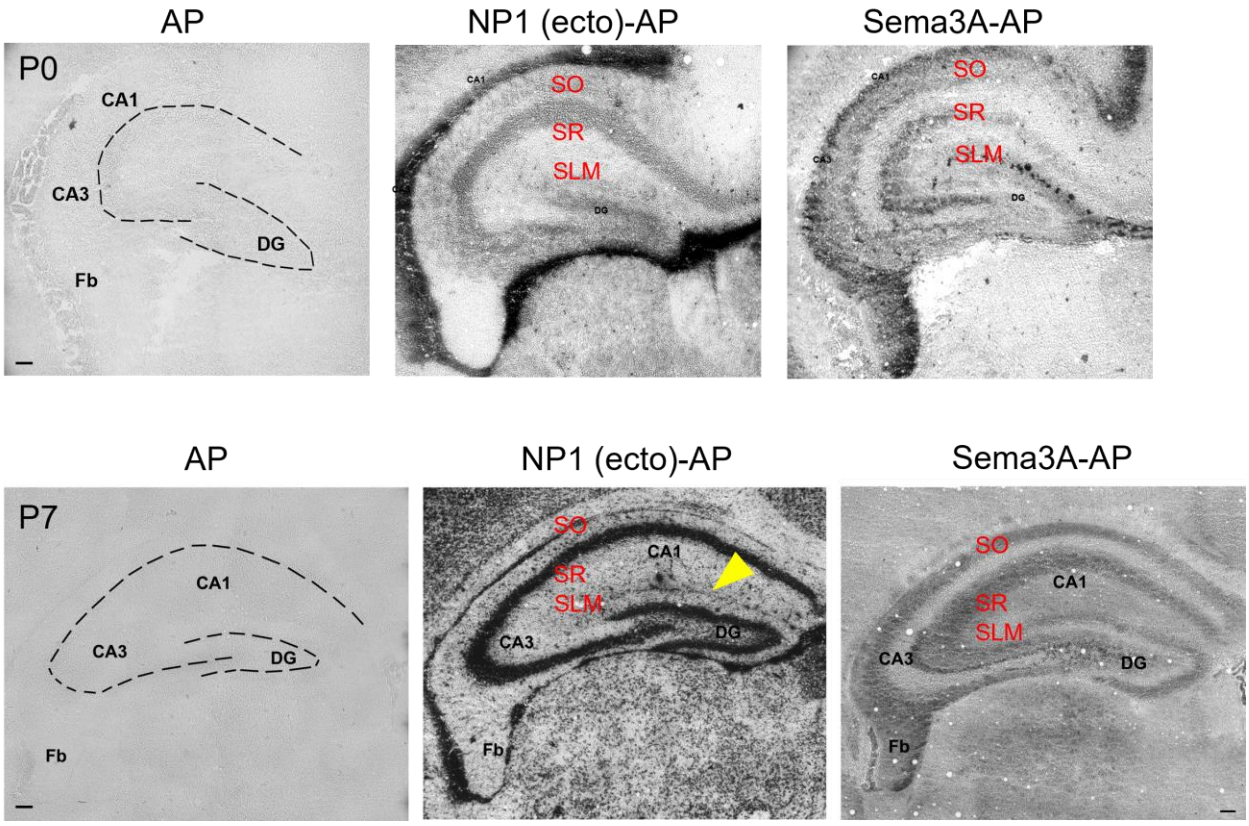

Figure S4. Expression of Sema3A and NP-1 in the hippocampus
Expression of Sema3A and NP-1 in P0 or P7 mouse hippocampus. Alkaline phosphatase (AP) fused to Sema3A (AP-Sema3A) or NP-1 ectodomain (NP-1(ecto)-AP), was bound to sagittal hippocampal sections of WT P0 or P7 mice, to test endogenous NP-1 or Sema3A expression, respectively, using AP activity assay. AP alone served as control. Depicted are: CA1; CA3; DG, Dentate Gyrus; Fb, Fimbria. SO, stratum oriens; SR, stratum radiatum; SLM, Stratum lacunosum moleculare. Sema3A was expressed in CA1 pyramidal cell-layer. NP-1 had complementary expression to Sema3A, mainly at the SR and SO. Sema3A also found to be expressed at the SLM (P7, arrowhead), where CA1 apical dendrites terminate. Scale bar is 100 µm.
Related to Figure 1, 2 and 4.

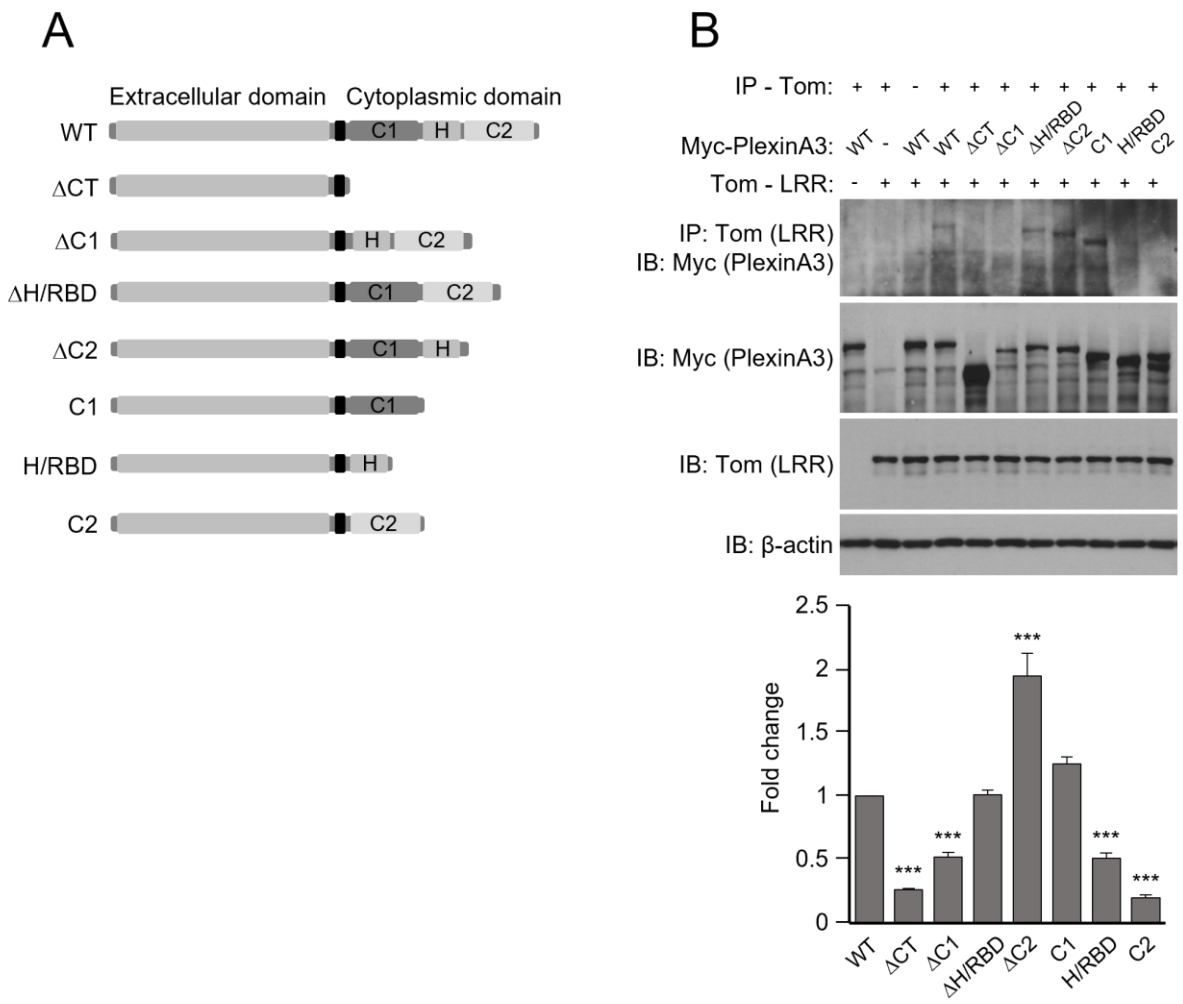

Figure S5. Mapping the Scribble association domain in PlexinA3

(A, B) Mapping the Scribble association domain in the cytoplasmic region of PlexinA3. (A) Schematic depiction of WT PlexinA3 containing an extracellular domain and a cytoplasmic region, and the generated cytoplasmic region deletion mutants. The cytoplasmic region of PlexinA receptors contains two domains, C1 and C2 with similarity to GTPase-activating proteins, that are separated by a Hinge or Rho GTPase binding domain (H/RBD). Cytoplasmic region mutants included those in which the different domains, C1, C2 and H/RBD, were either internally deleted (C1, ΔC1; H/RBD, ΔH/RBD, C2, ΔC2) or expressed in isolation (C1; H/RBD; C2). Also generated is a PlexinA3 mutant with the entire cytoplasmic region deleted (ΔCT). We predict that all PlexinA3 mutants are expressed at the cell surface, form a functional complex with NP-1, and respond to Sema3A (Mlechkovich et al., 2014). (B) Co-IP of WT PlexinA3 or PlexinA3 mutants with Scribble-LRR domain from HEK-293 cell lysates co-expressing N-terminal Myc-tagged PlexinA3 variants and dTom-LRR. Cell extracts were subjected to IP with Tom and IB with Myc. Total cell-lysates were also immunoblotted for Myc or Tom to test protein expression. Association of Myc-PlexinA3 mutants with dTom-LRR was quantified as fold change (n=3, One-way ANOVA; Dunnett's Multiple Comparison Test, ***p≤0.001) relative to WT-PlexinA3, normalized to total levels of the respective proteins. These analyses showed that LRR associated with the C1 domain of PlexinA3, and the association was further promoted in the presence of both C1 and H/RBD domains (ΔC2).

***p≤0.001. Error bars represent SEM.

Related to Figure 3.

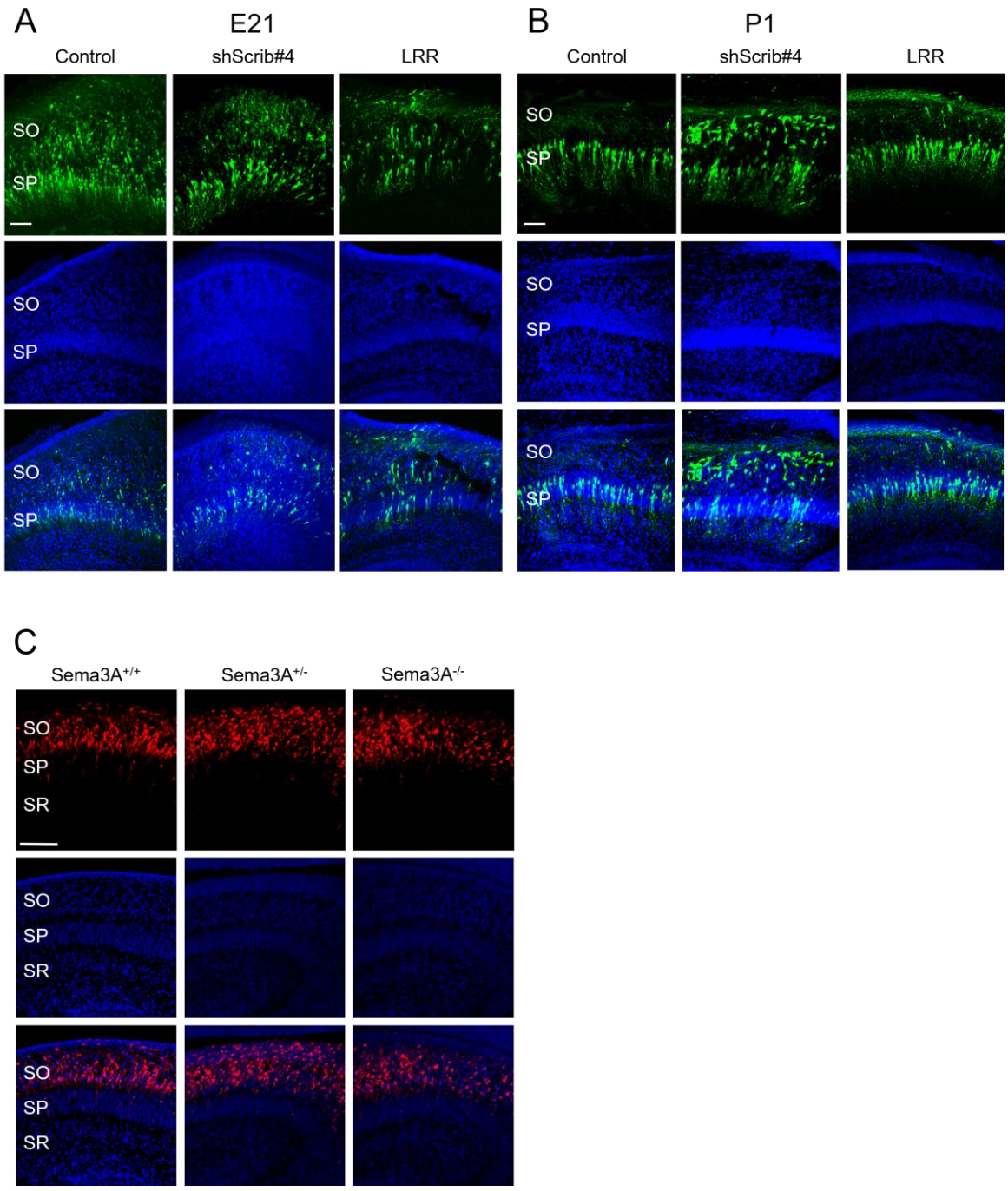

Figure S6. DAPI labeling for layer determination for neurons following LRR overexpression of Scribble knockdown at E21 and P1 or neurons following Sema3A deletion at E18

(A, B) Representative images of rat CA1 pyramidal neurons, as in Figure 4A (A) and Figure 4F (B), including DAPI labeling for layer determination.

(C) Representative images of CA1 pyramidal neurons at E18, from WT ($Sema3A^{+/+}$), heterozygous ($Sema3A^{+/-}$), or homozygous ($Sema3A^{-/-}$) mouse littermate brains for Sema3A deletion, as in Figure 4J, including DAPI labeling for layer determination.

Related to Figure 4.