
Figures and figure supplements

Plexins promote Hedgehog signaling through their cytoplasmic GAP activity

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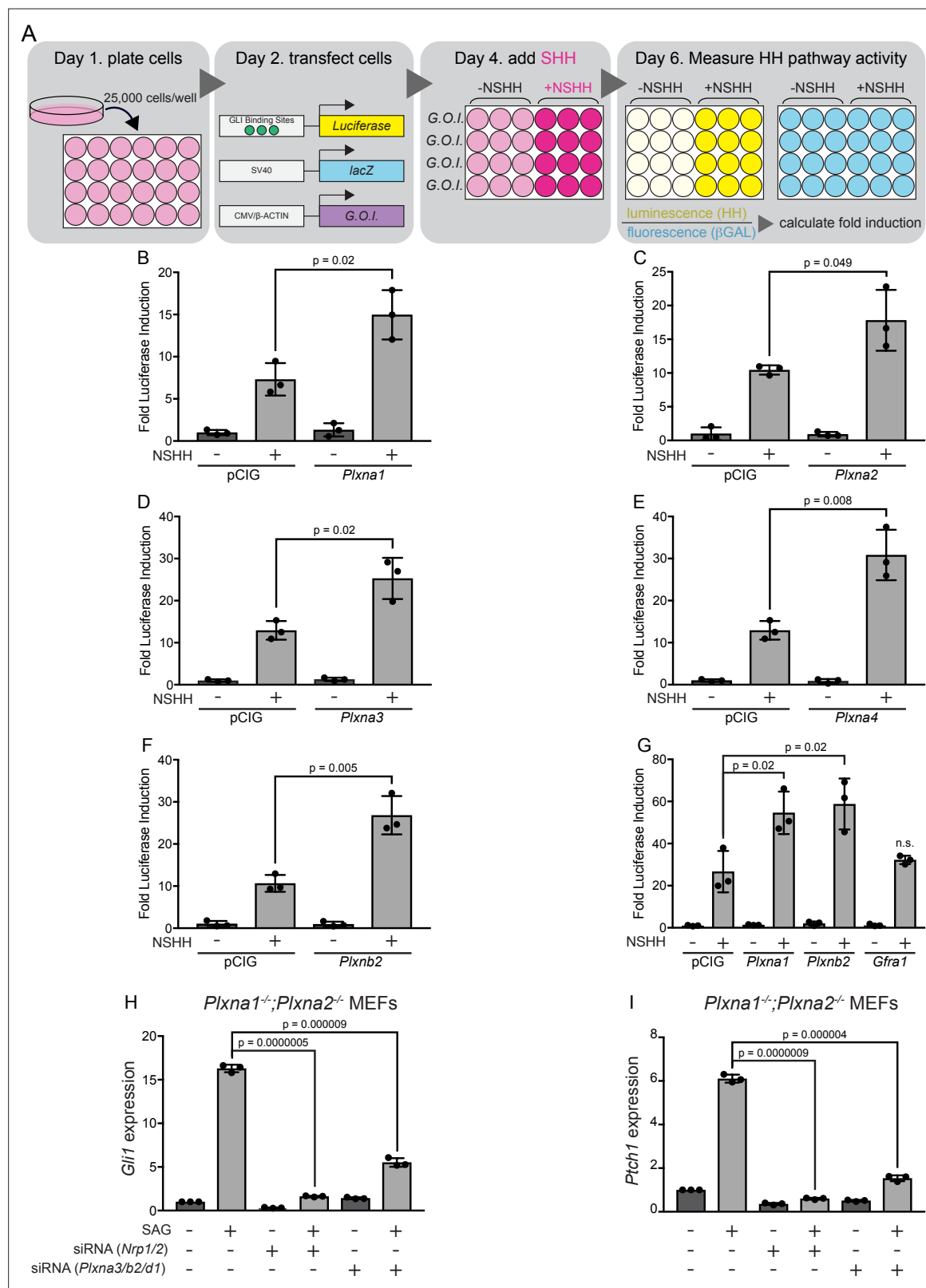


Figure 1. Multiple plexins (PLXNs) promote Hedgehog (HH) signaling. **(A)** Schematic of HH-responsive NIH/3T3 luciferase assays. G.O.I., gene of interest. **(B–F)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs or empty vector control (pCIG) and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). **(G)** Direct analysis of PLXNA1- and PLXNB2-mediated HH pathway promotion, compared with the unrelated cell surface protein GFRα1. **(H, I)** qRT-PCR analysis of *Gli1* and *Ptch1* in response to HH pathway

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Figure 1 continued

activation via the Smoothened agonist, SAG. *Plxna1*^{-/-};*Plxna2*^{-/-} mouse embryonic fibroblasts (MEFs) were treated with siRNA oligos for either *Nrp1* and *Nrp2* or *Plxna3*, *Plxnb2*, and *Plxnd1*, as indicated. Data points indicate technical replicates. Fold changes were determined using the $\Delta\Delta\text{CT}$ method. Data are reported as mean fold induction \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant.

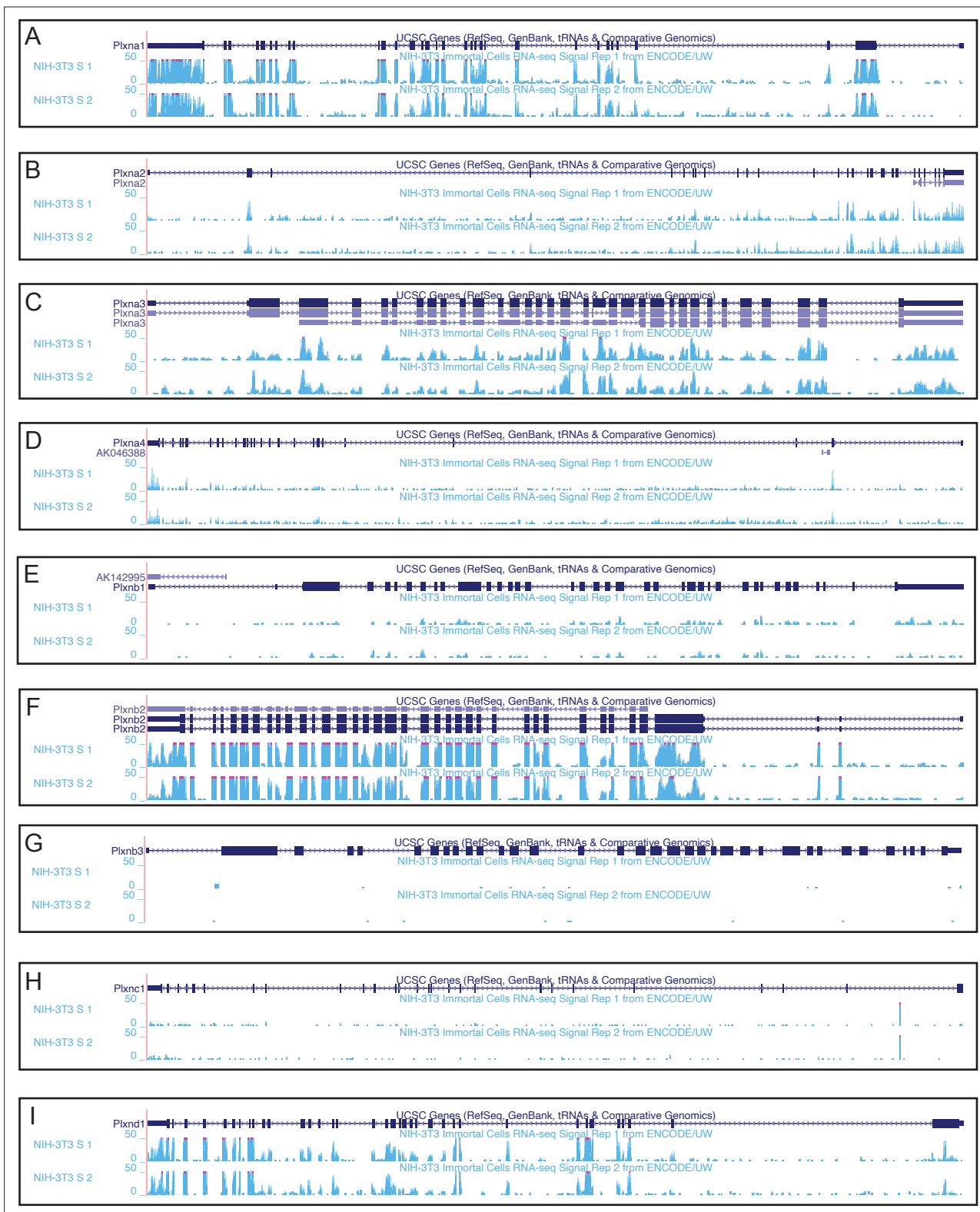


Figure 1—figure supplement 1. *Plxn* expression in NIH/3T3 fibroblasts. (A-I) RNA sequencing data from the ENCODE project indicating *Plxn* expression in NIH/3T3 cells. Data were aligned to the mouse GRCm38/mm10 assembly using the UCSC Genome Browser (<https://genome.ucsc.edu>).

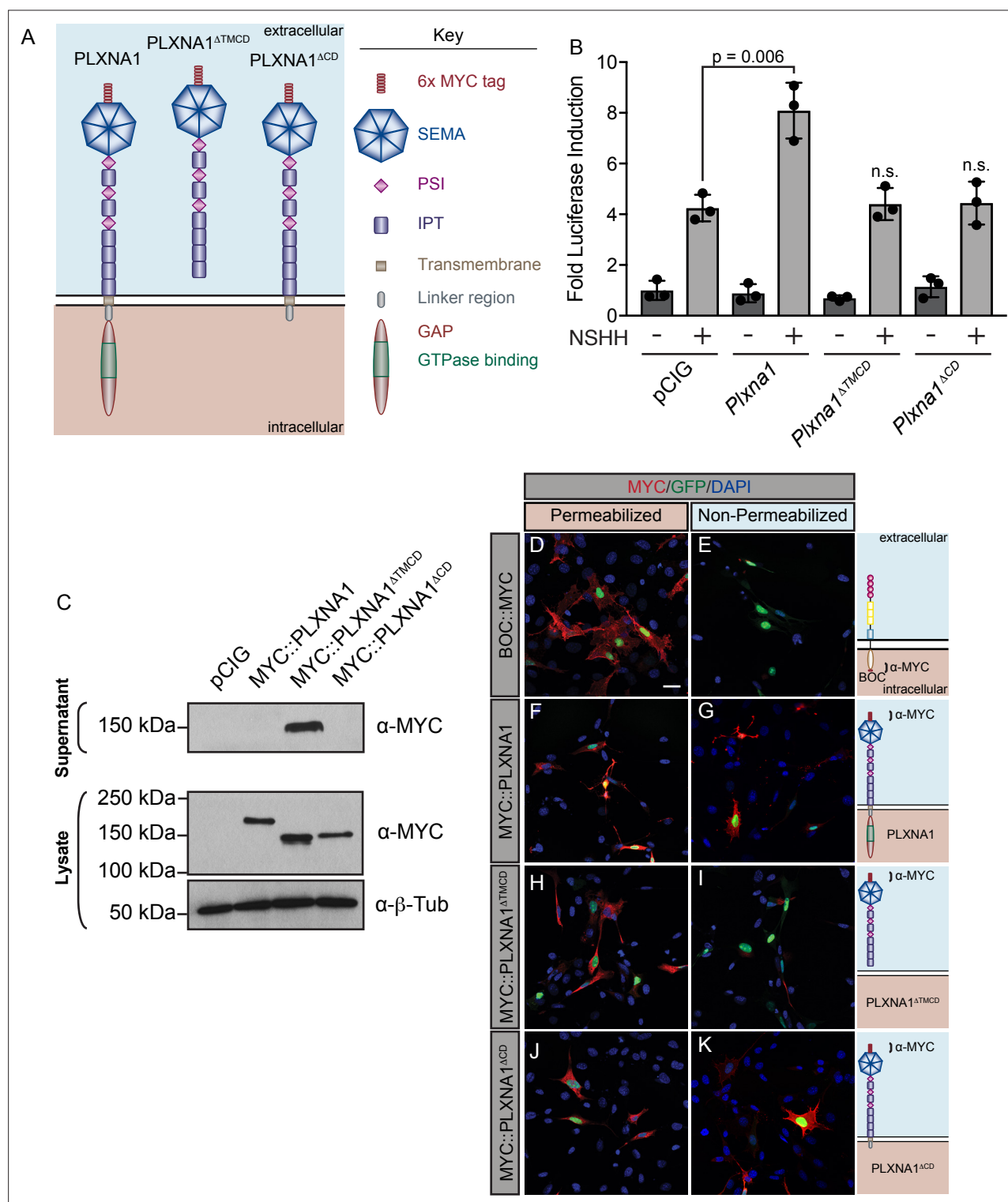


Figure 2. The PLXNA1 cytoplasmic and transmembrane domains are required for Hedgehog (HH) pathway promotion. **(A)** Schematic of different PLXNA1 proteins. **(B)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant. **(C)** Western blot analysis confirming expression of MYC-tagged PLXNA1 proteins in NIH/3T3 cells. Note that MYC::PLXNA1^{ΔTMCD} is detected in the supernatant, consistent with its predicted secretion. Anti-beta-tubulin (α - β -Tub) was used as a loading control. **(D-K)** Antibody detection of MYC (red) in permeabilized (left panels) and non-permeabilized (right panels) NIH/3T3 cells to assess cell surface localization of the indicated MYC-tagged proteins. Note that BOC, which contains a C-terminal MYC tag, is only detected under permeabilized conditions, while

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PLXNA1^{ΔTMC^D}, which is secreted, is also largely undetected under non-permeabilized conditions. Nuclear GFP (green) indicates transfected cells, whereas DAPI (blue) stains all nuclei. Diagrams (right) describe each construct, with brackets indicating antibody-binding sites. Scale bar = 10 μm.

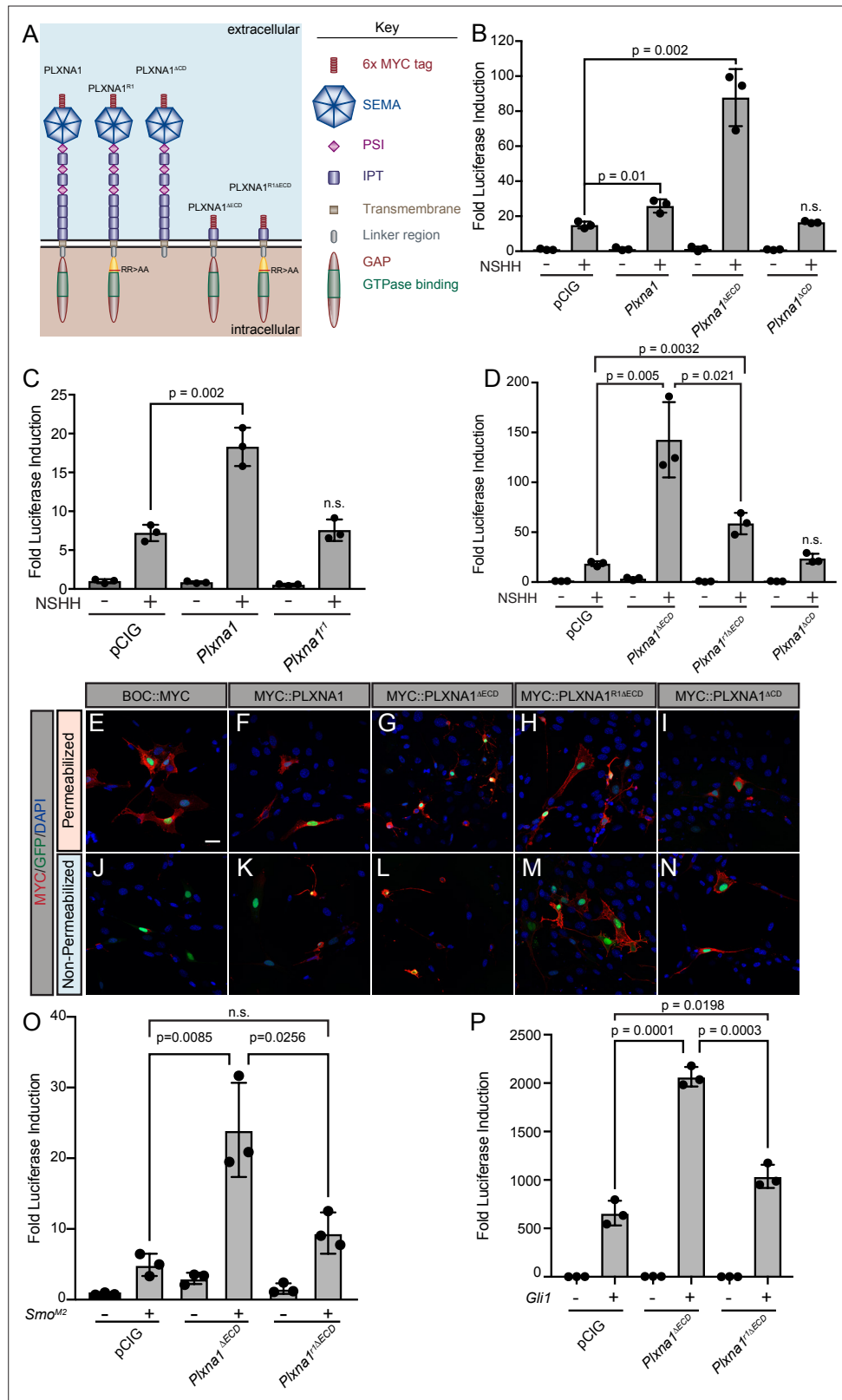


Figure 3. The plexin (PLXN) GTPase-activating protein (GAP) domain is required to promote Hedgehog (HH) signaling at the level of GLI transcription factors. **(A)** Schematic of different PLXNA1 proteins. **(B–D)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction \pm SD, with *Figure 3 continued on next page*

Figure 3 continued

p-values calculated using two-tailed Student's t-tests. n.s., not significant. **(E–N)** Antibody detection of MYC-tagged proteins (red) in permeabilized (top panels) and non-permeabilized (bottom panels) NIH/3T3 cells to assess cell surface localization of the indicated constructs. Nuclear GFP (green) indicates transfected cells, whereas DAPI (blue) stains all nuclei. Note that constitutive PLXN GAP activity leads to cell collapse, as is observed with PLXNA1^{ΔECD} and, to some extent, PLXNA1. For PLXNA1R1 localization, please see **Figure 3—figure supplement 1D and E**. Scale bar = 10 μm. **(O, P)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs and stimulated by co-transfecting cells with pCIG, *Smo*^{M2} **(O)**, or *Gli1* **(P)**. Data are reported as mean fold induction ± SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant.

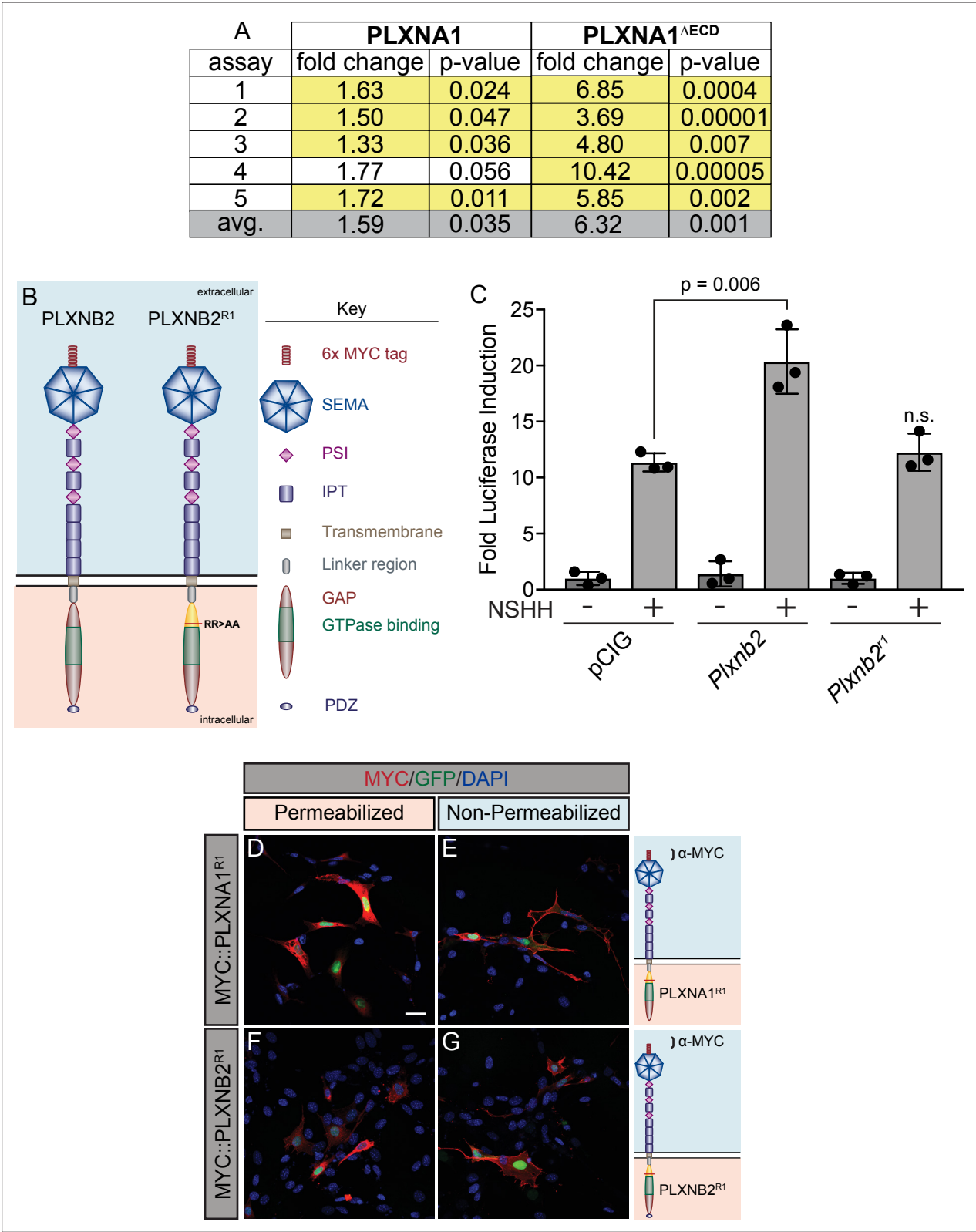


Figure 3—figure supplement 1. Constitutively active PLXNA1 reproducibly increases Hedgehog (HH) pathway activity. **(A)** Summary of luciferase assay data in which PLXNA1 and PLXNA1^{ΔECD} were directly compared in five independent assays. Fold change reported between ligand-stimulated vector only (pCIG) triplicate wells and ligand-stimulated *Plxna1*- or *Plxna1*^{ΔECD} - transfected triplicate wells. Yellow highlight denotes statistical significance (p<0.05). **(B)** Schematic of different PLXNB2 proteins. **(C)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the

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indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant. **(D–G)** Antibody detection of MYC (red) in permeabilized (left panels) and non-permeabilized (right panels) NIH/3T3 cells to assess cell surface localization of the indicated MYC-tagged proteins. Nuclear GFP (green) indicates transfected cells, whereas DAPI (blue) stains all nuclei. Diagrams (right) describe each construct, with brackets indicating antibody binding sites. Scale bar = 10 μ m.

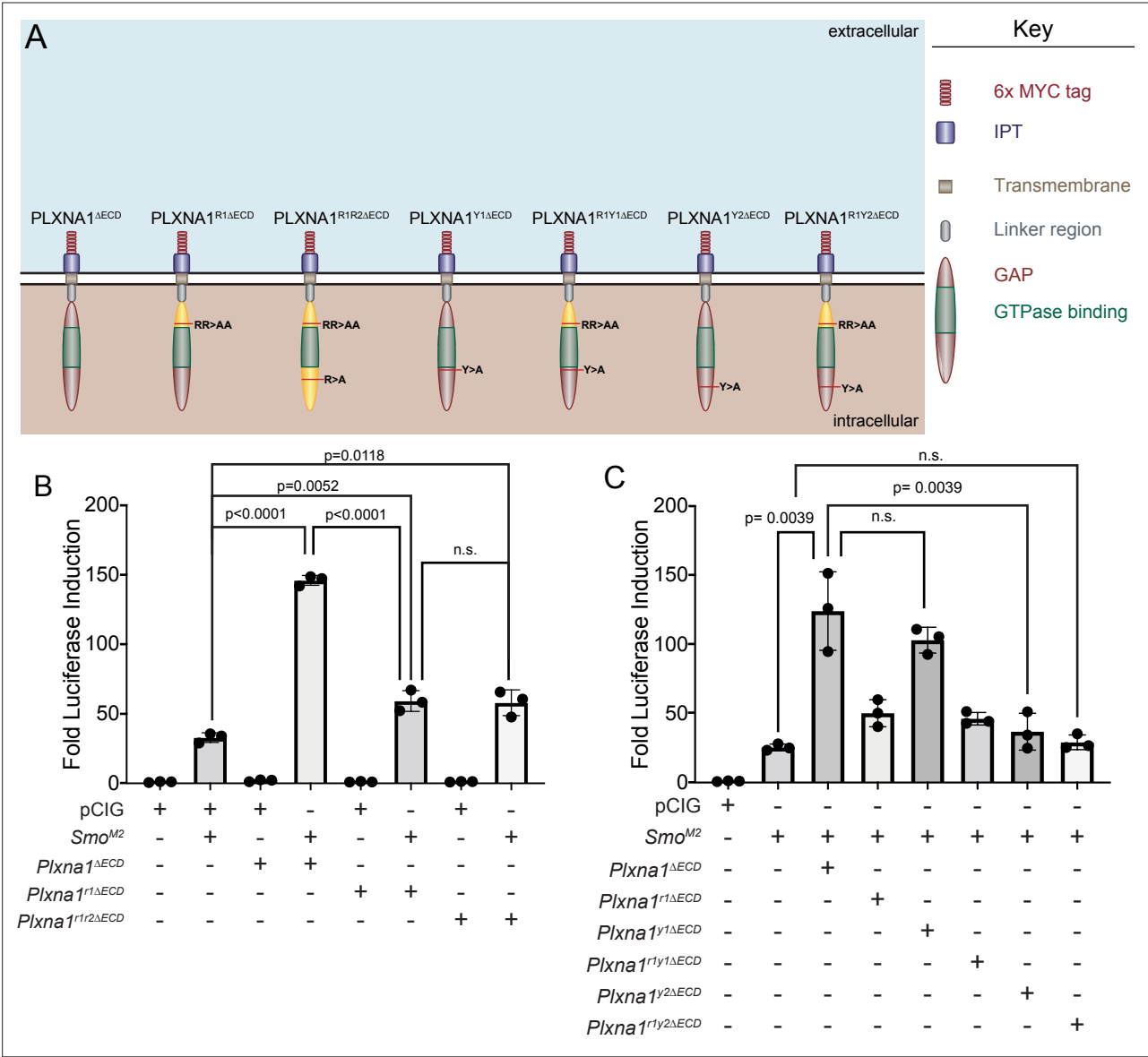
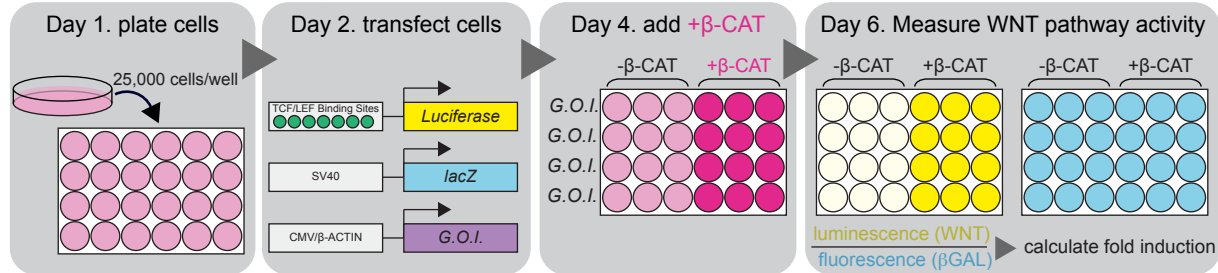
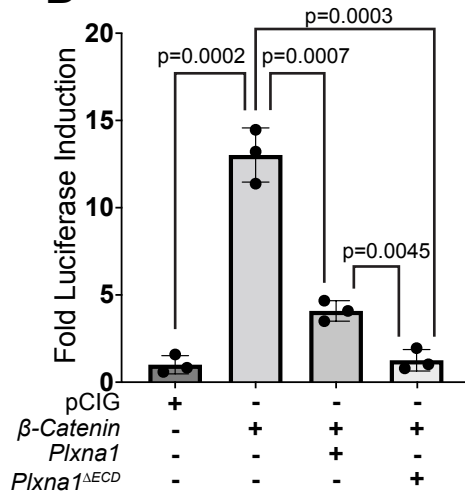


Figure 3—figure supplement 2. The plexin (PLXN) GTPase-activating protein (GAP) domain and a FYN kinase phosphorylation site contribute to PLXN-mediated promotion of Hedgehog (HH) signaling. **(A)** Schematic of different PLXNA1 proteins. **(B, C)** HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with pCIG or Smo^{M2} and co-transfected with indicated constructs. Data are reported as mean fold induction \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant.

A TOP-FLASH Assay



B



C

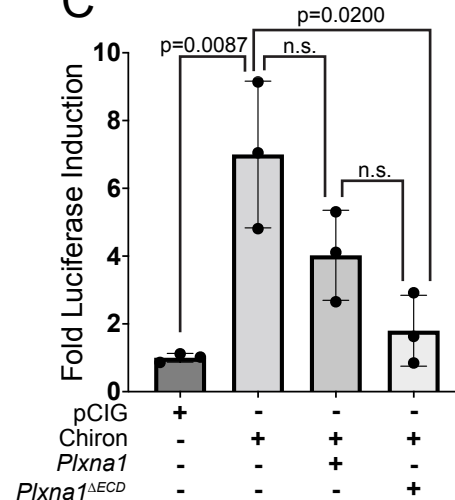


Figure 3—figure supplement 3. Plexins (PLXNs) inhibit WNT signaling. (A) Schematic of WNT-responsive NIH/3T3 TOP-FLASH luciferase assays. G.O.I., gene of interest. (B, C) WNT-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs or empty vector control (pCIG) and stimulated by co-transfection of β -catenin (B) or treatment with Chiron (C). Data are reported as mean fold induction \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant.

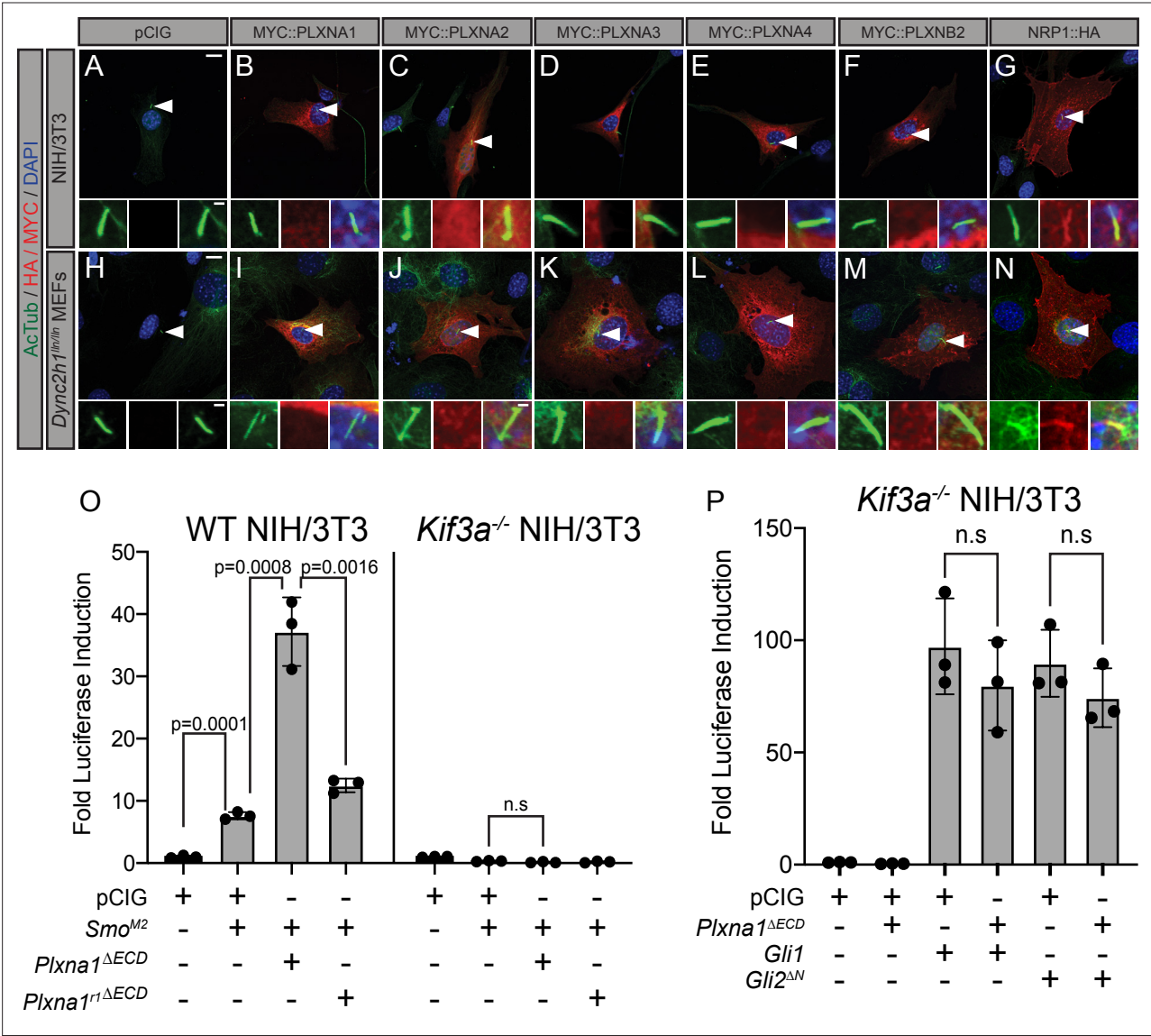


Figure 4. Plexins (PLXNs) do not localize to primary cilia, but do require primary cilia to promote Hedgehog (HH) pathway activity. **(A–N)** Antibody detection of MYC and HA-tagged constructs (red) in NIH/3T3 cells **(A–G)** and *Dync2h1*^{fln/fln} mouse embryonic fibroblasts (MEFs) **(H–N)**. Acetylated tubulin (AcTub, green) indicates the primary cilium and DAPI (blue) stains nuclei. Compared to NRP1, PLXNs are not enriched in primary cilia. Scale bar = 10 μm. Inset scale bar = 1 μm. **(O)** WT NIH/3T3 cells or *Kif3a*^{-/-} NIH/3T3 cells were co-transfected with *Smo*^{M2} and *Plxna1*^{ΔECD} or *Plxna1*^{tr1ΔECD}. **(P)** *Kif3a*^{-/-} NIH/3T3 were transfected with *Gli1* or *Gli2*^{ΔN} and co-transfected with *Plxna1*^{ΔECD}. Data are reported as mean fold induction ± SD, with p-values calculated using two-tailed Student's *t*-tests. n.s., not significant.

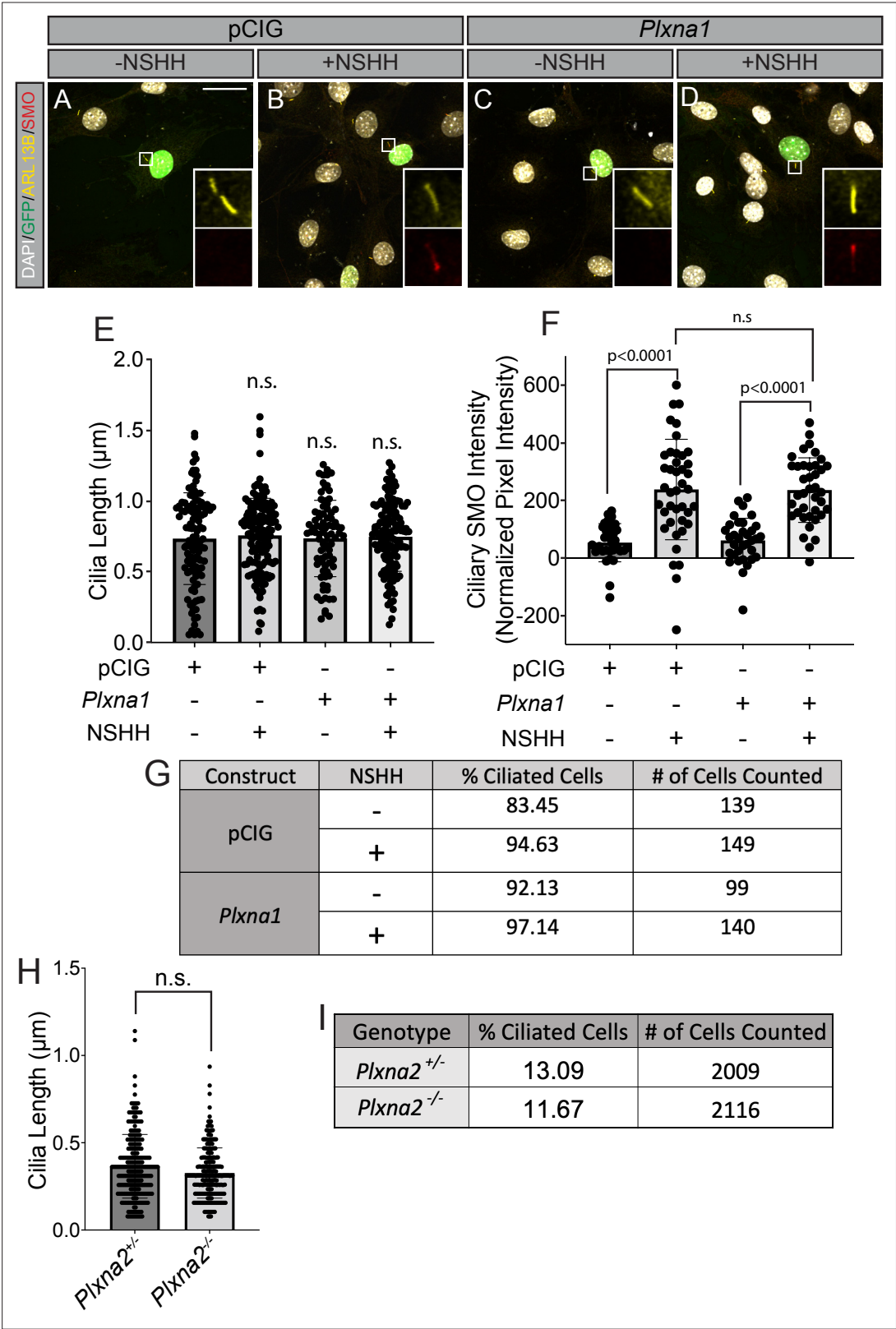


Figure 4—figure supplement 1. Plexins (PLXNs) do not affect Smoothed (SMO) accumulation in primary cilia, ciliary length, or ciliation frequency. (A–D) Antibody detection of ARL13B (yellow) and SMO (red) in NIH/3T3 cells transfected with pCIG or *Plxna1*. Nuclear GFP (green) indicates transfected cells and DAPI (gray) stains nuclei. Average cilia length (E), normalized intensity of SMO staining within cilia (F), and rate of ciliation (G) of cells transfected with pCIG or *Plxna1* and treated with control (-NSHH) or NSHH-conditioned media (+NSHH) were measured. No significant differences in

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ciliation frequency were observed between pCIG and *Plxna1* expressing cells in the absence ($\chi^2 = 3.658$, $p=0.0558$) or presence ($\chi^2 = 1.144$, $p=0.2847$) of NSHH (E). Average ciliary length (H) and rate of ciliation (I) were measured using microscope images of sections from the hippocampus of postnatal day 7 (P7) *Plxna2*^{-/-} mice (n = 4) compared to heterozygous littermates (n = 4) stained with antibodies directed against ARL13B in order to visualize cilia. Ciliation frequency between *Plxna2*^{+/-} and *Plxna2*^{-/-} littermates was not significantly different ($\chi^2 = 1.9128$, $p=0.1667$). Data are reported as average value \pm SD, with p-values calculated using two-tailed Student's t-tests. n.s., not significant.

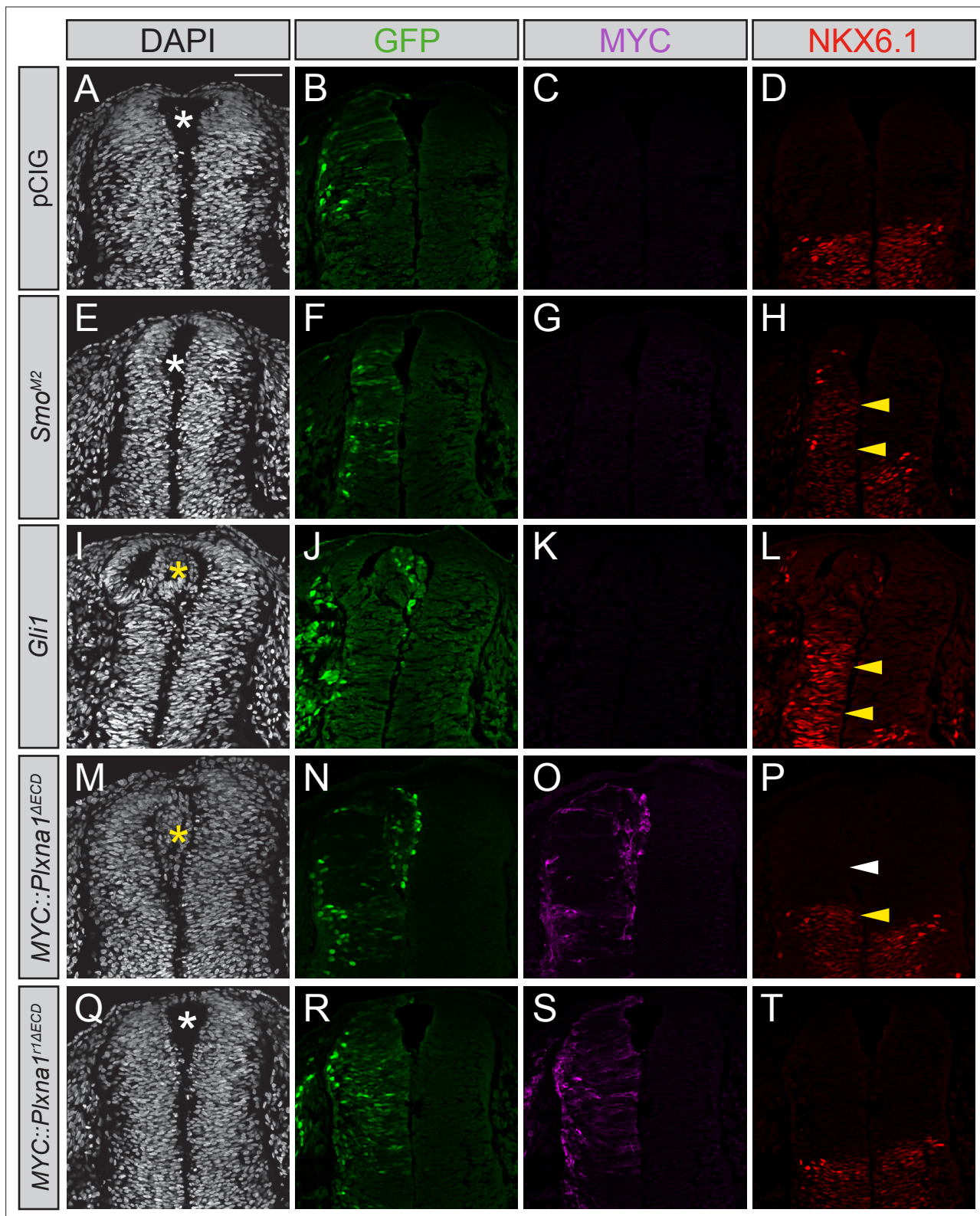


Figure 5. Constitutively active PLXNA1 induces ectopic cell migration into the lumen of the developing chicken neural tube. (A–T) Immunofluorescent analysis of neural patterning in forelimb-level sections from Hamburger–Hamilton stage 21–22 chicken embryos. Embryos were electroporated at Hamburger–Hamilton stage 11–13 with pCIG (A–D, n = 6 embryos), *Smo^{M2}* (E–H, n = 7 embryos), *Gli1* (I–L, n = 4 embryos), *MYC::Plxna1^{ΔECD}* (M–P, n = 17 embryos), or *MYC::Plxna1^{r1ΔECD}* (Q–T, n = 6 embryos). Transverse sections were stained with GFP, MYC, and NKX6.1 antibodies. DAPI stain labels nuclei (gray). Electroporated cells are labeled with GFP. Asterisks denote the presence (yellow) or absence (white) of ectopic cells within the lumen of the neural tube. Arrowheads denote the presence (yellow) or absence (white) of ectopic NKX6.1. Scale bar = 50 μm.

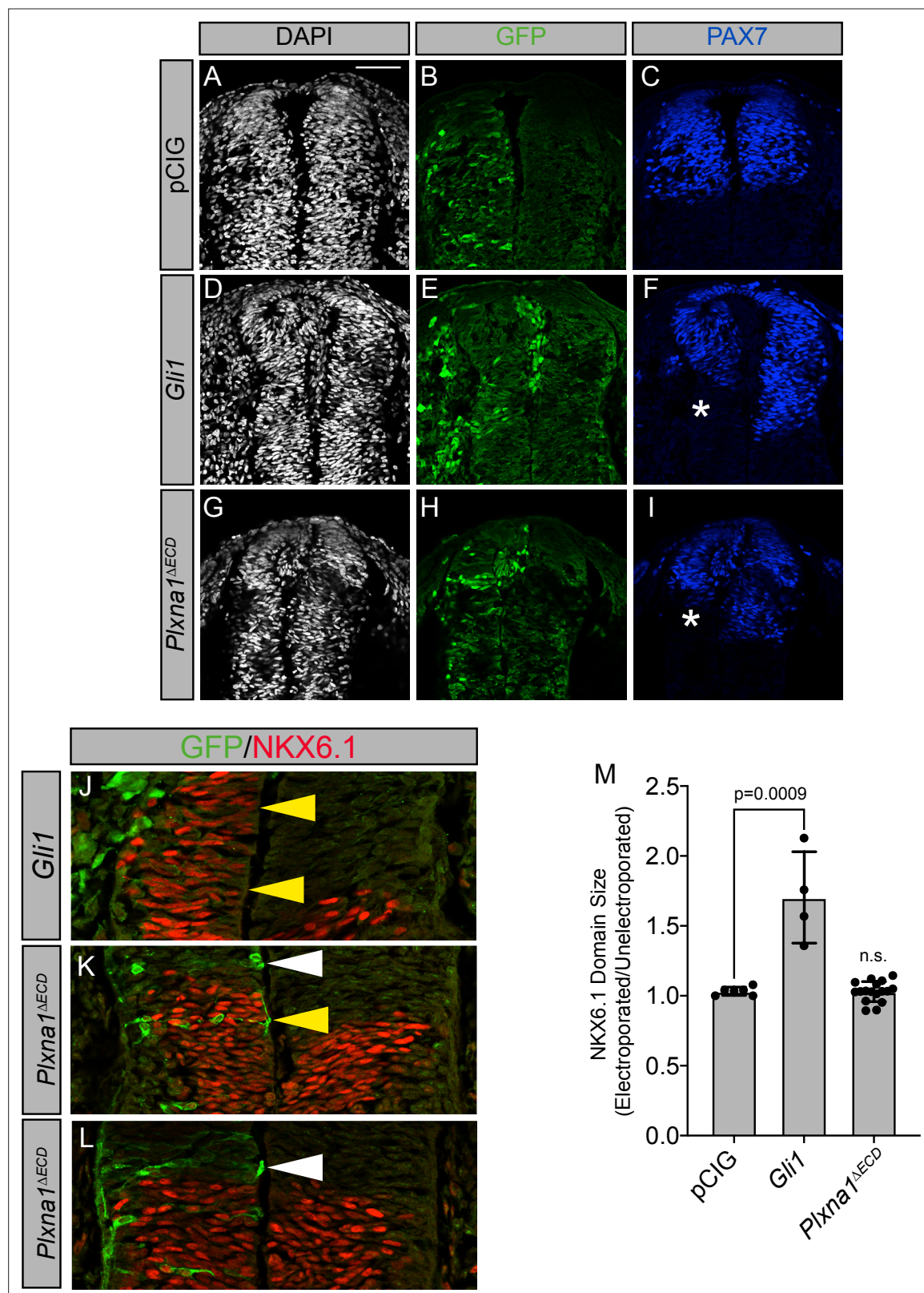


Figure 5—figure supplement 1. Constitutively active PLXNA1 does not significantly alter Hedgehog-dependent neural tube patterning in the developing chicken embryo. (A–M) Immunofluorescent analysis of neural patterning in forelimb-level sections from Hamburger–Hamilton stage 21–22 chicken embryos. Embryos were electroporated at Hamburger–Hamilton stage 11–13 with pCIG (A–C), *Gli1* (D–F, J), or *Plxna1 Δ ECD* (G–I, K, L). Transverse sections were stained with antibodies directed against GFP (green), PAX7 (blue), and NKX6.1 (red). DAPI stain labels nuclei (gray). Electroporated

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cells are labeled with GFP. Asterisks denote the loss of PAX7. Scale bar = 50 μ m. Yellow arrowheads indicate the presence of ectopic NKX6.1. White arrowheads (**K**, **L**) denote the absence of ectopic NKX6.1 in *Plxna1 Δ ECD*-electroporated embryos as compared to *Gli1* (**J**). Quantitation of NKX6.1 domain size normalizing electroporated and unelectroporated sides of developing chicken neural tubes electroporated with pCIG, *Gli1*, or *Plxna1 Δ ECD*. Values are reported as mean \pm SD, with p-values calculated using two-tailed Student's t-test.

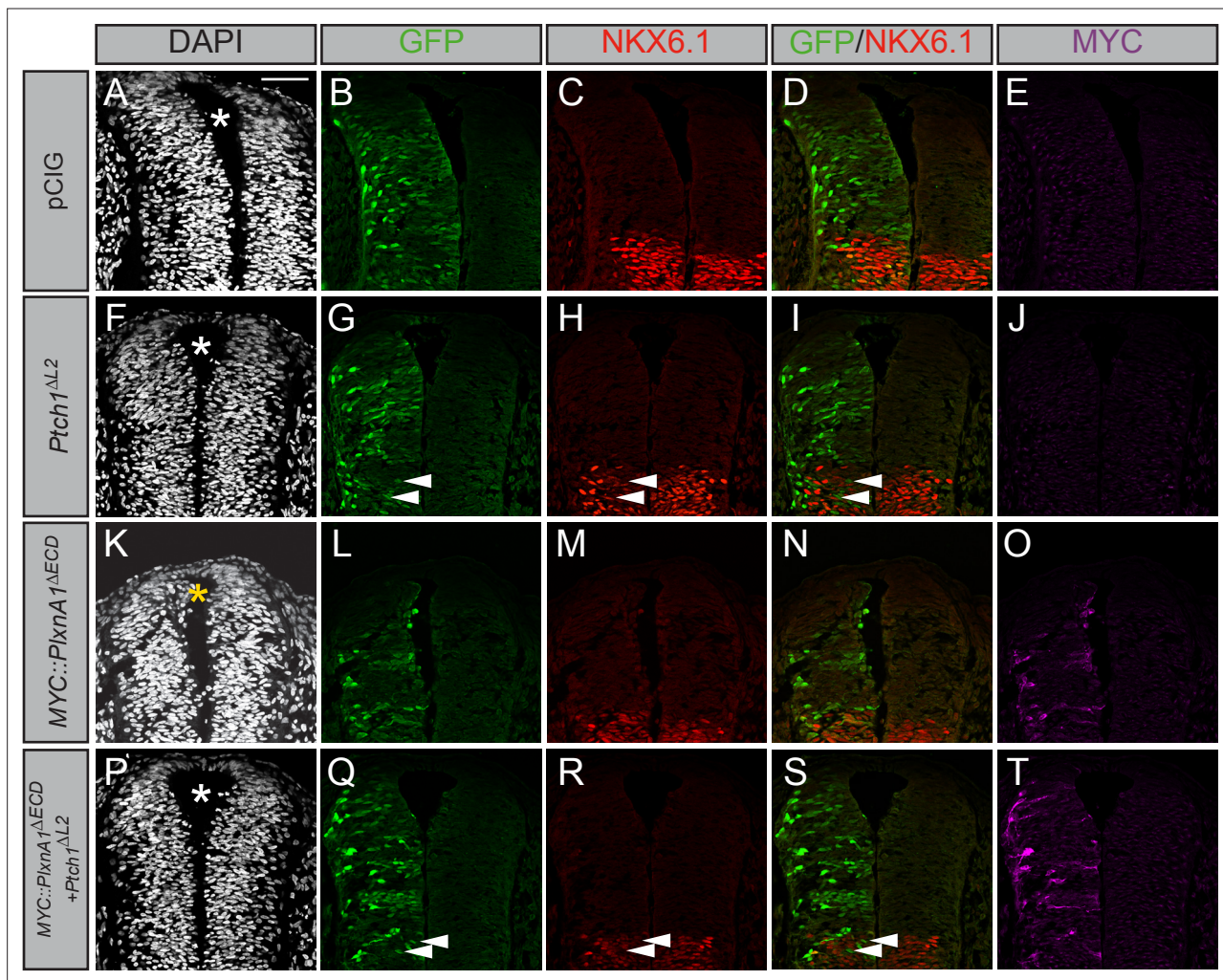


Figure 6. Plexin (PLXN)-mediated ectopic cell migration is Hedgehog (HH)-dependent. (A–T) Immunofluorescent analysis of neural patterning in forelimb-level sections from Hamburger–Hamilton stage 21–22 chicken embryos. Embryos were electroporated at Hamburger–Hamilton stage 11–13 with pCIG (A–E, $n = 6$ embryos), MYC::*Plxna1*^{ΔECD} (F–J, $n = 7$ embryos), *Ptch1*^{ΔL2} (K–O, $n = 5$ embryos), or MYC::*Plxna1*^{ΔECD} and *Ptch1*^{ΔL2} (P–T, $n = 8$ embryos). Transverse sections were stained with GFP, MYC, and NKX6.1 antibodies. DAPI stain labels nuclei (gray). Electroporated cells are labeled with GFP. Asterisks denote the presence (yellow) or absence (white) of ectopic cells within the lumen of the neural tube. Arrowheads denote absence of NKX6.1 in electroporated cells. Scale bar = 50 μ m.

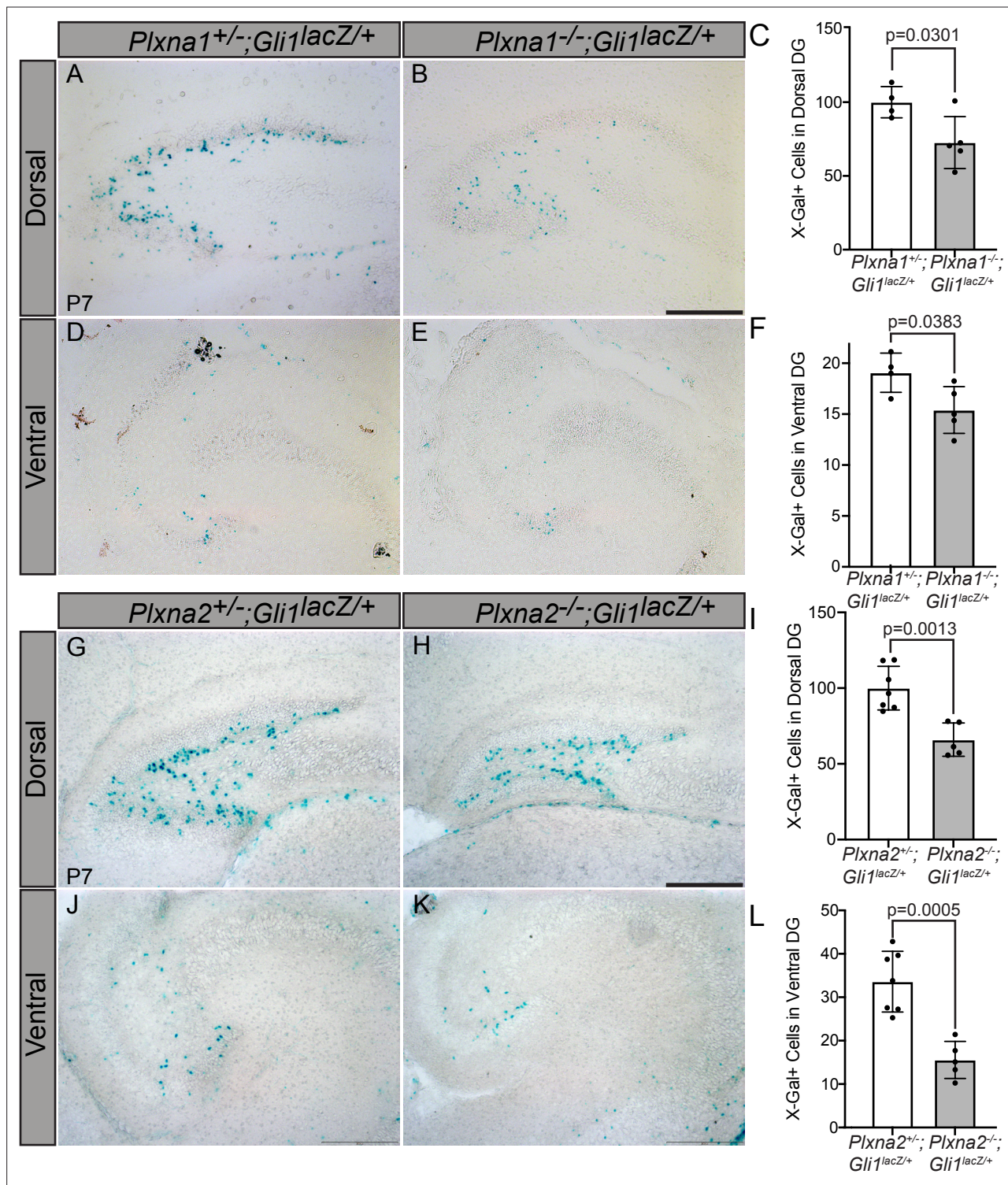


Figure 7. Reduced *Gli1^{lacZ}* expression in the dentate gyrus (DG) of mice lacking either *Plxna1* or *Plxna2*. X-Gal staining in coronal sections through the dorsal (A, B, G, H) and ventral (D, E, J, K) hippocampus of postnatal day 7 (P7) mice. The following numbers of pups were analyzed: *Plxna1^{+/-}; Gli1^{lacZ/+}* (n = 4); *Plxna1^{-/-}; Gli1^{lacZ/+}* (n = 5); *Plxna2^{+/-}; Gli1^{lacZ/+}* (n = 7); *Plxna2^{-/-}; Gli1^{lacZ/+}* (n = 5). Quantitation of *Gli1^{lacZ}*-positive cells (C, F, I, L) reported as mean \pm SD, with p-values calculated using two-tailed Student's t-test. Scale bar = 200 μ m.

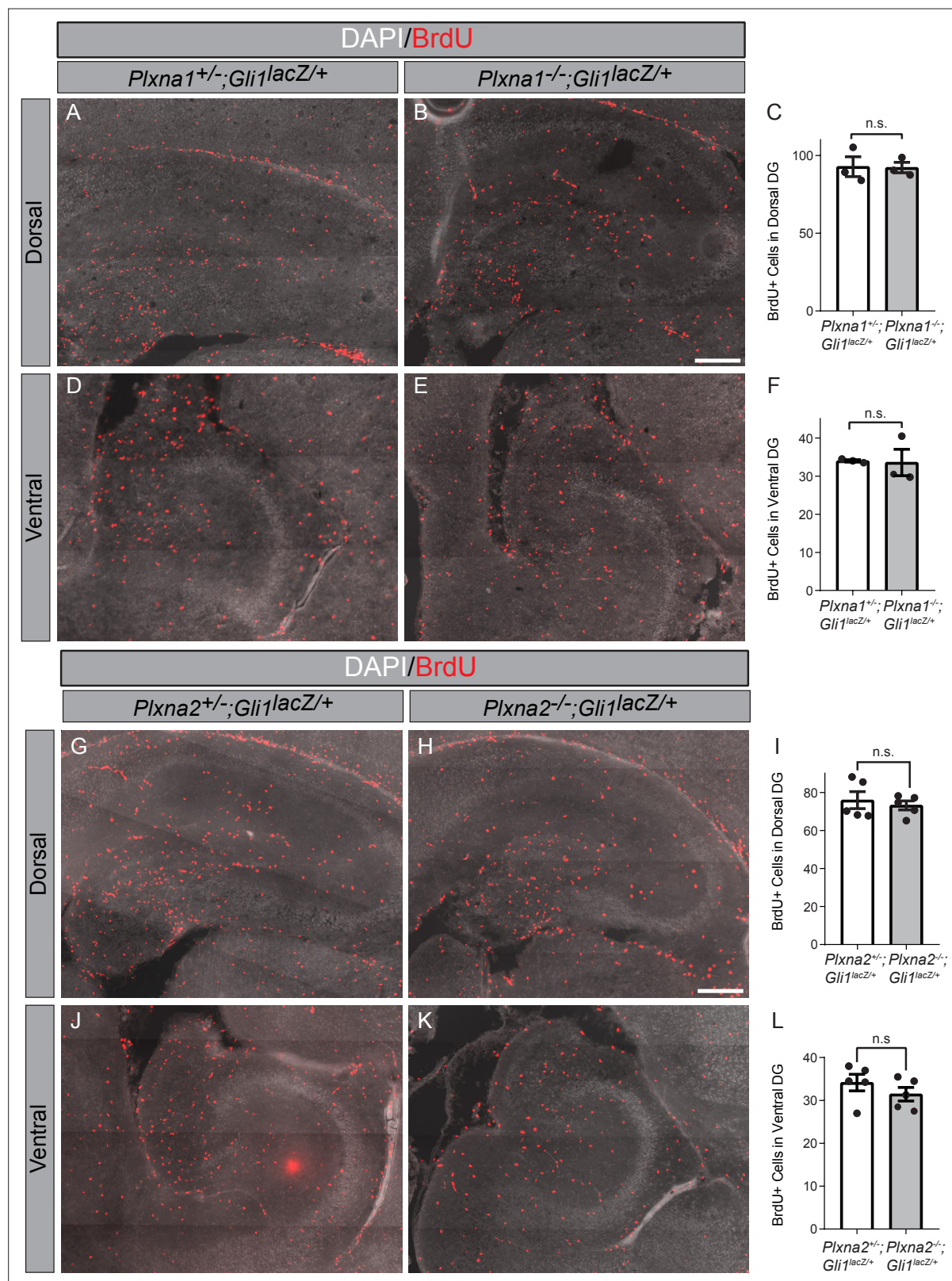


Figure 7—figure supplement 1. *Plxna1* and *Plxna2* deletion do not alter cell proliferation in the hippocampus. BrdU staining in coronal sections through the dorsal (A, B, G, H) and ventral (D, E, J, K) hippocampus of postnatal day 7 (P7) mice. The following numbers of pups were analyzed: *Plxna1*^{+/-}; *Gli1*^{lacZ/+} (n = 3); *Plxna1*^{-/-}; *Gli1*^{lacZ/+} (n = 3); *Plxna2*^{+/-}; *Gli1*^{lacZ/+} (n = 5); *Plxna2*^{-/-}; *Gli1*^{lacZ/+} (n = 5). Quantitation of BrdU-positive cells (C, F, I, L) reported as mean ± SD, with p-values calculated using two-tailed Student's t-test. n.s., not significant. DG, dentate gyrus. Scale bar = 200 μm.

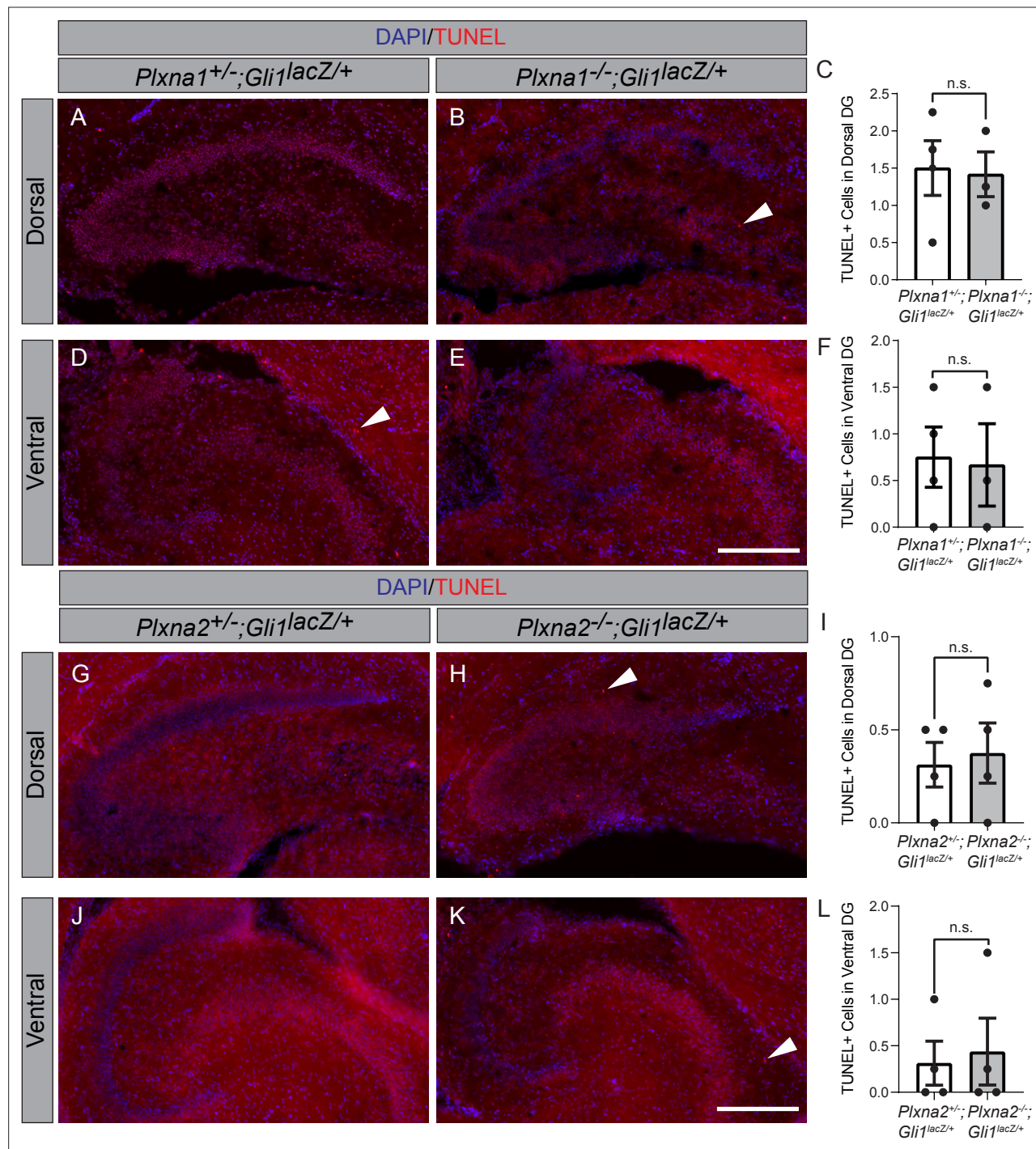


Figure 7—figure supplement 2. *Plxna1* and *Plxna2* deletion does not alter apoptosis in the hippocampus. TUNEL staining in coronal sections through the dorsal (A, B, G, H) and ventral (D, E, J, K) hippocampus of postnatal day 7 (P7) mice. The following numbers of pups were analyzed: *Plxna1*^{+/-}; *Gli1*^{lacZ/+} (n = 4); *Plxna1*^{-/-}; *Gli1*^{lacZ/+} (n = 3); *Plxna2*^{+/-}; *Gli1*^{lacZ/+} (n = 4); *Plxna2*^{-/-}; *Gli1*^{lacZ/+} (n = 4). Quantitation of TUNEL-positive cells (C, F, I, L, arrowheads) reported as mean ± SD, with p-values calculated using two-tailed Student's t-test. n.s., not significant. DG, dentate gyrus. Scale bar = 200 μm.

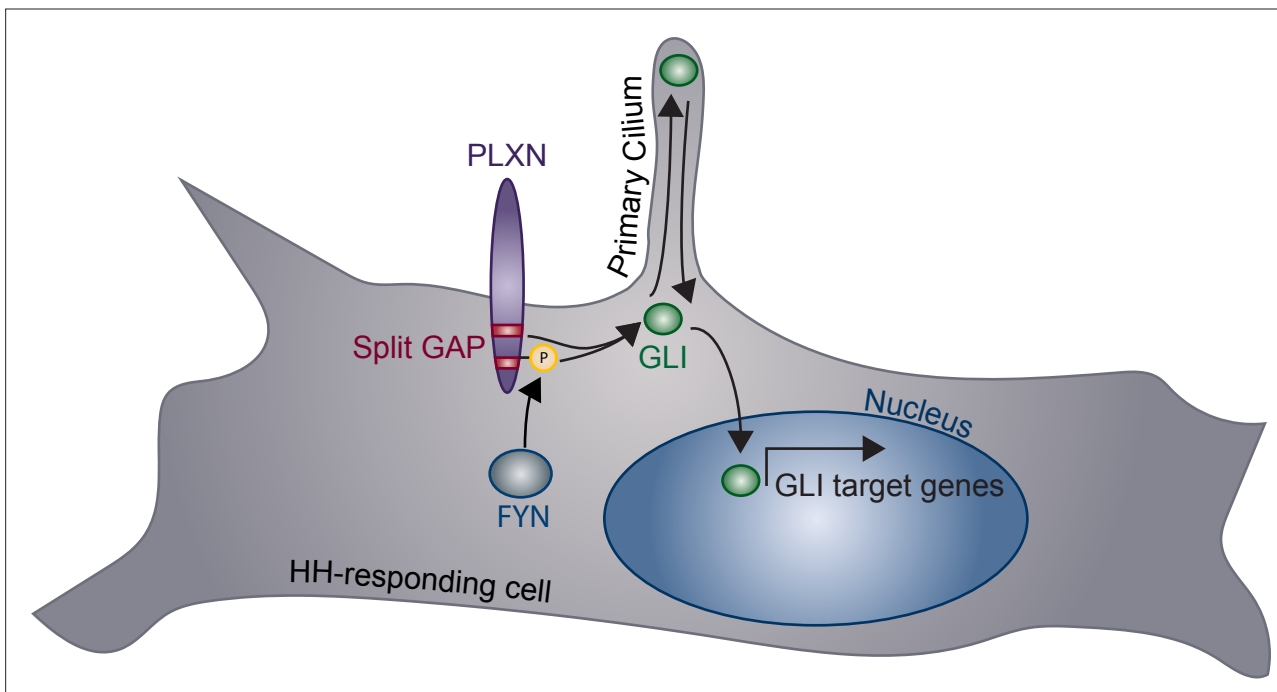


Figure 8. Model of plexin (PLXN)-mediated promotion of Hedgehog (HH) pathway activity. PLXNs (purple) at the cell surface promote HH signaling through GLI transcription factor (green) activation, mediated by their cytoplasmic GTPase-activating protein (GAP) activity (red) and FYN kinase phosphorylation (yellow). Notably, this PLXN-dependent promotion requires primary cilia to induce GLI target gene expression in the nucleus.