

1 **Plexins Promote Hedgehog Signaling Through Their Cytoplasmic GAP Activity**

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Abstract

Hedgehog signaling controls tissue patterning during embryonic and postnatal development and continues to play important roles throughout life. Characterizing the full complement of Hedgehog pathway components is essential to understanding its wide-ranging functions. Previous work has identified Neuropilins, established Semaphorin receptors, as positive regulators of Hedgehog signaling. Neuropilins require Plexin co-receptors to mediate Semaphorin signaling, but a role for Plexins in Hedgehog signaling has not yet been explored. Here, we provide evidence that multiple Plexins promote Hedgehog signaling in NIH/3T3 fibroblasts and that Plexin loss-of-function in these cells results in significantly reduced Hedgehog pathway activity. Catalytic activity of the Plexin GTPase activating protein (GAP) domain is required for Hedgehog signal promotion, and constitutive activation of the GAP domain further amplifies Hedgehog signaling. Additionally, we demonstrate that Plexins promote Hedgehog signaling at the level of GLI transcription factors and that this promotion requires intact primary cilia. Finally, we find that Plexin loss-of-function significantly reduces the response to Hedgehog α pathway activation in the mouse dentate gyrus. Together, these data identify Plexins as novel components of the Hedgehog pathway and provide insight into their mechanism of action.

Introduction

The Hedgehog (HH) signaling pathway utilizes a core set of components to coordinate diverse cellular processes. In the absence of HH ligand, the twelve-pass transmembrane protein Patched 1 (PTCH1) inhibits pathway activity by repressing a second cell-surface protein Smoothened (SMO), a seven-pass transmembrane protein with GPCR-like activity (Alcedo et al. 1996; Marigo and Tabin 1996; Stone et al. 1996; van den Heuvel and Ingham 1996). HH ligand binding to PTCH1 leads to de-repression of SMO, which shifts the processing of GLI transcription factors from repressor to activator forms, thus altering the balance of HH target gene expression (Hui and Angers 2011). By balancing the activity of these key molecules, HH signaling directs embryonic and postnatal development as well as adult tissue homeostasis in a wide variety of cellular contexts. In contrast, HH pathway disruption can drive a number of diseases, including cancer (Teglund and Toftgard 2010; Briscoe and Therond 2013; Petrova and Joyner 2014).

Beyond these core pathway components, a growing list of additional proteins regulate HH signaling at the cell surface in a tissue- and stage-specific manner (Beachy et al. 2010). Some examples include growth arrest-specific 1 (GAS1); CAM-related/downregulated by oncogenes (CDON); brother of CDON (BOC); PTCH1 homolog Patched 2 (PTCH2); Hedgehog interacting protein (HHIP); Dispatched (DISP); Signal peptide, CUB domain, EGF-like 2 (Scube2); G-protein-coupled-receptor 161 (GPR161); glypicans (GPCs); and low-density lipoprotein receptor-related 2 (LRP2) (Burke et al. 1999; Caspary et al. 2002; Kawakami et al. 2002; Ma et al. 2002; Jeong and McMahon 2005; Kawakami et al. 2005; Woods and Talbot 2005; Hollway et al. 2006;

Vyas et al. 2008; Yan and Lin 2008; Christ et al. 2012; Creanga et al. 2012; Tukachinsky et al. 2012; Mukhopadhyay et al. 2013; Bandari et al. 2015; Christ et al. 2015). Notably, many of these components act redundantly to mediate HH signal transduction (Zhang et al. 2001; Jeong and McMahon 2005; Allen et al. 2007; Allen et al. 2011; Izzi et al. 2011; Holtz et al. 2013). As a result, previous genetic screens may have missed additional regulators of the HH pathway due to their redundant nature. Furthermore, gene duplication events and increased complexity within vertebrate HH signaling, including a requirement for the primary cilium, have made it difficult to study HH regulators that lack invertebrate counterparts, such as Scube2 and GAS1. Therefore, our overall understanding of HH regulation remains incomplete.

The Semaphorins (SEMA) are a large family of membrane-bound and secreted proteins that regulate cell migration, axon guidance, synapse assembly, angiogenesis, immune function, and cell death (Yazdani and Terman 2006; Jongbloets and Pasterkamp 2014; Koropouli and Kolodkin 2014; Fard and Tamagnone 2021). NRPs directly interact with class 3 SEMA ligands and require Plexin (PLXN) co-receptors to transduce SEMA signals intracellularly (Chen et al. 1997; He and Tessier-Lavigne 1997; Kolodkin et al. 1997; Takahashi et al. 1999; Tamagnone et al. 1999; Gu et al. 2005). Membrane-bound SEMA and Sema3E interact directly with PLXN extracellular domains to activate downstream signaling events, which lead to remodeling and disassembly of the cytoskeleton (Barberis et al. 2004; Neufeld and Kessler 2008; Jongbloets and Pasterkamp 2014; Rich et al. 2021). PLXNs are a family of conserved, single-pass transmembrane proteins containing nine different receptor types, which fall into four subfamilies based on homology (A, B, C, and D) (Tamagnone et al. 1999). The cytoplasmic domain of all

PLXN family members harbors a GTPase activating protein (GAP) domain (Rohm et al. 2000b; Wang et al. 2012). Catalytic activity of the PLXN GAP domain is necessary for SEMA mediated cytoskeletal remodeling and cell migration (Hota and Buck 2012; Wang et al. 2013; Zhao et al. 2018). Importantly, there is a mechanistic link between HH and NRPs. Multiple lines of evidence show that NRPs positively regulate HH signaling through their cytoplasmic domains (Ge et al., 2015; Hillman et al., 2011; Pinskey et al., 2017); however, a role for PLXNs in HH signaling remains unexplored.

Here, we investigated a role for PLXNs in HH pathway regulation. Our data suggest that multiple PLXNs, including members of the PLXN A and B subfamilies, positively regulate HH signaling. Similar to NRPs, we find that the PLXN cytoplasmic domain is necessary for HH regulation. Interestingly, while the mechanism of NRP action in HH signaling may diverge from its mechanism in SEMA signaling (Andreyeva et al. 2011; Ge et al. 2015; Pinskey et al. 2017), we discover that PLXNs function similarly in SEMA and HH cascades. Mutating key residues within the cytoplasmic PLXN GAP domain prevents PLXN from promoting HH signaling. Further, deleting the PLXN extracellular domain to create a constitutively active receptor augments HH promotion and alters HH-dependent tissue patterning and cell migration in the embryonic chicken neural tube, suggesting that PLXNs positively regulate HH signaling through GAP enzymatic activity. Additionally, we determine that PLXNs act at the level of the GLI transcription factors, and that PLXNs require intact primary cilia to promote HH pathway activity. In the developing mouse hippocampus, we observe PLXN dependent regulation of HH target gene expression in the dentate gyrus, *in vivo*. Taken together, these data

identify PLXNs as novel components of the HH pathway and contribute to our mechanistic understanding of HH regulation at the cell surface.

Results

Multiple Plxns promote HH signaling in NIH/3T3 fibroblasts

PLXNs consist of nine members that can be classified into four different subfamilies based on homology (PLXNA1-4, PLXNB1-3, PLXNC1, and PLXND1) (Tamagnone et al. 1999; Neufeld and Kessler 2008). PLXNs from the A and D subfamilies interact with NRP co-receptors (Takahashi et al. 1999; Neufeld and Kessler 2008), which have been previously identified as positive regulators of HH signaling (Hillman et al. 2011; Ge et al. 2015; Pinskey et al. 2017). We initially investigated whether *Plxna1* expression in HH-responsive NIH/3T3 fibroblasts would impact HH signaling using a luciferase reporter assay ((Nybakken et al. 2005); Figure 1A). Strikingly, and similar to what we previously observed with *Nrpl* (Pinskey et al. 2017), *Plxna1* expression significantly increases HH pathway activation compared to a vector-transfected control (Figure 1B). Of note, PLXNA1 does not promote HH signaling in the absence of pathway activation with HH ligand (Figure 1B). To address whether HH promotion was specific to PLXNA1, we also examined PLXNA2, PLXNA3, and PLXNA4. Our data suggest that all members of the PLXN A subfamily promote HH signaling following pathway activation with HH ligand (Figure 1C-E). We extended our analyses to include PLXNB2, which is not known to interact with NRPs (Neufeld and Kessler 2008). Surprisingly, PLXNB2 also promotes HH signaling to a similar extent as PLXNs from the A subfamily, suggesting that PLXN-mediated HH promotion may be

independent of NRP interaction (Figure 1F-G). Importantly, GFR α 1, an unrelated cell-surface protein within the glial cell line-derived neurotrophic factor receptor (GFR) family, does not promote HH signaling (Figure 1G). Taken together, these data suggest that multiple PLXN family members promote HH signaling in NIH/3T3 cells.

Plxn knockdown decreases HH-responsiveness in NIH/3T3 fibroblasts

According to RNA sequencing data from the ENCODE project (Consortium 2012; Davis et al. 2018), NIH/3T3 fibroblasts express a subset of Plxns at varying levels, with *Plxna1* and *Plxnb2* most highly expressed, followed by *Plxnd1*, *Plxna3*, and *Plxna2* (Figure S1). To address the effect of endogenous PLXNs on HH signaling in NIH/3T3 cells, we generated two different *Plxna1*^{-/-};*Plxna2*^{-/-} mouse embryonic fibroblast lines from embryonic day (E) 14.5 mouse embryos (Todaro and Green 1963). We then used quantitative, real-time polymerase chain reaction (RT-qPCR) to analyze HH target gene expression in fibroblasts treated with a SMO agonist (SAG; Figure 1H-I). In each experiment, we used BLOCK-iTTM fluorescent oligos to visually confirm transfection, and we compared each result to an internal BLOCK-iTTM transfected control (Figure 1H-I). Therefore, fold changes in expression are relative within each experiment and should not be compared across panels. Interestingly, both cell lines lacking *Plxna1* and *Plxna2* still respond to SAG activation of HH signaling, as measured by expression of the direct HH transcriptional targets, *Gli1* and *Ptch1* (Figure 1H-I). We hypothesized that this was likely due to the presence of other Plxn family members, which could compensate for the lack of PLXNA1 and PLXNA2.

To address the potential functional redundancy of other Plxn family members, we used siRNA reagents to reduce levels of *PlxnB2*, *PlxnA3*, and *PlxnD1* in *Plxna1*^{-/-}; *Plxna2*^{-/-} cells. Strikingly, both cell lines treated with the *Plxn* siRNAs listed above showed significantly reduced responses to SAG activation of *Gli1* and *Ptch1* compared to BLOCK-iT™ controls (Figure 1H-I). The degree of reduction following *Plxn* depletion is similar to that observed with *Nrp* depletion using previously published siRNA reagents targeting *Nrp1* and *Nrp2* (Hillman et al. 2011) (Figure 1H-I). Together, these data suggest that, like NRPs, PLXNs are required for HH signal transduction in NIH/3T3 fibroblasts.

The PLXNA1 transmembrane and cytoplasmic domains are necessary for HH signal promotion

PLXNs are single-pass transmembrane proteins containing an extracellular domain (ECD) that can interact with NRPs and SEMA ligands, a transmembrane (TM) domain that mediates dimerization, and a cytoplasmic domain (CD) through which PLXNs signal intracellularly (Neufeld and Kessler 2008). While many HH regulators at the cell surface bind to HH ligands through their ECD (Lee et al. 2001; Tenzen et al. 2006; Capurro et al. 2008; Chang et al. 2011; Izzi et al. 2011; Christ et al. 2012; Whalen et al. 2013), NRP1 acts through its CD to regulate HH signaling (Ge et al. 2015; Pinskey et al. 2017). To investigate the mechanism of PLXN action in HH signaling, we first asked whether the PLXN CD is required for HH promotion. Interestingly, deleting the PLXNA1 TM and CD (PLXNA1^{ΔTMCD}) or the CD alone (PLXNA1^{ΔCD}) abrogates PLXNA1-mediated promotion of HH signaling in NIH/3T3 cells (Figure 2A-B). Western blot analyses confirmed PLXNA1, PLXNA1^{ΔTMCD}, and PLXNA1^{ΔCD} expression and

PLXNA1^{ΔTMCD} secretion (Figure 2C). Further, immunofluorescence staining for an extracellular MYC epitope under permeabilizing and non-permeabilizing conditions confirmed the cell surface localization of PLXNA1 and PLXNA1^{ΔCD} as well as the secretion of PLXNA1^{ΔTMCD}, as compared to a control BOC construct with a C-terminal MYC tag (Figure 2D-K). These results suggest that the PLXNA1 TM and CD are required for promotion of HH signaling.

PLXN cytoplasmic GAP activity mediates HH signal promotion

Upon binding to the PLXN extracellular SEMA domain, SEMA ligand triggers a conformational change, releasing PLXN autoinhibition and allowing for the full activation of the intracellular GAP (Takahashi and Strittmatter 2001; Janssen et al. 2010; Nogi et al. 2010). As a result, deleting the autoinhibitory PLXN ECD results in constitutively GAP activity that induces robust cytoskeletal collapse through downstream signaling events (Takahashi and Strittmatter 2001; Hota and Buck 2012). To further test whether PLXN GAP function regulates HH signaling, we deleted the PLXNA1 ECD (PLXNA1^{ΔECD}) and measured HH-dependent luciferase reporter activity in NIH/3T3 cells (Figure 3A). Not only is PLXNA1^{ΔECD} still able to promote HH signaling, but the constitutively active PLXN GAP domain significantly augments the level of HH promotion (Figure 3B). While full-length PLXN boosts HH signaling one and a half to two-fold on average, PLXNA1^{ΔECD} consistently increases the level of HH signaling between four- and ten-fold, averaging an approximately six-fold increase (Figure S2A).

The PLXN CD is essential for intracellular SEMA signal transduction, acting through a split GAP domain to induce cytoskeletal collapse (Puschel 2007; Neufeld and

Kessler 2008; Duan et al. 2014). Arginine to alanine mutations in residues 1429 and 1430 of mouse PLXNA1 disrupt GAP activity, rendering PLXNA1 a nonfunctional SEMA receptor in a COS7 cell collapse assay (Rohm et al. 2000a). Strikingly, recapitulating these conserved arginine mutations within the PLXNA1 GAP domain also rendered PLXNA1 unable to promote HH signaling (PLXNA1R1; Figure 3C). Importantly, analogous mutations in PLXNB2 also abrogate the promotion of HH pathway activity (PLXNB2R1; Figure S2B-C). Further, the A1R1 arginine to alanine GAP mutations in the context of the PLXNA1 ECD deletion significantly reduces the level of HH promotion, though it does not completely abrogate PLXN-mediated HH pathway induction when compared with PLXNA1^{ΔCD} (Figure 3D). Immunofluorescence analyses indicated appropriate localization of these constructs to the cell surface, compared to a C-terminally tagged BOC control, as well as cytoskeletal collapse in PLXNA1^{ΔECD} and to some extent PLXNA1, with the expected lack of collapse in PLXNA1R1^{ΔECD} and PLXNA1^{ΔCD} (Figure 3E-N; Figure S2D-G). Together, these results suggest that GAP activity is necessary for PLXN-mediated promotion of HH signaling.

PLXNA1 promotes HH signaling at the level of GLI activation

HH signaling culminates in the differential processing and activation of the GLI family of transcription factors, which shuttle in and out of the primary cilium and are phosphorylated by several kinases to regulate their activity (Hui and Angers 2011). Transfecting *SmoM2*, a constitutively active form of Smoothened, or *Gli1*, an obligate HH activator, into our luciferase reporter assay in NIH/3T3 cells results in tens to thousands of fold induction of HH reporter activity, respectively. Still, co-transfecting

SmoM2 or *Gli1* with *Plxna1^{ΔECD}* results in a significantly greater HH response (Figure 3O-P). Notably, this promotion requires GAP activity as co-transfection of *SmoM2* or *Gli1* with a GAP-deficient *Plxn* (*Plxna1r1^{ΔECD}*) returns HH pathway activation to near-baseline levels (Figure 3O-P). These data suggest that PLXNs function downstream of HH ligand at the level of GLI regulation, and that full PLXN GAP activation via the release of extracellular autoinhibition is necessary for HH promotion with either *SmoM2* or *Gli1*.

PLXNs are not enriched in the primary cilium, but do require primary cilia for HH pathway promotion

The primary cilium is an important platform for HH signaling molecules (Wong et al. 2009; Goetz and Anderson 2010) and many HH pathway components, including NRP, are enriched there (Corbit et al. 2005; Haycraft et al. 2005; Rohatgi et al. 2007; Pinskey et al. 2017). Notably, molecules over 40 kDa are unable to freely diffuse into the primary cilium, requiring active transport to enter this highly regulated subcellular compartment (Kee et al. 2012). To test whether PLXNs localize to the primary cilium, we expressed MYC-tagged PLXNs in NIH/3T3 cells and performed immunofluorescent staining for MYC and Acetylated Tubulin (AcTub), which marks the primary cilium. PLXNs are broadly localized throughout the cell (Figure 4A-N), including the cell surface (cf. Figure 3E-N), but are largely excluded from the nucleus. Unlike NRP1, PLXN staining was not enriched within the primary cilium for any of the constructs we tested (Figure 4A-G). Mouse embryonic fibroblasts (MEFs) with a mutation in the dynein heavy chain (*Dync2h1^{lln/lln}*) exhibit impaired retrograde transport within the cilium,

allowing for more robust detection of accumulated proteins (Ocbina et al. 2011).
However, even in *Dync2h1^{ln/ln}* MEFs, PLXNs still do not accumulate in the primary cilium (Figure 4H-N). These data suggest that PLXN localization to primary cilia is not required to regulate HH signal transduction.

To examine a potential requirement for primary cilia in PLXN-dependent promotion of HH signaling, we performed luciferase assays in WT NIH/3T3 cells as well as *Kif3a^{-/-}* NIH/3T3 cells, which fail to assemble primary cilia (Engelke et al. 2019). As expected, WT NIH/3T3 cells activate HH signaling in response to *SmoM2* transfection, while *Kif3a^{-/-}* NIH/3T3 cells do not (Figure 4O). Notably, *Kif3a^{-/-}* NIH/3T3 cells also do not respond to co-transfection with *SmoM2* and *Plxna1^{ΔECD}* (Figure 4O). Both GLI1 and GLI2ΔN have been reported to promote HH pathway activation in the absence of primary cilia (Haycraft et al. 2005; Wong et al. 2009). We confirmed these data by transfecting *Kif3a^{-/-}* NIH/3T3 cells with either *Gli1* or *Gli2ΔN* (Figure 4P). Strikingly, and distinct from what we observe in WT NIH/3T3 cells, co-transfecting *Kif3a^{-/-}* NIH/3T3 cells with either *Gli1* or *Gli2ΔN* and *Plxna1^{ΔECD}* displayed no further promotion of HH-signaling (Figure 4P; cf. Figure 3P). These data suggest that, while PLXNs do not localize to the primary cilium, primary cilia are required for PLXN-dependent promotion of HH signaling.

Constitutive Plxn GAP activity drives ectopic cell migration in the embryonic chicken neural tube

The developing spinal cord requires HH signaling for proper patterning and development (Dessaud et al. 2008). SHH, which is initially secreted from the notochord,

261 signals in a ventral-dorsal gradient to specify distinct cell fates in the neural tube.

262 Notably, SHH also controls cell proliferation and cell migration in this tissue (Cayuso

263 and Marti 2005; Cayuso et al. 2006). Previous work demonstrated that multiple *Plxns* are

264 expressed in the developing chicken neural tube concomitant with SHH-dependent tissue

265 patterning (Mauti et al. 2006). To investigate potential contributions of PLXNs to these

266 SHH-dependent outcomes, we employed chicken *in ovo* neural tube electroporation.

267 While electroporation with an empty vector (*pCIG*) does not impact neural tube

268 patterning (Fig 5A-D), *SmoM2* electroporation drives ectopic expression of NKX6.1 in

269 the dorsal neural tube, a direct target of HH signaling that is normally restricted ventrally

270 (Fig 5E-H). Similarly, electroporation with *Gli1*, an obligate activator of the HH pathway

271 that drives high levels of HH signaling, also results in expansion of the NKX6.1 domain

272 (Figure 5I-L). However, *Gli1* expression also results in ectopic migration of cells into the

273 dorsal lumen of the neural tube, which is typically completely devoid of cells (Figure 5I;

274 yellow asterisk). Electroporation of *Plxna1^{ΔECD}* phenocopies *Gli1*-induced migration into

275 the lumen of the neural tube (Figure 5M; yellow asterisk). In some *Plxna1^{ΔECD}*-

276 electroporated embryos, we observed a minor shift in the NKX6.1 domain (Figure 5P;

277 yellow arrowhead). We also observed a similar trend in the PAX7 domain, which is

278 largely devoid of HH signaling (Figure S3A-I). However, quantitation of the NKX6.1

279 domain size revealed no significant differences between *pCIG*- and *Plxna1^{ΔECD}*-

280 electroporated embryos (Figure S3J-M). Further, cells electroporated with *Plxna1^{ΔECD}* at

281 the periphery of the endogenous NKX6.1 domain do not express NKX6.1, while cells in

282 this same region that were electroporated with *Gli1* are NKX6.1 positive (Figure S3J-L).

283 Importantly, PLXN-dependent ectopic cell migration is lost upon mutation of the

intracellular PLXN GAP domain (Figure 5Q-T). These data are consistent with our cell signaling assays, which indicate that PLXNs can promote GLI-dependent cellular responses.

Plxna1 or Plxna2 deletion results in decreased numbers of HH-responding cells within the dentate gyrus

Plxns are expressed widely throughout the developing mouse embryo, particularly in the central nervous system (Perala et al. 2005). Interestingly, developing neurons and progenitor cells in the hippocampus express *Plxns* (Cheng et al. 2001) and neuronal progenitor cells rely on HH signaling for proliferation and maintenance, particularly within the dentate gyrus (Machold et al. 2003; Ahn and Joyner 2005). To determine whether PLXNs impact HH signaling in the hippocampus, we crossed *Plxna1* and *Plxna2* deficient mice with a HH-responsive *Gli1lacZ* reporter allele and examined β -galactosidase activity along the rostro-ventral axis of dentate gyrus at postnatal day 7 (P7). Strikingly, *Plxna1*^{-/-} mice have significantly fewer *Gli1*-positive cells in both the dorsal and ventral dentate gyrus compared to their heterozygous littermates (Figure 6A-F). In addition, *Gli1*-positive cells in *Plxna1*^{-/-} mice fail to properly migrate, similar to previously published HH loss-of-function models (Figure 6A-B) (Machold et al. 2003). *Plxna2* deletion has a similar effect on both *lacZ* expression and migration of *Gli1*-positive cells. Significantly fewer β -galactosidase positive cells are detected in the hilus and subventricular zone of the dorsal and ventral dentate gyrus (Figure 6G-L). Together, these data show that PLXNs can regulate HH pathway activation *in vivo* and suggest that multiple PLXNs regulate HH signaling in the developing mouse hippocampus.

307

308 **Discussion**

309 HH signaling plays important roles in tissue formation, homeostasis and repair,
 310 coordinating a number of cellular processes including proliferation, fate specification,
 311 and survival (Briscoe and Thérond 2013). Canonical SEMA receptors, the NRPs and
 312 PLXNs, are expressed in a wide variety of tissues during active HH regulation (Kawasaki
 313 et al. 1999; Peralá et al. 2005; Mauti et al. 2006; Peralá et al. 2012). Here, we present
 314 evidence that PLXNs positively regulate HH signaling. Unlike many previously
 315 described cell surface HH regulators, which interact directly with HH ligands, PLXNs
 316 promote HH signaling through their cytoplasmic domains at the level of GLI regulation
 317 (Figure 7). More specifically, we find that GAP enzymatic activity within the PLXN
 318 cytoplasmic domain is required for HH promotion, and that constitutive GAP activity
 319 further amplifies the HH response. This shows that the PLXN GAP domain is important
 320 for canonical SEMA signaling as well as amplification of HH signaling. Further, we find
 321 that, while PLXNs themselves do not localize to primary cilia, they require primary cilia
 322 to promote HH pathway activity. Finally, our data indicate that increased *Plxn* activity *in*
 323 *ovo* increases cell migration into the neural tube lumen, and *Plxn* deletion *in vivo* results
 324 in reduced HH pathway activity in mice. Taken together, we provide multiple lines of
 325 evidence for a novel role of PLXNs in HH pathway regulation.

326

327 *Semaphorin Receptors Act Promiscuously in Multiple Signaling Pathways*

328 While NRPs and PLXNs were first described as SEMA receptors, they also
 329 function within other signaling pathways (He and Tessier-Lavigne 1997; Kolodkin et al.

1997; Takahashi et al. 1999; Tamagnone et al. 1999). NRPs play roles in VEGF signaling to regulate angiogenesis, and they interact with a wide variety of proteins, including PIGF-2, heparan sulfate, TGF- β 1, HGF, PDGF, FGF, L1-CAM, integrins, and SARS-CoV-2 spike protein (Roth et al. 2008; Prud'homme and Glinka 2012; Muhl et al. 2017; Sarabipour and Mac Gabhann 2021). PLXNs also form complexes with off-track, MET, Ron, scatter factor, and VEGFR2 in various cellular contexts (Winberg et al. 2001; Giordano et al. 2002; Conrotto et al. 2004; Toyofuku et al. 2004). This raises many questions about the nature of these receptors' activities within individual and overlapping signaling contexts. For example, what factors determine whether PLXNs and NRPs function as SEMA receptors and whether they regulate HH signaling? Can these processes happen simultaneously, and if so, how do they influence one another? → Multiple lines of evidence link altered SEMA/PLXN signaling to cancer. Depending on context, aberrant SEMA signaling may promote or suppress tumor growth and lead to various types of cancer (Neufeld et al. 2016). The mechanisms by which altered PLXN signaling influence tumor growth are incompletely understood. A link to increased HH signaling is intriguing because of the well-established role of elevated HH signaling in malignancies.

Another outstanding question is how SEMA ligand impacts HH signaling. A role for SEMA ligands in HH pathway promotion remains unclear as conflicting pieces of evidence exist in the literature. In one study, addition of SEMA ligands in combination with HH ligand or SAG increased HH signaling in NIH/3T3 cells (Ge et al. 2015). Conversely, blocking NRP interaction with SEMA ligand reduces GLI expression (Ge et al. 2015). This model suggests that SEMA ligand increases recruitment of PDE4D to the cell membrane, which interacts with the NRP CD and inhibits PKA, a negative regulator

of GLI proteins (Ge et al. 2015). However, other studies suggest that addition of SEMA ligand has no effect on HH signaling (Hillman et al. 2011), and that NRPs still promote HH signaling when co-transfected with a version of GLI2 that cannot be phosphorylated by PKA at seven important sites (Pinsky et al. 2017). It is important to consider that NIH/3T3 cells, in which these studies were performed, express endogenous PLXNs (Figure S1). Given the results presented here, an alternate explanation of SEMA-mediated HH promotion is that SEMA ligands act through endogenous PLXNs to increase HH reporter activity by stimulating GAP activity. Another discrepancy in the literature concerns the requirement for the NRP ECD in HH promotion (Ge et al. 2015; Pinsky et al. 2017). Again, given that PLXNs promote HH signaling and that the NRP ECD mediates interactions with PLXN co-receptors, the variable effects that have been reported could be explained by the presence of endogenous PLXNs, the level of NRP overexpression, and the sensitivity of the assay.

NRP and PLXN Cooperation in HH Signaling

We previously reported that NRPs promote HH signaling through a novel cytoplasmic motif (Pinsky et al. 2017), within a region of the protein that is dispensable for SEMA signaling (Fantin et al. 2011). This suggests that NRPs may act very differently within SEMA and HH signaling contexts. PLXNs on the other hand, seem to function similarly in HH and SEMA signaling, through cytoplasmic GAP activity. Together, these data raise the question: do NRPs and PLXNs function together or separately in HH signaling? The answer may be both. Several pieces of evidence suggest that NRPs function independently of PLXNs in HH signaling. First, deleting the NRP

ECD, which mediates interaction between NRPs, PLXNs, and SEMA, does not disrupt HH pathway promotion (Pinsky et al. 2017). Furthermore, we report here that PLXNB2 can promote HH signaling, despite its lack of reported interactions with NRPs (Neufeld and Kessler 2008). However, we cannot exclude the possibility that PLXN A subfamily members bind to endogenous NRPs to mediate HH promotion in our assays. Therefore, the ideas that NRPs and PLXNs function independently and together in HH signaling are not mutually exclusive, and additional studies will be required to elucidate their independent and/or cooperative roles.

Connecting PLXN GAP Activity to the HH Pathway

We find that HH pathway activity is regulated by enzymatic activity of the PLXN GAP domain. However, it remains unclear how GAP downstream signaling intersects with the HH signal cascade. The PLXN cytoplasmic domain interacts with a plethora of intracellular proteins, including collapse-response-mediator protein (CRMP) family phosphoproteins, protein kinases, MICAL redox proteins, and small intracellular GTPases from the Rho, Ras, and Rap superfamilies (Puschel 2007; Yang and Terman 2013; Jongbloets and Pasterkamp 2014). Further, our understanding of the cellular mechanisms downstream of the PLXN GAP domain remains incomplete, including which GTPases are regulated by various PLXN family members. This makes it difficult to identify candidates that might mediate HH signaling. Here, we find that PLXNs from both the A and B subfamilies can promote HH signaling, which may be an important clue in answering this question. While we cannot exclude the possibility that each PLXN or PLXN subfamily regulates HH differently, it is likely that they converge upon a common

protein or set of proteins that mediate HH promotion. Our data suggest that this convergence takes place at the level of GLI transcription factors and requires intact primary cilia. Therefore, candidates for future study should have common demonstrated roles downstream of all PLXNs.

PLXN Redundancy in HH Pathway Promotion

As previously discussed, the PLXN family of proteins is comprised of nine members with distinct and overlapping functions (Neufeld and Kessler 2008). One shared feature between all PLXN proteins is the conserved cytoplasmic GAP domain (Neufeld and Kessler 2008), which we find mediates HH signal promotion. Therefore, our results are complicated by the presence of endogenous PLXN proteins that may act redundantly in the HH signaling cascade, particularly given that PLXNs from multiple subfamilies promote HH signaling. Though technically challenging, a PLXN null background would be necessary to truly study the function of individual PLXN family members in HH signaling. It is also important to consider that PLXNs exhibit largely overlapping expression patterns *in vivo*, further complicating loss-of-function studies (Perala et al. 2005; Mauti et al. 2006). Notably, our results suggest that deleting *Plxna1* or *Plxna2* alone is sufficient to reduce HH target gene expression in the dentate gyrus (Figure 6), despite the widespread expression of additional *Plxns* in the central nervous system (Perala et al. 2005). Additional HH-responsive tissues that express a smaller subset of *Plxns*, including the olfactory epithelium, the tooth bud, and the lung (Perala et al. 2005), should be considered for broader *in vivo* studies.

Our study and many others highlight the complex, entangled nature of cell signaling molecules and pathways. While they are typically studied in isolation, it may be useful to instead consider signaling pathways as broader signaling networks, with overlapping inputs and outputs that combine to elicit cellular behaviors. By better understanding these systems, we can begin to decode the factors influencing cellular decision-making in developmental, homeostatic, and diseased states.

Materials and Methods

Plxn Constructs

Plxn constructs were derived from full length cDNAs using standard molecular biology techniques. All constructs were cloned into the *pCIG* vector, which contains a CMV enhancer, a chicken beta actin promoter, and an internal ribosome entry site (IRES) with a nuclear enhanced green fluorescent protein reporter (3XNLS-EGFP) (Megason and McMahon 2002). C-terminal or N-terminal 6X MYC tags (EQKLISEEDL) were added to constructs as indicated. Deletion and mutation variants were generated using standard cloning techniques and the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, 200521).

Cell Culture and MEF generation

Cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, 11965-118) supplemented with 10% bovine calf serum (ATCC, 30-2030) and 1X Penicillin-Streptomycin-Glutamine (Life Technologies, 10378016).

Cultures were maintained at 37 °C with 5% CO₂ and 95% humidity. MEFs were generated as previously described (Todaro and Green 1963).

Cell Signaling Assays

Luciferase-based reporter assays for HH pathway activity in NIH/3T3 cells were performed as previously described using a *ptcΔ136-GL3* reporter construct (Nybakken et al. 2005). Briefly, cells were seeded at 2.5 X 10⁴ cells/well into 0.5% gelatin-coated 24-well plates. The next day, cells were transfected with empty vector (*pCIG*) or experimental constructs along with the *ptcΔ136-GL3* luciferase reporter construct and beta-galactosidase transfection control (pSV-β-galactosidase; Promega, E1081).

Transfections were performed using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum media (Invitrogen, 31985). 48h after transfection, culture media was replaced with low-serum media (0.5% bovine calf serum, 1% Penicillin Streptomycin L-Glutamine) containing either control or N-terminal SHH (NSHH)-conditioned media. Luciferase reporter activity and Beta Galactosidase activity were measured 48h later on a Spectramax M5^e Plate reader (Molecular Devices) using the Luciferase Assay System (Promega, E1501) and the Betafluor Beta Galactosidase Assay Kit (EMD Millipore, 70979), respectively. Luciferase values were divided by beta galactosidase activity to control for transfection, and data were reported as fold induction relative to the vector-transfected control. All treatments were performed in triplicate (each data point indicates a technical replicate) and averaged (bar height), with error bars representing the standard deviation between triplicate wells. Each experiment was repeated a minimum of three times (biological replicates); representative results are

shown. Student's t-tests were used to determine whether each treatment was significantly different from the control, with P-values of 0.05 or less considered statistically significant.

Immunofluorescent Analyses for cultured cells

NIH/3T3 fibroblasts were plated at 1.5×10^5 cells/well onto glass coverslips in a 6-well dish. Cells were transfected 24h after plating using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum media (Invitrogen, 31985). To assess expression and collapse, cells were incubated for 24-48h at 37 °C as indicated. To image cilia, cells were placed in low serum media approximately 6h after transfection (0.5% bovine calf serum, 1% Penicillin Streptomycin L-Glutamine) for 48h. All cells were fixed in 4% paraformaldehyde for 10min at room temperature and washed with PBS. A 5min permeabilization step with 0.2% Triton X-100 in PBS was performed as indicated, prior to staining. Primary antibodies included: mouse IgG2a anti-MYC (1:1000, Cell Signaling, 2276), goat IgG anti-PLXNA1 (1:250, R&D Systems, AF4309), and mouse IgG2b anti-acetylated tubulin (1:2500, Sigma Aldrich, T7451), all diluted in IF blocking buffer (list reagents please – I don't have the recipe on hand!). Coverslips were incubated with primary antibodies overnight, followed by a 10min DAPI stain (1:30,000 in PBS at room temperature, Invitrogen, D1306) and 1h incubation with secondary antibodies including: AlexaFluor 555 goat anti-mouse IgG2a, AlexaFluor 488 donkey anti-goat IgG, AlexaFluor 488 goat anti-mouse IgG2b, and AlexaFluor 555 goat anti-mouse IgG2b (1:500, Invitrogen, A21137, A11055, A21141, and A21147, respectively). Coverslips were mounted to glass slides using Shandon Immu-Mount Mounting Medium (Fisher,

9990412). Immunofluorescent analysis and imaging were performed on a Leica SP5X Upright 2-Photon Confocal microscope using LAS AF software (Leica) and a Leica 63X (type: HC Plan Apochromat CS2; NA1.2) water immersion objective.

Western Blot Analysis

NIH/3T3 cells were transfected using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum media (Invitrogen, 31985). Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA) 48h after transfection, sonicated using a Fisher Scientific Sonic Dismembrator, Model 500 (4 pulses at 20%), and centrifuged at 14,000 x g for 25min at 4 °C to remove the insoluble fraction. Protein concentrations were determined using the BCA Protein Assay Kit (Fisher, PI23225). After boiling for 10min, 50µg of protein from each sample were separated using SDS-PAGE with 7.5-12.5% gels and transferred onto Immun-Blot PVDF membranes (Bio-Rad, 162-0177). Membranes were washed in tris-buffered saline (TBS) with 0.5% OmniPur Tween-20 (TBST; EMD Millipore, 9480) and blocked in western blocking buffer (30 g/L Bovine Serum Albumin with 0.2% NaN₃ in TBST) for 1h to overnight. Blots were probed with the following antibodies: rabbit IgG anti-MYC (1:10,000, Bethyl Labs, A190-105A), goat IgG anti-PLXNA1 (1:200, R&D Systems, AF4309), and Mouse IgG1 anti-Beta Tubulin (1:10,000, generously provided by Dr. Kristen J. Verhey, University of Michigan). Secondary antibodies from Jackson ImmunoResearch were diluted 1:10,000, and included: peroxidase-conjugated AffiniPure goat anti-mouse IgG, light chain specific (115-035-174), peroxidase-conjugated AffiniPure F(ab)₂ Fragment

donkey anti-rabbit IgG (711-036-152), and peroxidase-conjugated AffiniPure donkey anti-goat IgG, light chain specific (705-035-147). Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, WBKLS0500) was added for 10min before membranes were exposed to HyBlot CL Autoradiography Film (Denville, E3018) and developed using a Konica Minolta SRX-101A Medical Film Processor.

RNAi

RNAi was performed using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific, 13778150) with BLOCK-iT Fluorescent Oligo as a transfection control (ThermoFisher Scientific, 13750062). *Plxn* knockdown was performed using Dharmacon ON-TARGET^{plus} SMARTpool reagents with catalog numbers L-040789-01-0005, L-040790-01-0005, L-040791-01-0005, L-040980-00-0005, and L-056934-01-0005 for *Plxna1*, *Plxna2*, *Plxna3*, *Plxnb2*, and *Plxnd1*, respectively. *Nrp* oligos included *Nrp1*: GCACAAAUCUCUGAAACUA; and *Nrp2*: GACAAUGGCUGGACACCCA.

RT-qPCR

NIH/3T3 cells were cultured as previously described and treated with low-serum media (0.5% bovine calf serum, 1% Penicillin Streptomycin L-Glutamine) containing SAG as indicated. RNA was isolated using the RNAqueous kit (ThermoFisher Scientific, AM1912). cDNA was generated using 1 µg of template RNA (iScript RT Supermix, BioRad, 1708841). cDNA was diluted 1:100, and qPCR was performed using SYBR green master mix (ThermoFisher Scientific, AM9780) on an Applied BioSystems StepOnePlus Real-Time PCR System with the following primers: *Gli1* forward:

535 GTGCACGTTTGAAGGCTGTC; *Gli1* reverse: GAGTGGGTCCGATTCTGGTG; *Ptch1*
 536 forward: GAAGCCACAGAAAACCCTGTC; *Ptch1* reverse:
 537 GCCGCAAGCCTTCTCTAGG; *Cyclophilin* forward:
 538 TCACAGAATTATTCCAGGATTCATG; and *Cyclophilin* reverse:
 539 TGCCGCCAGTGCCATT. *Cyclophilin* expression was used for normalization.

540

541 *Chicken in ovo Neural Tube Electroporation*

542 Electroporations were performed as previously described (Tenzen et al. 2006),
 543 using *Plxn*, *SmoM2*, and *Gli1* constructs cloned into the pCIG vector (Megason and
 544 McMahon 2002). Briefly, DNA constructs (1.0 $\mu\text{g}/\mu\text{l}$) were mixed with 50 ng/ μl Fast
 545 green FCF dye (Millipore Sigma, F7252) and injected into the neural tube of Hamburger
 546 Hamilton stage 11-13 chicken embryos (Hamburger and Hamilton 1951). Embryos were
 547 dissected 48-hours post-injection and screened for GFP expression before being fixed in
 548 4% PFA and prepared for immunofluorescent analysis. Embryos were embedded in
 549 Tissue-Tek OCT compound (Thermo Fisher Scientific, NC9806257), rapidly frozen over
 550 dry ice, and cryo-sectioned at a thickness of using a Leica cryostat. Twelve micron thick
 551 samples were affixed to glass slides and immunostained using the following antibodies:
 552 mouse IgG1 anti-PAX7 (1:20, Developmental Studies Hybridoma Bank, DSHB), mouse
 553 IgG1 anti-NKX6.1 (1:20, DSHB), goat IgG anti-GFP (1:200, Abcam, ab6673), rabbit
 554 IgG anti-MYC (1:100, Bethyl Laboratories, A190-205A). Slides were incubated with
 555 primary antibody overnight at 4°C followed by a 10min DAPI stain (1:30,000 at room
 556 temperature, Invitrogen, D1306) and 1h incubation with secondary antibodies including:
 557 AlexaFluor 555 donkey anti-mouse IgG, AlexaFluor 488 donkey anti-goat IgG,

AlexaFlour 647 donkey anti-rabbit IgG (1:500, Invitrogen, A31570, A11055, A31573, respectively). Samples were visualized on a Leica Upright SP5X Light Laser Confocal Microscope, and figures were generated using Adobe Photoshop and Illustrator. The size of the NKX6.1 domain was measured using Adobe Illustrator in chicken neural tubes electroporated with *pCIG* (n=6), *Gli1* (n=4), and *Plxna1^{ΔECD}* (n=17). These measurements were then normalized to the NKX6.1 domain size of the unelectroporated side of the neural tube.

Mice

Plxna1 (Yoshida et al. 2006) and *Plxna2* (Suto et al. 2007; Duan et al. 2014) mice, both on mixed genetic backgrounds, were generously provided by Dr. Alex Kolodkin. *Gli1^{lacZ}* animals were maintained on a mixed CD1 and C57BL/6J background (Bai et al. 2002). All mice were housed and cared for according to NIH guidelines, and all animal research was approved by the University of Michigan Medical School Institutional Animal Care and Use Committee. *Plxn* genotyping was performed using the following primers: *Plxna1* WT_F: CCTGCAGATTGATGACGACTTCTG; *Plxna1* WT_R: TCATGAGACCCAGTCTCCCTGTC; *Plxna1* MT_F: GCATGCCTGTGACACTTGGCTCACT; *Plxna1* MT_R: CCATTGCTCAGCGGTGCTGTCCATC; *Plxna2* WT_F: GCTGGAACCATGTGAGAGCTGATC; *Plxna2* WT_R: GGTCATCTAGTCGCAGGAGCTTGC; *Plxna2* MT_F: GGTCATCTAGTCGCAGGAGCTTGC; *Plxna2* MT_R: TACCCGTGATATTGCTGAAGAGCTTGG. Tissue preparation and X-gal staining

were performed as previously described (Duan et al. 2014; Holtz et al. 2015). Briefly, serial sagittal sections (16µm) were collected from P7 brains and mounted onto six slides. One slide from each animal was used for X-gal staining. The total number of X-gal positive cells was quantified from eight serial sections per slide to yield the average number of X-gal positive cells per animal; each data point represents a single animal.

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We are grateful to Dr. A. L. Kolodkin (Johns Hopkins University, MD, USA) for providing *Plxn* constructs. Members of the Allen and Giger labs contributed technical assistance, insightful comments, and helpful suggestions. We are also thankful to Drs. K. S. O'Shea, K. J. Verhey, and J. D. Engel for sharing equipment and reagents. Confocal imaging was performed in the Microscopy Core at the University of Michigan. We acknowledge the ENCODE consortium, and particularly the lab of Dr. John Stamatoyannopoulos at the University of Washington for sharing their RNA-seq dataset on NIH/3T3 cells (GEO: GSM970853). J.M.P. was supported by a Rackham Merit Fellowship, Benard Maas Fellowship, Bradley Merrill Patten Fellowship, Organogenesis Training Grant (T32 HD007505), and Ruth L. Kirschstein National Research Service Award (F31 NS096734). R.J.G. is supported by the Adelson Medical Foundation, Craig H. Neilsen Foundation, and funding from the National Institutes of Health (R01 MH119346). B.L.A. is supported by funding from the National Institutes of Health (R01 DC014428, R01 CA198074 and R01 GM118751). B.L.A. and R.J.G. are supported by an MCubed Research Grant from The University of Michigan.

604 **Author Contributions**

605 J.M. Pinskey: Conceptualization, Validation, Formal Analysis, Investigation, Writing-
 606 Original Draft, Writing- Review & Editing. T.M. Hoard: Conceptualization, Validation,
 607 Formal Analysis, Investigation, Writing- Original Draft, Writing- Review & Editing. X-F
 608 Zhao: Formal Analysis, Investigation. N.E. Franks: Formal Analysis, Investigation. Z.C.
 609 Frank: Investigation. A.N. McMellen: Investigation. R.J. Giger: Conceptualization,
 610 Resources, Formal Analysis, Investigation, Writing- Original Draft, Writing- Review and
 611 Editing. B.L. Allen: Conceptualization, Resources, Formal Analysis, Supervision,
 612 Funding Acquisition, Investigation, Methodology, Project Administration, Writing-
 613 Original Draft, Writing- Review and Editing.

Pinsky and Hoard et al., 2021, Figure 1

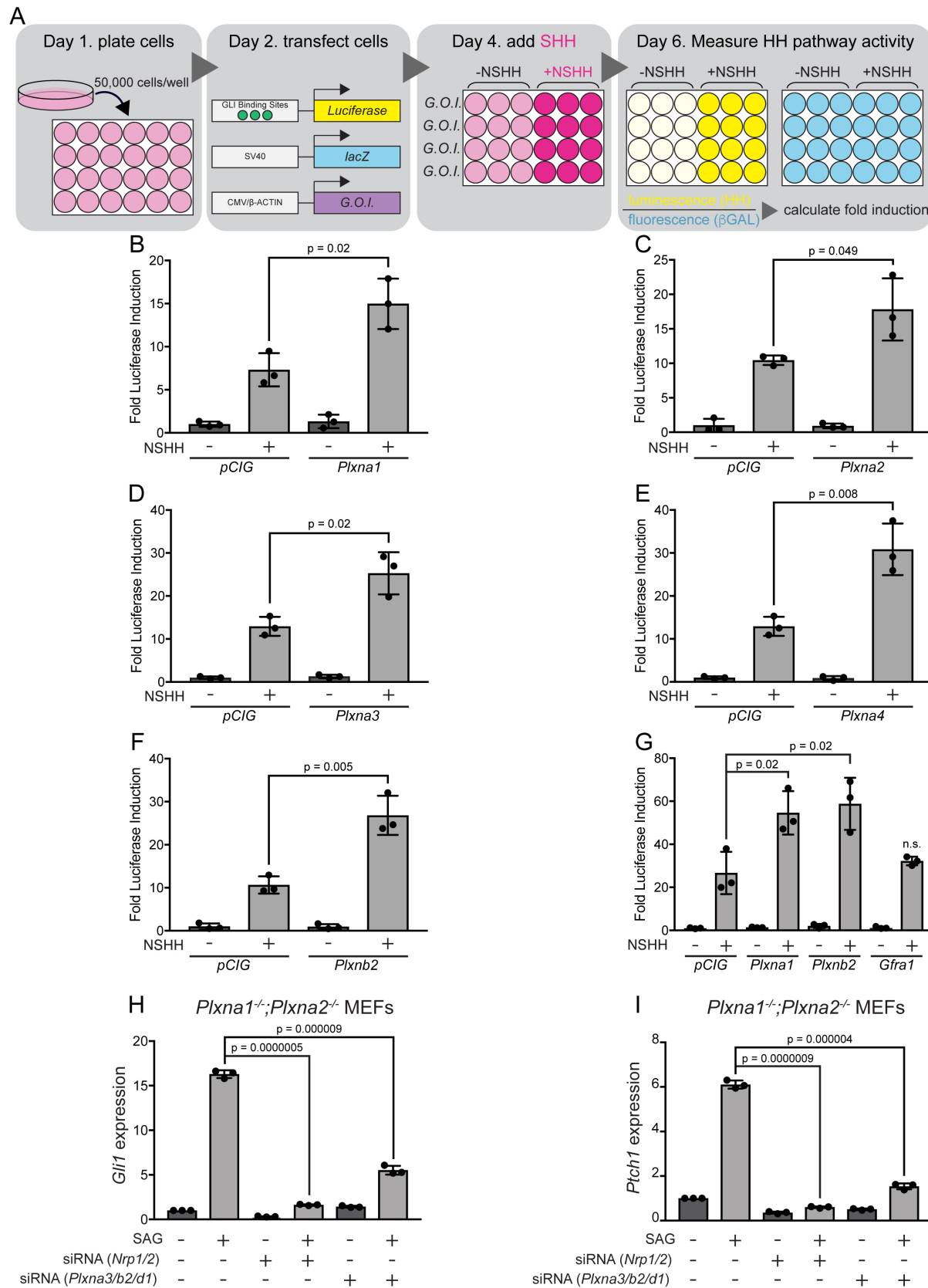


Figure 1. Multiple PLXNs promote HH signaling.

(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 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 GFR α 1. (H-I) qRT-PCR analysis of *Gli1* and *Ptch1* in response to HH pathway activation via the Smoothed agonist, SAG. *Plxna1*^{-/-}; *Plxna2*^{-/-} 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 $\Delta\Delta$ CT method. Data are reported as mean fold induction $\pm$ S.D., with p-values calculated using two-tailed Student's t tests. n.s. = not significant.

Figure 1- Source Data 1

Raw Data for Figure 1B-I

Pinskey and Hoard et al., 2021, Figure 2

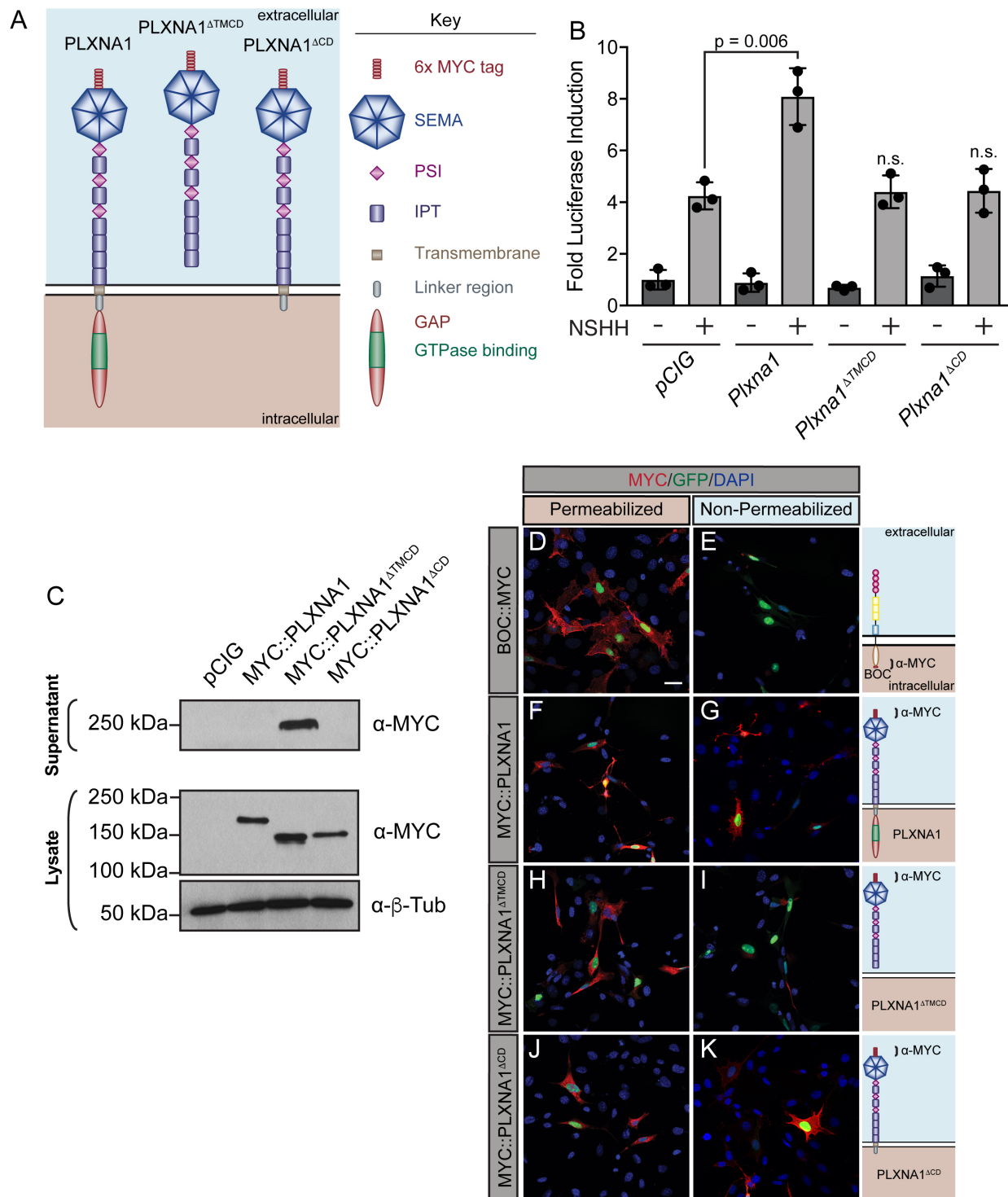


Figure 2. The PLXNA1 cytoplasmic and transmembrane domains are required for HH pathway promotion. (A) Schematic of different PLXNA1 proteins. (B) HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction $\pm$ S.D. 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 PLXNA1^{ΔTMCD}, 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 μ m.

Figure 2- Source Data 1

Raw data for Figure 2B

Figure 2- Source Data 2

Raw, unedited blot from Figure 2C

Figure 2- Source Data 3

Raw, unedited blot from Figure 2C

654 **Figure 2- Source Data 4**

655 Raw, unedited blot from Figure 2C

656 **Figure 2- Source Data 5**

657 Raw, labeled blot from Figure 2C

658 **Figure 2- Source Data 6**

659 Raw, labeled blot from Figure 2C

660 **Figure 2- Source Data 7**

661 Raw, labeled blot from Figure 2C

Pinsky and Hoard et al., 2021, Figure 3

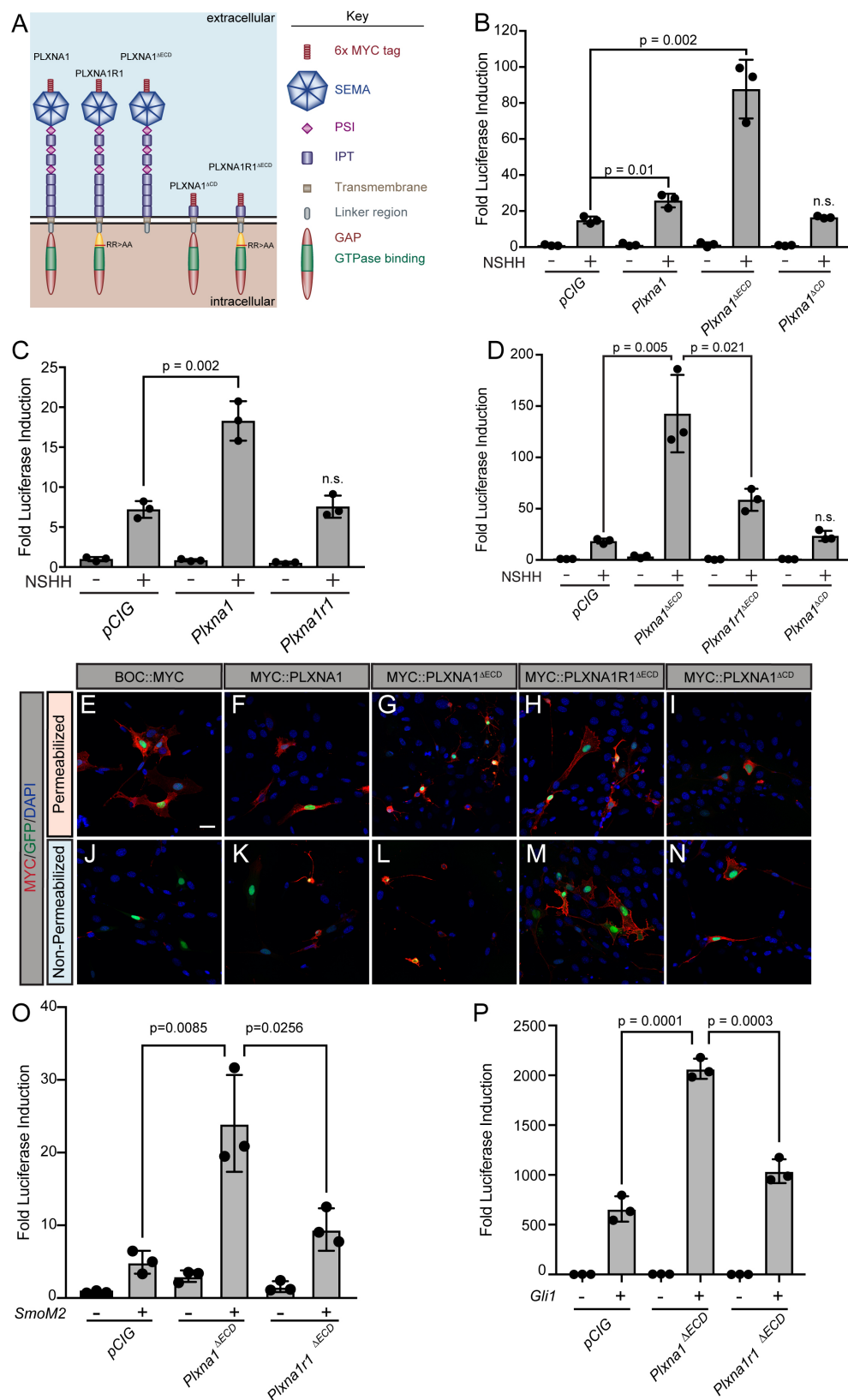


Figure 3. The Plexin GAP domain is required to promote Hedgehog signaling at the level of GLI transcription factors. (A) Schematic of different PLXNA1 proteins. (B-D) HH-dependent luciferase reporter activity was measured in NIH/3T3 cells transfected with the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction $\pm$ S.D. with 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 ^{Δ ECD} and, to some extent, PLXNA1. For PLXNA1R1 localization, please see Figure S2, D-E. Scale bar = 10 μ m. (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*, *SmoM2* (O), or *Gli1* (P). Data are reported as mean fold induction $\pm$ S.D., with p-values calculated using two-tailed Student's t tests. n.s. = not significant.

Figure 3- Source Data 1

Raw data for Figure 3B-D and Figure 3O-P

Pinskey and Hoard et al., 2021, Figure 4

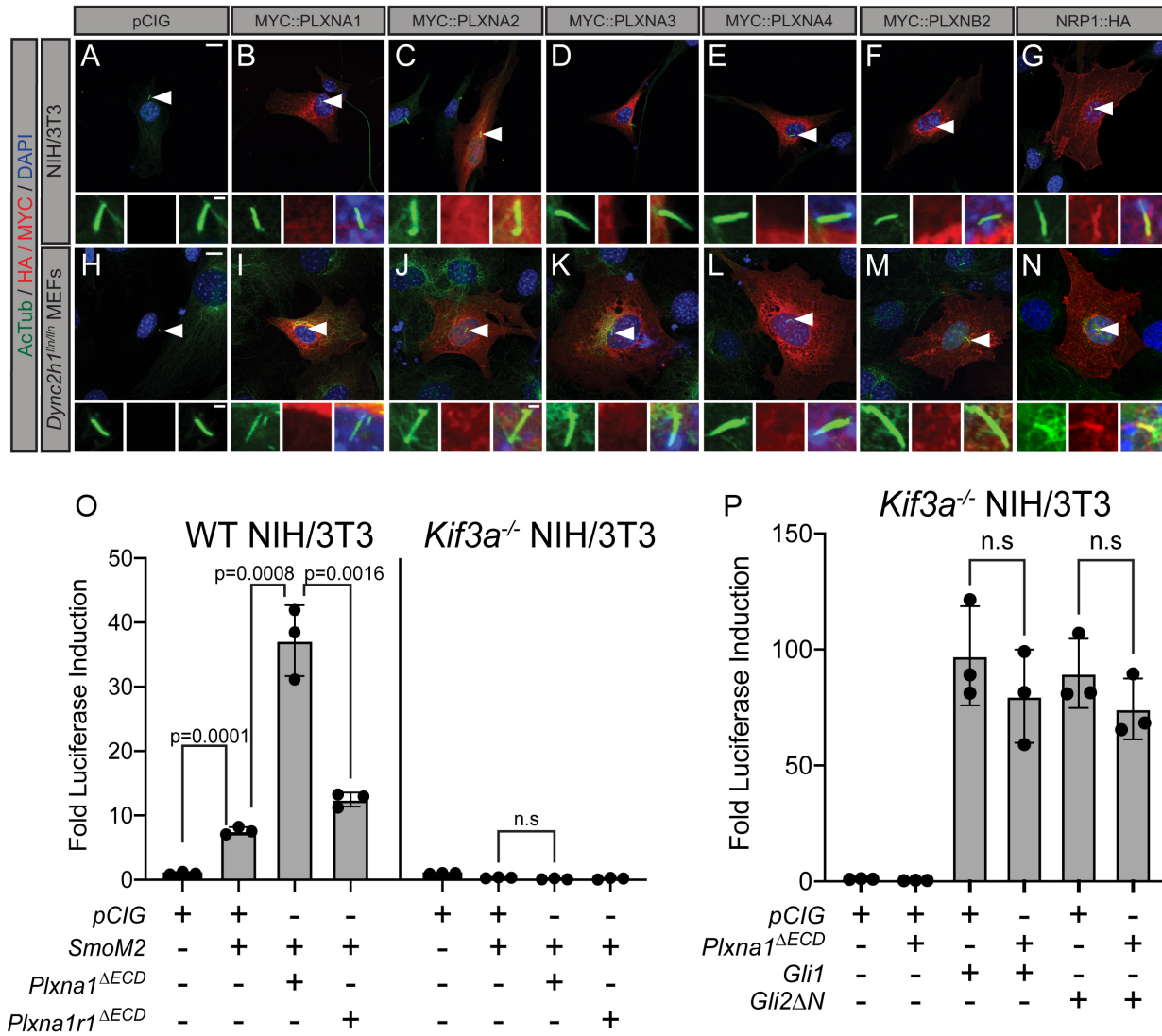
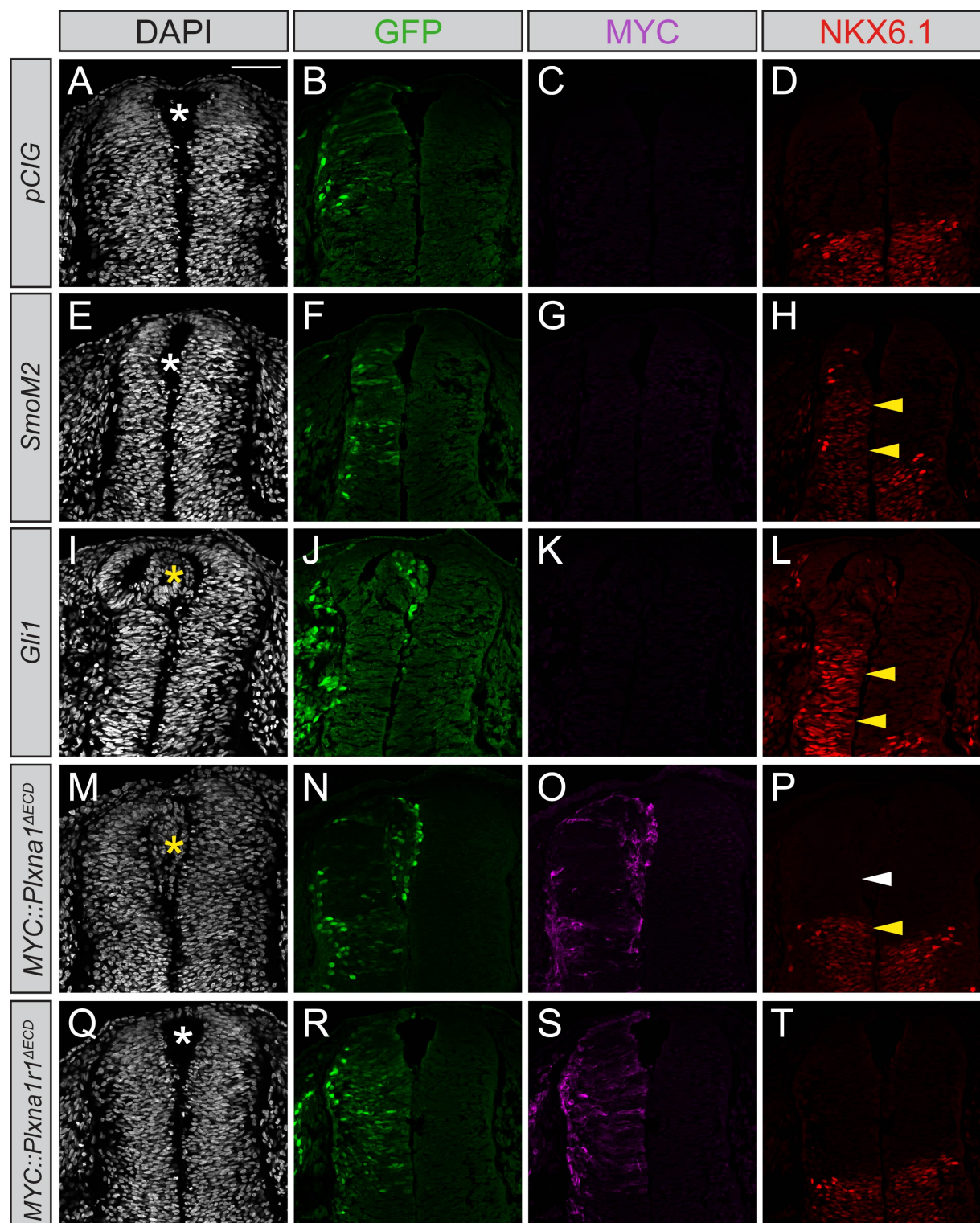


Figure 4. PLXNs do not localize to primary cilia, but do require primary cilia to promote HH pathway activity. (A-N) Antibody detection of MYC and HA tagged constructs (red) in NIH/3T3 cells (A-G) and *Dync2h1^{ln/ln}* MEFs (H-N). Acetylated tubulin (AcTub, green) indicates the primary cilium and DAPI (blue) stains nuclei. Compared to NRP1, PLXNs are not enriched in primary cilia. Scale bar = 10 μ m. Inset scale bar = 1 μ m. (O) WT NIH/3T3 cells or *Kif3a^{-/-}* NIH/3T3 cells were co-transfected with *SmoM2* and *Plxna1^{ΔECD}* or *Plxna1r1^{ΔECD}*. (P) *Kif3a^{-/-}* NIH/3T3 were transfected with *Gli1* or *Gli2ΔN* and co-transfected with *Plxna1^{ΔECD}*. Data are reported as mean fold induction $\pm$ S.D., with p-values calculated using two-tailed Student's t tests. n.s. = not significant.

Figure 4- Source Data 1

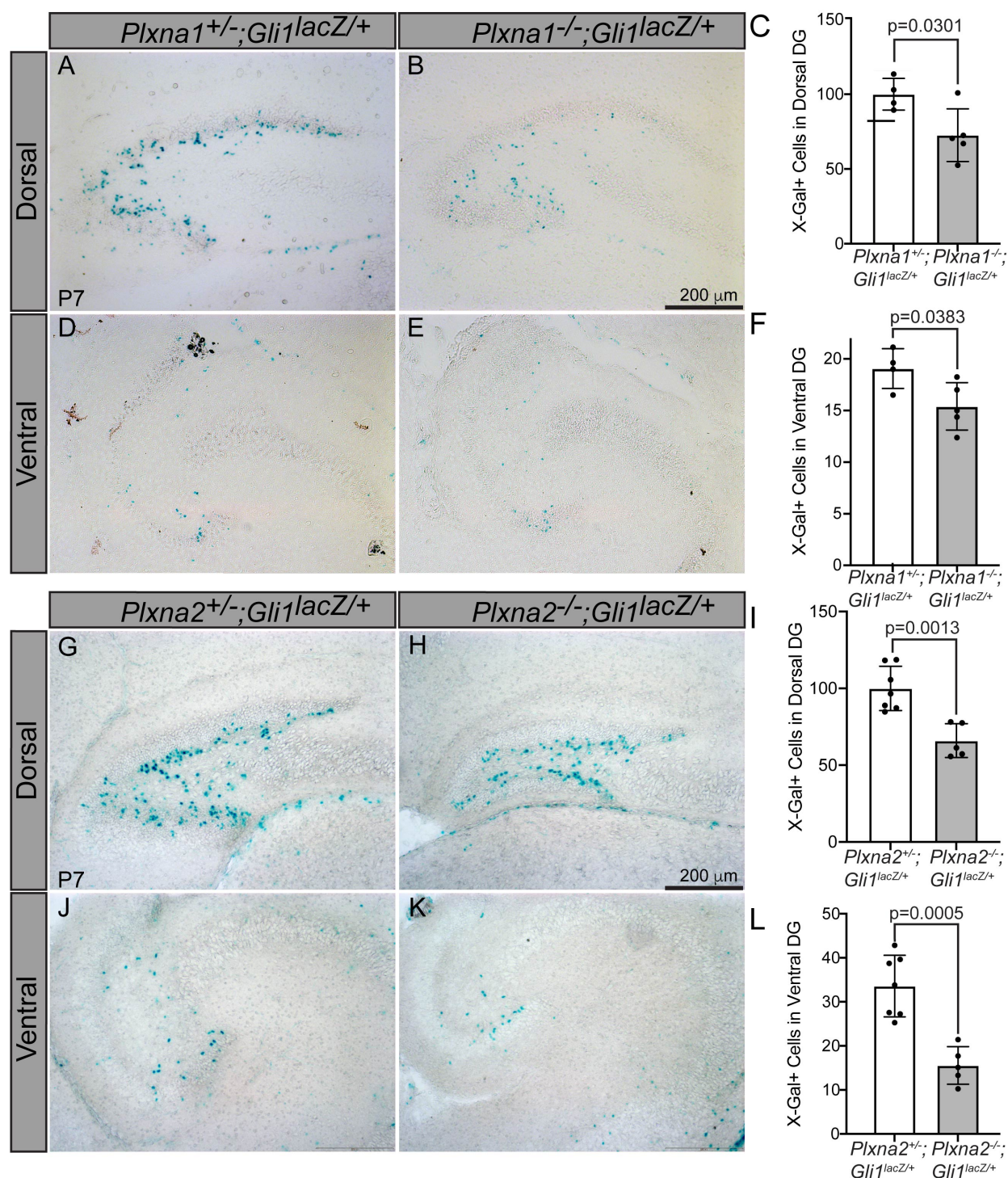
Raw data for Figure 4 O-P

Pinskey and Hoard et al., 2021, Figure 5



697 **Figure 5. Constitutively active PLXNA1 induces ectopic cell migration into the**
698 **lumen of the developing chicken neural tube.** (A-T) Immunofluorescent analysis of
699 neural patterning in forelimb-level sections from Hamburger-Hamilton stage 21-22
700 chicken embryos. Embryos were electroporated at Hamburger-Hamilton stage 11-13 with
701 *pCIG* (A-D, n= 6 embryos), *SmoM2* (E-H, n = 7 embryos), *Gli1* (I-L, n = 4 embryos),
702 *MYC::Plxna1^{ΔECD}* (M-P, n = 17 embryos), or *MYC::Plxna1r1^{ΔECD}* (Q-T, n = 6 embryos).
703 Transverse sections were stained with GFP, MYC, and NKX6.1 antibodies. DAPI stain
704 labels nuclei (gray). Electroporated cells are labeled with GFP. Asterisks denote the
705 presence (yellow) or absence (white) of ectopic cells within the lumen of the neural tube.
706 Arrowheads denote the presence (yellow) or absence (white) of ectopic NKX6.1. Scale
707 bar = 50 μm.

Pinsky and Hoard et al., 2021, Figure 6



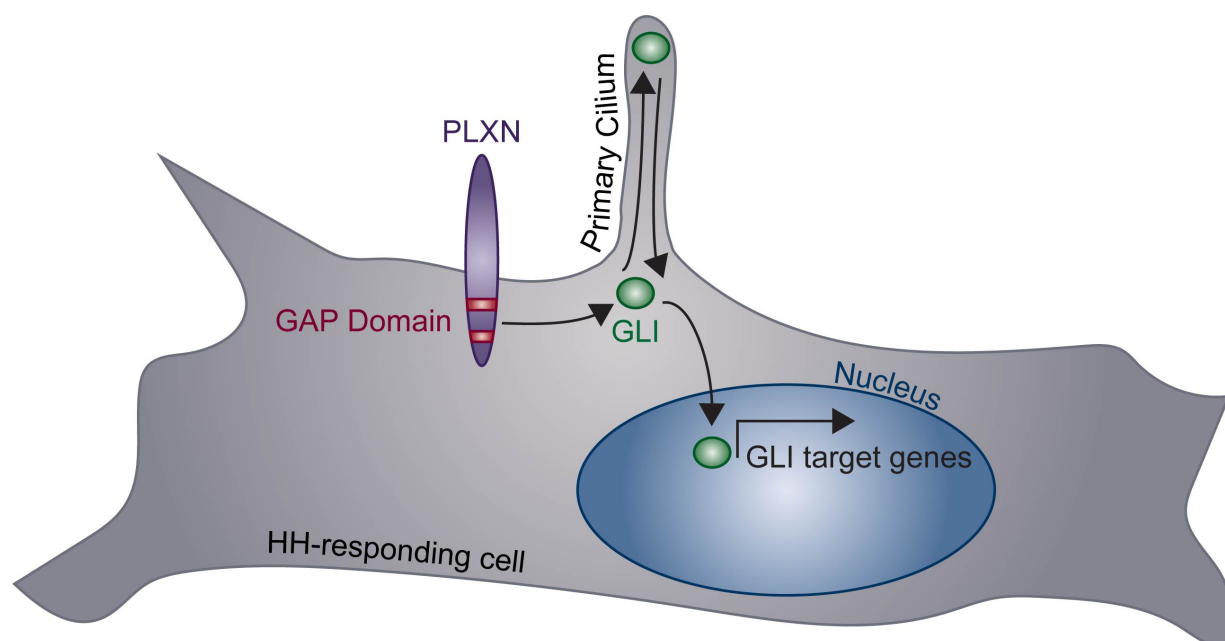
709 **Figure 6. Reduced *Gli1^{lacZ}* expression in the dentate gyrus of mice lacking either**
710 ***Plxna1* or *Plxna2*.** X-Gal staining in coronal sections through the dorsal (A, B, G, and H)
711 and ventral (D, E, J, and K) hippocampus of postnatal day 7 (P7) mice. The following
712 numbers of pups were analyzed: *Plxna1*^{+/-};*Gli1^{lacZ}*⁺ (n=4); *Plxna1*^{-/-};*Gli1^{lacZ}*⁺ (n=5);
713 *Plxna2*^{+/-};*Gli1^{lacZ}*⁺ (n=7); *Plxna2*^{-/-};*Gli1^{lacZ}*⁺ (n=5). Quantitation of *Gli1^{lacZ}* positive cells
714 (C, F, I, and L) reported as mean ± S.D. with p-values calculated using two-tailed
715 Student's t test. DG = dentate gyrus. Scale bar = 200 μm.

716

717 **Figure 6- Source Data 1**

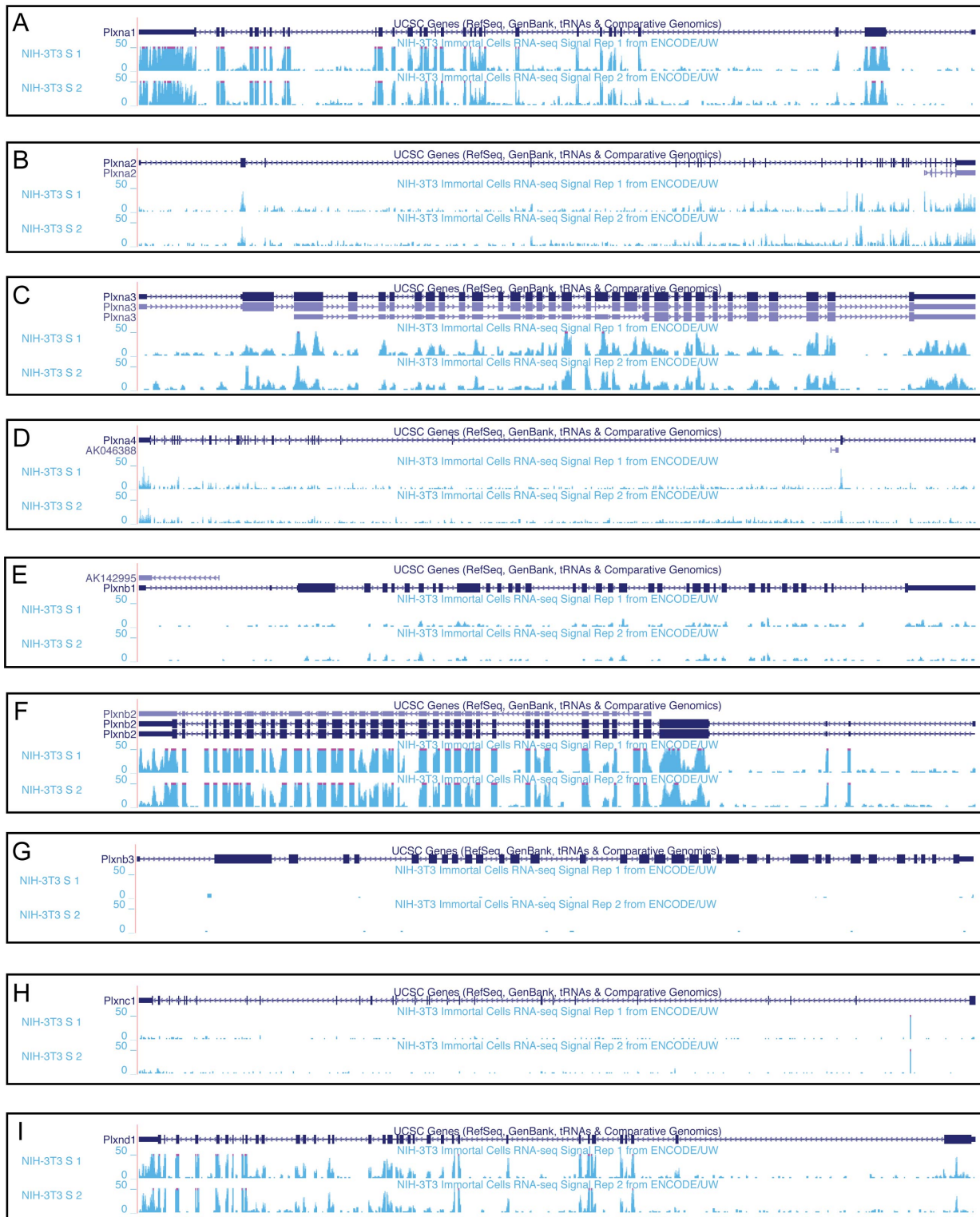
718 Raw data for Figure 6C, F, I, L

Pinskey and Hoard et al., 2021, Figure 7



720 **Figure 7. Model of PLXN-mediated promotion of HH pathway activity.**
 721 PLXNs (purple) at the cell surface promote HH signaling through GLI transcription
 722 factor (green) activation, mediated by their cytoplasmic GAP activity (red). Notably, this
 723 PLXN-dependent promotion requires primary cilia to induce GLI target gene expression
 724 in the nucleus.

Pinsky and Hoard et. al., 2021, Figure S1



726 **Figure S1. *Plxn* expression in NIH/3T3 fibroblasts.**

727 RNA sequencing data from the ENCODE project indicating *Plxn* expression in NIH/3T3

728 cells. Data were aligned to the mouse GRCm38/mm10 assembly using the UCSC

729 Genome Browser (<https://genome.ucsc.edu>).

Pinsky and Hoard et al., 2021, Figure S2

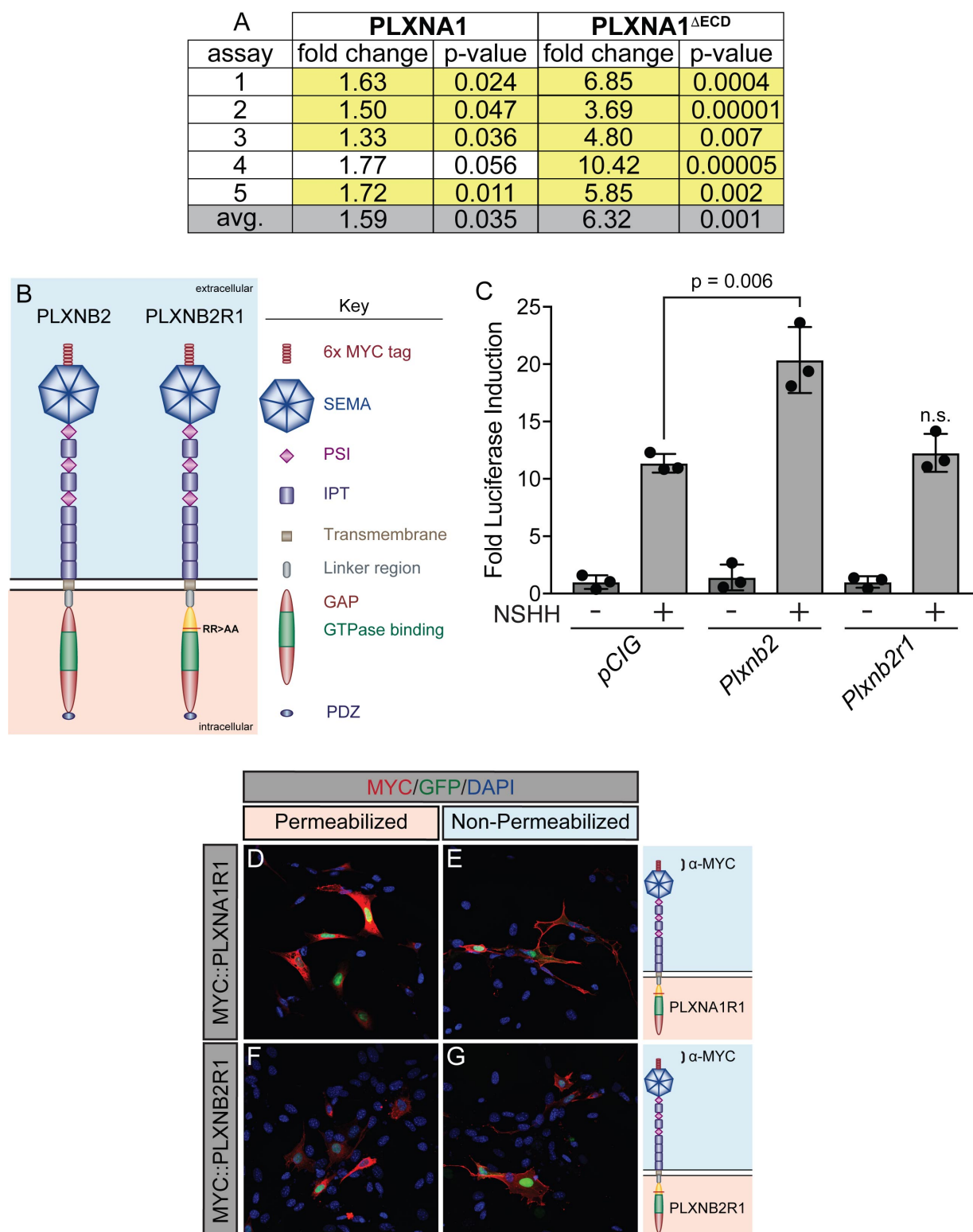


Figure S2. Constitutively active PLXNA1 reproducibly increases HH pathway activity. (A) Summary of luciferase assay data in which PLXNA1 and PLXNA1^{ΔECD} were directly compared in five independent assays. Fold change reported between ligand-stimulated vector only (*pCIG*) triplicate wells and ligand-stimulated *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 indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction ± S.D. 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 = 10μm.

Figure S2- Source Data 1

Raw data for Figure S2C

Pinsky and Hoard et al., 2021, Figure S3

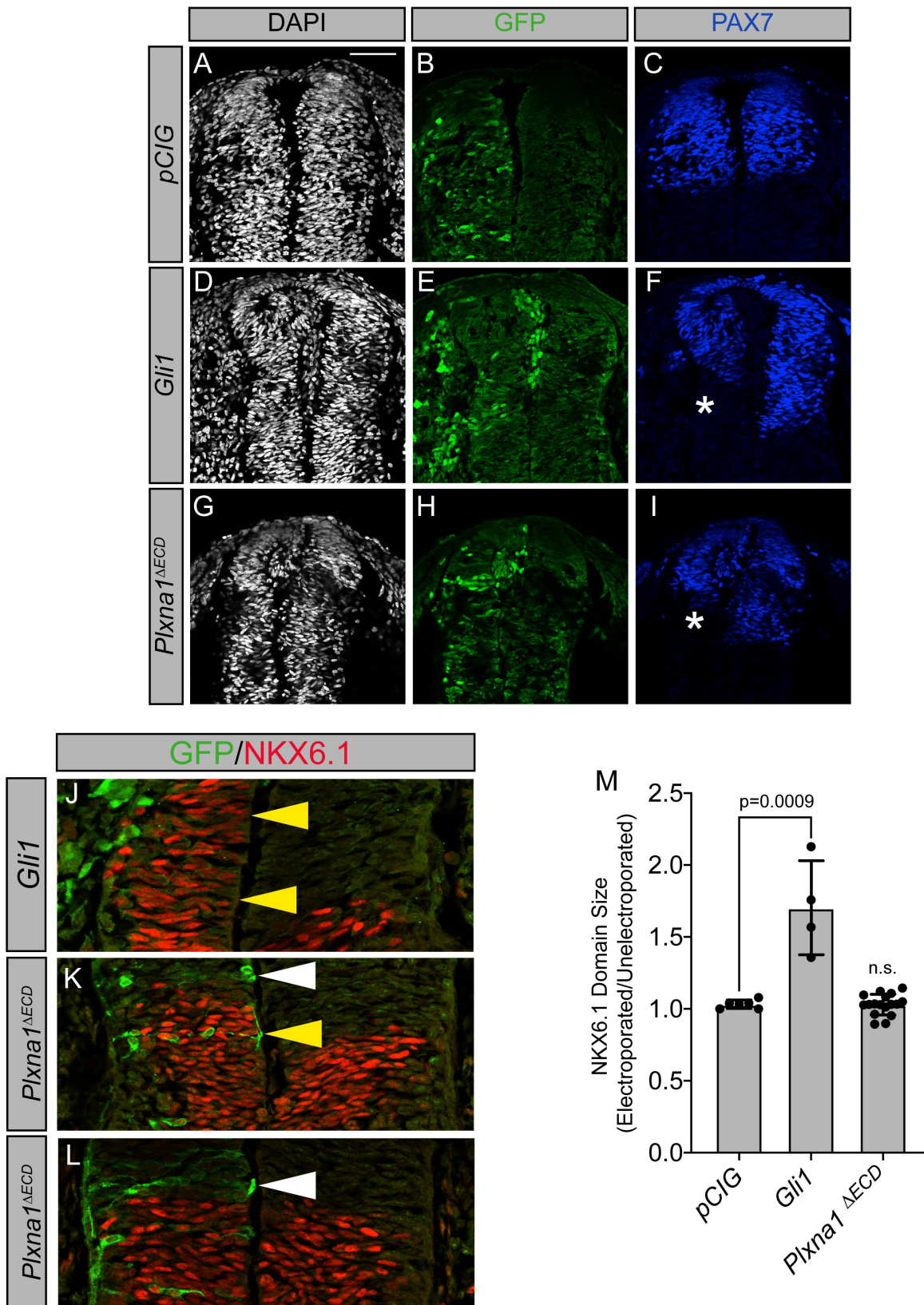


Figure S3. Constitutively active PLXNA1 does not significantly alter Hedgehog-dependent neural tube patterning in the developing chicken embryo. (A-M)

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^{ΔECD}* (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 cells are labeled with GFP. Asterisks denote the loss of PAX7. Scale bar = 50 μm. Yellow arrowheads indicate the presence of ectopic NKX6.1. White arrowheads (K-L) denote the absence of ectopic NKX6.1 in *Plxna1^{ΔECD}*-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^{ΔECD}*. Values are reported as mean ± S.D. with p-values calculated using two-tailed Student's t-test.

Figure S3- Source Data 1

Raw data for Figure S3M

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