Semaphorin Receptor Function in Hedgehog Signal Transduction

by

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Dedication

To my family.

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Abstract

Hedgehog signaling is essential for embryonic and postnatal development, while perturbation of Hedgehog pathway function can lead to a variety of developmental diseases, birth defects, and cancers. Neuropilins, which have well-characterized roles in Semaphorin and VEGF signaling, have recently been implicated in the regulation of Hedgehog signaling. Neuropilins contain short, catalytically inactive cytoplasmic domains, requiring Plexin receptors to regulate small intracellular GTPases during Semaphorin signal transduction. However, the mechanism of Neuropilin function in Hedgehog signal transduction remains unclear, and a role for Plexins in Hedgehog signaling has not been explored. In this thesis, I present evidence suggesting that the Neuropilin-1 cytoplasmic and transmembrane domains are both necessary and sufficient to regulate Hedgehog pathway activity. Neuropilin-1 also enters the primary cilium, an important subcellular compartment for Hedgehog regulation; however, this cilia localization does not correlate with Hedgehog signal promotion. Instead, Neuropilin-1 selectively regulates GLI transcriptional activator function through a novel 12-amino acid cytoplasmic motif. Strikingly, I also find that multiple Plexin family members promote Hedgehog signaling. Point mutations in the GTPase activating (GAP) domain of Plexins prevent Hedgehog pathway promotion, suggesting that GAP function is required for Plexin-dependent Hedgehog regulation. Furthermore, deletion of the autoinhibitory Plexin A1 extracellular domain significantly increases Hedgehog pathway activity, providing additional evidence that Plexin GAP activity regulates Hedgehog signaling. Together, these data suggest that Neuropilins and Plexins regulate Hedgehog signaling downstream of ligand activation through distinct cytoplasmic mechanisms.

Therapeutic approaches targeting these Semaphorin receptors may be useful to correct deregulated Hedgehog signaling in cancer and other diseases.

Chapter 1: Introduction

1.1 Abstract

Hedgehog signaling is a conserved cell-cell communication pathway that plays essential and diverse roles during embryonic development and adult tissue homeostasis. Many cell surface-associated molecules critically regulate the Hedgehog signaling pathway, coordinating the secretion and distribution of Hedgehog ligands as well as signal reception and downstream signal transduction. Recent evidence suggests that Neuropilins, single-pass transmembrane receptors for Semaphorin and Vascular endothelial growth factor ligands, positively regulate Hedgehog signaling. However, the mechanism of Neuropilin action in Hedgehog signal transduction remains unclear. Neuropilins require Plexin co-receptors to transduce Semaphorin signals, although a role for Plexins in HH signaling has not been explored. These questions are particularly interesting given overlapping expression of HH and Semaphorin components in both development and disease. Investigating how Semaphorin receptors function within the Hedgehog signaling cascade will provide important insight into the regulation of this key developmental pathway. Furthermore, therapeutic approaches targeting Semaphorin receptors may be useful to regulate deregulated Hedgehog signaling in cancer and other diseases. In this chapter, I review both the Hedgehog and Semaphorin signal transduction pathways, emphasizing areas of overlap between these two pathways which could be mediated by Neuropilin and Plexin receptors.

1.2 Introduction

For many years, scientists thought that the complexity of human biology was a result of our expansive and sophisticated genetics. Accordingly, when the human genome was first being sequenced in the 1990s, most scientists estimated that the number of protein-coding genes would be greater than 100,000 (Pennisi, 2003). Many were shocked, therefore, when initial estimates from the sequencing of the human genome reduced that number to between 26,000 and 30,000 genes (Lander et al., 2001; Venter et al., 2001), a number which was reduced even further by the time human genome sequencing was completed, to between 20,000 and 25,000 genes (International Human Genome Sequencing, 2004). To date, our best estimates suggest that the human genome actually only contains about 19,000 protein-coding genes (Ezkurdia et al., 2014), approximately the same number of genes as a *C. elegans* roundworm (Hillier et al., 2005).

Similarly, the complex process of developing from a single cell to a fully-grown, functional human comprised of trillions of cells with highly specialized functions requires instructions from a surprisingly small number of proteins associated with a handful of signaling pathways. Among the most well-studied are Wingless-type mouse mammary tumor virus integration site (WNT), Notch, Transforming growth factor-β (TGFβ), Janus kinase (JAK)-signal transducer and activator of transcription (STAT) kinases, Retinoic acid (RA), Hedgehog (HH), and Receptor tyrosine kinase (RTK) pathways, which act in overlapping and distinct populations of cells at different times to elicit a multitude of cellular behaviors (Basson, 2012; Ingham et al., 2011; Sanz-Ezquerro et al., 2017). But how does this limited set of pathways regulate such a variety of cells and tissues? How is such a small genetic blueprint interpreted to achieve the incredible diversity of function required for human life, and indeed of life in general?

The complexity of biological life arises not from a large number of genes and proteins, but rather from countless combinations generated by interactions between a much smaller number of key players. These proteins are reused in different organisms and different tissues, and they are adapted for specific functions depending on molecular and temporal context. Each player has more than one role, and depending on how the players interact with one another, the same protein can achieve many different functions. We are currently transitioning from a time in developmental biology when our focus has been on identifying these key players and their associated pathways, into an exploration of how these molecular players function as part of vast networks of inputs and outputs that are decoded by cells in various ways to elicit behavior.

The focus of this dissertation is to explore one axis of this complex network by investigating how the cell surface receptors, Neuropilins (NRPs) and Plexins (PLXNs), which have been previously studied as axon guidance cues in the Semaphorin (SEMA) signaling pathway, also function to regulate HH signaling through distinct cytoplasmic mechanisms. NRPs have been previously identified as HH pathway regulators (Ge et al., 2015; Hillman et al., 2011), but their mechanism of action is not well understood, and a role for their PLXN co-receptors within the HH signaling cascade has not been described. In this chapter, I will review pertinent information about HH signal transduction, including what is known about the cell-surface regulation of the pathway, its intracellular signaling cascade, and its importance in both development and disease. I then summarize SEMA signal transduction and its importance, highlighting known roles and downstream effectors for NRP and PLXN co-receptors. In the final section of this chapter, I discuss the overlap between HH and SEMA signaling pathways, highlighting major gaps in our knowledge that will be further explored in Chapters 2, 3, and 4.

1.3 Overview of Hedgehog Signal Transduction

1.3.1 Discovery and Conservation of Hedgehog Pathway Components

The HH signaling pathway was identified as a result of a genetic screen for patterning mutants in *Drosophila melanogaster* larvae (Nusslein-Volhard and Wieschaus, 1980). One of the mutants displayed an expansion of the dorsal denticle stripes into a broad swath, resembling hedgehog spines (Nusslein-Volhard and Wieschaus, 1980). The mutant was therefore called "Hedgehog," although it would be over a decade until the gene encoding the HH ligand was cloned and characterized (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993)

Since its initial discovery in *Drosophila*, conserved homologs of the *Hh* gene have been identified in a wide variety of vertebrate species, including zebrafish, chicken, mice, rats, and humans (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Marigo et al., 1995; Riddle et al., 1993; Roelink et al., 1994). Duplication events gave rise to three *Hh* genes within the mammalian and avian lineages, *Sonic Hedgehog* (*Shh*), *Indian Hedgehog* (*Ihh*), and *Desert Hedgehog* (*Dhh*), with further duplication events resulting in additional *Shh* and *Ihh* homologs (Echelard et al., 1993; Ingham and McMahon, 2001). HH ligands are highly conserved, with vertebrate SHH and IHH most closely related to one another, and DHH most closely resembling the *Drosophila* HH ligand (Varjosalo and Taipale, 2008). Importantly, core components of the downstream HH signaling cascade, including Patched (PTCH) receptors and cubitus interruptus (CI)/glioma-associated oncogene (GLI) family transcription factors, are also highly conserved (Ingham et al., 2011; Platt et al., 1997), although several differences exist between vertebrate and invertebrate signal transduction (Hooper and Scott, 2005; Varjosalo et al., 2006). One major difference is that vertebrate HH regulation requires a microtubule-based structure called the

primary cilium, which protrudes from the apical surface of the cell (Goetz and Anderson, 2010; Huangfu et al., 2003). Many components of the vertebrate HH signal cascade localize to the primary cilium in a highly-regulated way, and mutations that disrupt ciliary structure impact the processing of GLI transcription factors, as discussed below (Corbit et al., 2005; Haycraft et al., 2005; Huangfu et al., 2003; Hui and Angers, 2011; Liu et al., 2005; Rohatgi et al., 2007). For the purpose of this dissertation, I will focus on mammalian HH signal transduction, where HH signaling is a critical regulator of embryonic development as well as adult tissue homeostasis.

1.3.2 Hedgehog Pathway Contribution to Development and Disease

The HH signaling pathway elicits a multitude of cellular responses across nearly every mammalian tissue and organ to instruct embryonic development (McMahon et al., 2003). Proper levels of HH signaling are vital for embryonic growth and patterning, controlling processes ranging from digit specification (McGlinn and Tabin, 2006) to central nervous system formation (Dessaud et al., 2008) and craniofacial development (Xavier et al., 2016a; Xavier et al., 2016b). Defective HH signaling results in severe birth defects, including neural tube closure defects and holoprosencephaly (Hooper and Scott, 2005; Murdoch and Copp, 2010; Schachter and Krauss, 2008), while overactive HH signaling can cause a variety of cancers, including basal cell carcinoma and medulloblastoma (Barakat et al., 2010; Teglund and Toftgard, 2010). Given the importance of HH signaling in development and disease, understanding the mechanisms that regulate this pathway is central to discovering novel treatments for a growing number of HH-dependent pathologies. In particular, the characterization of new HH pathway regulators at the cell surface provides attractive targets for the development of novel therapeutic approaches to treat HH-driven diseases.

1.3.3 Hedgehog Pathway Regulation at the Cell Surface

Current models of HH signaling invoke a defined set of cell surface proteins that regulate HH ligand secretion, bind to HH ligands, and transmit signals to intracellular pathway components (Figure 1-0-1). These intracellular proteins then relay information to the nucleus, leading to altered gene expression. HH signaling is regulated by a number of different proteins to achieve ligand secretion, ligand reception, and ultimately, signal transduction.

In ligand-producing cells, immature HH proteins are translated in the form of approximately 45 kDa pro-proteins, which undergo signal sequence cleavage to enter the secretory pathway, followed by autocleavage catalyzed by the carboxyl-terminal (C-terminal) portion of the protein (Ingham et al., 2011; Lee et al., 1994). The C-terminal fragment is then thought to undergo rapid proteasomal degradation (Chen et al., 2011), while the N-terminal portion mediates most signaling activity (Briscoe and Therond, 2013). This N-terminal portion is conjugated to cholesterol at its C-terminal end during autocleavage and palmitoylated at its N-terminal end by skinny hedgehog (SKI) before undergoing secretion and distribution (Amanai and Jiang, 2001; Chamoun et al., 2001; Chen et al., 2004; Feng et al., 2004; Mann and Beachy, 2004; Pepinsky et al., 1998; Taylor et al., 2001).

Several cell-surface proteins are required for secretion and long-range distribution of HH ligands. Dispatched (DISP) family proteins are 12-pass transmembrane proteins required for secretion of cholesterol-modified HH ligands (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002). Interestingly, HH ligand lacking cholesterol modification does not require DISP for secretion (Burke et al., 1999). At least two different DISP homologues are present in mice, DISP1 and DISP2, but deletion of DISP1 is sufficient to cause a

near-complete loss of HH signaling (Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002; Tian et al., 2005). DISP1 binds directly to the cholesterol moiety of HH ligands, but requires Signal sequence, CUB domain, EGF-related (SCUBE) family proteins to liberate HH ligands from the cell surface (Creanga et al., 2012; Hollway et al., 2006; Kawakami et al., 2005; Tukachinsky et al., 2012; Woods and Talbot, 2005). Secreted SCUBE proteins shield the cholesterol moiety of HH ligands in order to maintain HH solubility in the extracellular space (Creanga et al., 2012; Tukachinsky et al., 2012). Similar to DISP, deletion of the three zebrafish SCUBE proteins causes a complete loss of HH signaling, highlighting the importance of proper HH packaging and secretion (Johnson et al., 2012).

To maintain their solubility while traveling through the extracellular space, HH ligands utilize several strategies, including assembly into multivalent ligand complexes. As a result, HH proteins are often observed as large punctate structures via immunofluorescent microscopy (Gallet et al., 2003; Gallet et al., 2006). While monomeric ligands are observed, multimeric complexes are more stable and exhibit significantly more potent signaling activity (Chen et al., 2004; Feng et al., 2004; Zeng et al., 2001). Packaging of HH ligands into multimeric structures requires cholesterol modification, palmitoylation, and interactions with heparan sulfate proteoglycans (HSPGs) at the cell-surface (Chen et al., 2004; Gallet et al., 2003; Gallet et al., 2006; Vyas et al., 2008). The exact nature of HH secretion remains controversial, and many studies suggest the involvement of exovesicles or lipoprotein particles to facilitate HH distribution (Eugster et al., 2007; Liegeois et al., 2006; Panakova et al., 2005; Tanaka et al., 2005; Therond, 2012). Other studies propose a mechanism by which HH ligands are transported via long filopodial cellular extensions, thus circumventing the need for long-range diffusion (Bischoff et al., 2013; Kornberg and Roy, 2014; Ramirez-Weber and Kornberg, 1999; Rojas-

Rios et al., 2012; Sanders et al., 2013). These various models of HH secretion and distribution are not mutually exclusive, but further work is needed to understand the complexities of HH ligand dispersal.

On receiving cells, HH ligands interact with a variety of proteins, which act to restrict ligand distribution and mediate downstream signaling events (Figure 1-0-1). Patched 1 (PTCH1) is a twelve-pass transmembrane protein that is the canonical receptor for HH ligands (Marigo et al., 1996; Stone et al., 1996). Prior to the onset of HH signaling, PTCH1 inhibits the activity of a second cell surface protein, Smoothened (SMO), a seven-pass transmembrane protein with GPCR-like activity (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). As a result, transcription factors from the GLI family are processed into repressors that inhibit target gene expression. HH ligand binding to PTCH1 results in de-repression and ciliary localization of SMO, which activates an intracellular signaling cascade that results in the activation of GLI transcription factors and the modulation of target gene expression (Hui and Angers, 2011).

Beyond PTCH1 and SMO, several additional classes of cell-surface proteins regulate HH signaling in a tissue- and time-specific manner (Figure 1-0-1). The HH co-receptors, which include growth arrest-specific 1 (GAS1), CAM-related/downregulated by oncogenes (CDON), and brother of CDON (BOC), promote HH signaling in a ligand-dependent manner (Allen et al., 2011; Allen et al., 2007; Lee et al., 2001; Tenzen et al., 2006). CDON and BOC are single-pass transmembrane proteins containing extracellular Ig repeats and fibronectin type III (FN III) domains, while GAS1 contains a GPI-anchor which mediates membrane association (Cabrera et al., 2006; Kang et al., 1997; Kang et al., 2002; Stebel et al., 2000). Importantly, all three HH co-receptors bind directly to HH ligands and can interact with PTCH1 to form different receptor complexes (Allen et al., 2011; Allen et al., 2007; Izzi et al., 2011; Lee et al., 2001; Martinelli and

Fan, 2007; Tenzen et al., 2006). The single-pass transmembrane protein Low-density lipoprotein receptor-related protein 2 (LRP2) also directly binds HH ligands, regulating uptake and intracellular trafficking of SHH to critically regulate HH in a variety of developmental tissues (Christ et al., 2015; Christ et al., 2012; Christ et al., 2016). In addition to their roles in HH ligand distribution, members of the HSPG family of glycoproteins also regulate HH signaling in ligand-receiving cells (Bandari et al., 2015; Yan and Lin, 2008). Similar to the HH co-receptors, HSPGs can directly interact with HH ligands and other pathway components at the cell surface (Bumcrot et al., 1995; Capurro et al., 2008; Chang et al., 2011; Lee et al., 1994; Rubin et al., 2002; Whalen et al., 2013; Williams et al., 2010; Zhang et al., 2007). Examples include Glypican-3 (GPC3), which antagonizes HH signaling (Capurro et al., 2009; Capurro et al., 2008), and Glypican-5 (GPC5), which can either promote or inhibit HH signaling depending on context (Li et al., 2011; Witt et al., 2013). Furthermore, an additional set of proteins antagonizes HH signaling at the cell surface. Patched 2 (PTCH2), a structural homolog of PTCH1, acts in a ligand-dependent manner to antagonize HH signaling (Carpenter et al., 1998; Holtz et al., 2013; Motoyama et al., 1998). Hedgehog interacting protein 1 (HHIP1) also binds HH ligand to antagonize HH signaling, although HHIP1 is unique in that it is a secreted antagonist that maintains membrane association through interactions with HSPGs (Chuang et al., 2003; Chuang and McMahon, 1999; Holtz et al., 2015; Holtz et al., 2013; Jeong and McMahon, 2005). Together, these diverse groups of cell-surface proteins are essential to control HH pathway activity in a context-dependent manner.

1.3.4 Intracellular Hedgehog Signaling Events

HH signal transduction ultimately culminates in the modulation of GLI transcriptional activity. In mammals, there are three GLI family members, GLI1, GLI2, and GLI3, each of

which contains five C2H2 zinc-finger DNA binding domains (Aza-Blanc et al., 1997; Barnfield et al., 2005; Briscoe and Therond, 2013; Chen et al., 1999; Hepker et al., 1997; Kinzler and Vogelstein, 1990; Wang and Holmgren, 1999). GLI2 and GLI3 are proteolytically processed into activator and repressor forms, with HH pathway activity regulating the balance between the two in order to modulate target gene expression (Briscoe and Therond, 2013; Dai et al., 1999). In mammals, GLI1 exclusively functions as a transcriptional activator, GLI2 mainly function as an activator, and GLI3 mainly functions as a repressor (Bai et al., 2004; Hui and Angers, 2011; Litingtung and Chiang, 2000; Matise et al., 1998). However, there is evidence that GLI2 selectively functions as a transcriptional repressor (Chang et al., 2016; Pan et al., 2006), while GLI3 can operate as a transcriptional activator in certain tissues (Bai and Joyner, Cell, 2004).

Intracellular GLI2/3 regulation is complex, relying on the integration of a number of different biochemical processes. In the absence of HH pathway activation, GLI2 and GLI3 are phosphorylated by protein kinase A (PKA), casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3β), leading to near complete degradation of GLI2 and proteolytic cleavage of GLI3 into its repressor form (Pan et al., 2006; Pan and Wang, 2007; Pan et al., 2009; Tempe et al., 2006; Wang et al., 2000; Wang and Li, 2006). Interestingly, PKA phosphorylation at six highly conserved residues is required for processing and degradation of GLI2/3, while dephosphorylation at those sites combined with phosphorylation at additional partial consensus PKA sites is required for GLI2/3 to act as transcriptional activators (Niewiadomski et al., 2014). Thus, PKA phosphorylation regulates both GLI activator and GLI repressor states to modulate HH signaling (Hammerschmidt et al., 1996; Kaesler et al., 2000; Niewiadomski et al., 2014). GLI1, on the other hand, is regulated and degraded by proteins numb and itch (Di Marcotullio et al., 2006). In addition, DYRK family kinases control nuclear localization and transcriptional

activity of GLI1, while simultaneously promoting degradation of GLI2 and GLI3 (Mao et al., 2002). When HH ligand is present, SMO activation results in a change in the balance of GLI processing, favoring activator forms. While the mechanisms by which SMO regulates GLI proteins are incompletely understood, negative regulators of GLI activity, including kinesin family protein 7 (KIF7) and suppressor of fused (SUFU), are inhibited when SMO is activated (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009; Raisin et al., 2010; Robbins et al., 1997; Ruel et al., 2007; Sisson et al., 1997; Tay et al., 2005).

Interestingly, several core components of the HH signaling cascade localize to the primary cilium, including GLI proteins (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). The primary cilium is a microtubule-based structure that protrudes from the apical surface of most mammalian cell types during growth arrest (Sorokin, 1968). While once thought to be a vestigial structure, we now understand that the cilium plays important roles in regulating developmental signaling, and HH signaling in particular (Goetz and Anderson, 2010). Within primary cilia, proteins are trafficked by two types of motor proteins, with kinesins carrying cargo in the anterograde direction and dyneins transporting cargo in the retrograde direction (Goetz and Anderson, 2010). Importantly, a specialized pore complex at the base of primary cilia tightly regulates movement of molecules into and out of this subcellular compartment which are too large to diffuse through this barrier (Gilula and Satir, 1972; Kee et al., 2012; Rosenbaum and Witman, 2002; Takao et al., 2014). Notably, GLI1, GLI2, and GLI3 all localize to primary cilia, although GLI3 is not detected after processing into its repressor form (Haycraft et al., 2005). When HH signaling is off, GLI2 and GLI3 cycle through the primary cilium at low levels, while pathway activation results in their rapid accumulation at the tips of cilia (Chen et al., 2009; Kim et al., 2009; Wen et al., 2010). PKA localizes to and is thought to modify GLI proteins at the

base of the primary cilium, and loss of PKA increases the amount of GLI at cilia tips (Barzi et al., 2010; Tukachinsky et al., 2010; Tuson et al., 2011; Zeng et al., 2010). Furthermore, many studies indicate that mutations disrupting ciliary structure and transport impact GLI processing and activity, causing defective HH signaling (Gorivodsky et al., 2009; Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Hui and Angers, 2011; Keady et al., 2012; Liu et al., 2005; Ocbina et al., 2011; Qin et al., 2011; Takeda et al., 1999; Yang et al., 2015). Surprisingly, mutations in ciliary transport processes can result in HH loss-of-function and gain-of-function phenotypes, depending on context (Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005; Wong et al., 2009). One model suggests that active HH signaling leads to SMO accumulation in primary cilia and localization of SUFU-GLI complexes to the tips of cilia, leading to their dissociation and subsequent processing into GLI activator forms (Tukachinsky et al., 2010). Together, these studies highlight the importance of primary cilia in GLI regulation, although many questions still remain in this area of research.

Ultimately, the complex regulation and processing of GLI proteins into activator and repressor forms, the cycling of GLI proteins through the primary cilium, and the accessibility of chromatin all influence HH target gene regulation within the nucleus. GLI proteins bind to both high- and low-affinity sites within tissue-specific promoter and enhancer elements of a wide variety of genes throughout the genome to regulate cell behaviors (Lorberbaum et al., 2016; Ramos and Barolo, 2013). HH transcriptional targets vary in a context- and developmental stage-specific manner (Aberger and Ruiz, 2014; Oosterveen et al., 2012; Peterson et al., 2012; Vokes et al., 2007; Vokes et al., 2008). However, many HH targets promote cell survival and growth – processes essential for development and tissue maintenance, although potentially detrimental when activated in cancerous cells. Common examples of GLI target genes include the HH

pathway members *Ptch1* (Agren et al., 2004), *Gli1* (Dai et al., 1999), *Ptch2* (Holtz et al., 2013; Vokes et al., 2007), *Hhip1* (Vokes et al., 2007), and *Boc* (Lee et al., 2010). However, a multitude of additional HH target genes regulate cell fate specification, differentiation, proliferation, and survival in a stage- and tissue-specific manner.

A variety of proteins regulate HH signaling, both intracellularly and at the plasma membrane. However, our understanding of these regulatory mechanisms, including which proteins are active under specific conditions and how these inputs are simultaneously incorporated to inform cell behavior, is far from complete. Furthermore, how HH signaling overlaps with other signaling pathways within in the same cells, and how those various signals are integrated are questions that remain largely unexplored. Recent evidence suggests that receptors from the SEMA signaling pathway, which is important for axon guidance and other processes in the developing embryo, also regulate HH signaling (Ge et al., 2015; Hillman et al., 2011). Understanding the process of SEMA signal transduction and how it might influence HH signaling will be the focus of the next two sections of this chapter.

1.4 Overview of Vertebrate Semaphorin Signaling

1.4.1 Discovery and Diversity of Semaphorin Ligands

SEMA ligands were originally described in the early 1990s for their role in axon guidance and growth (Kolodkin et al., 1992; Luo et al., 1993; Raper and Grunewald, 1990). While studying nervous system development in grasshopper embryos, Kolodkin et. al., cloned and characterized a novel transmembrane protein called fasciclin IV, which regulates axon guidance in the grasshopper limb bud and central nervous system (Kolodkin et al., 1992). Simultaneously, another group identified a biochemical extract from chicken brains that causes

growth cone collapse in cultured cells, naming the protein "collapsin" (Luo et al., 1993; Raper and Kapfhammer, 1990). Later, it was discovered that fasciclin and collapsin were two members of the same protein family, containing a unique, conserved SEMA domain that would come to define SEMA ligands as well as their PLXN receptors.

Since the original discovery of fasciclin (SEMA1A) and collapsin (SEMA3A), 30 different SEMA ligands have been identified, separated into eight classes based on structural homology and species-specific expression (Alto and Terman, 2017). While most of the SEMA ligands have been described in vertebrates, class one and two SEMAs along with SEMA5C are expressed only in invertebrates, and class V SEMAs are found in viruses (Alto and Terman, 2017). Interestingly, both vertebrate and non-vertebrate SEMAs can be transmembrane, glycosylphosphatidylinositol (GPI)-anchored, or secreted, and some contain Ig domains, basic domains, and thrombospondin repeats (Alto and Terman, 2017). Despite the diversity of SEMA ligand expression and structure, their defining feature is a cysteine-rich, 7-blade beta propeller fold motif known as the SEMA domain, and with the exception of some viral SEMAs, almost all have a cysteine-rich plexin-semaphorin-integrin PSI domain (Alto and Terman, 2017). PLXN family proteins as well as some receptor tyrosine kinases also contain a SEMA domain, and therefore are considered part of the SEMA superfamily (Siebold and Jones, 2013). The SEMA domain mediates dimerization between SEMA ligands, which is thought to be required for certain members to function, as well as receptor-ligand interactions (Klostermann et al., 1998; Koppel and Raper, 1998).

1.4.2 Semaphorin Regulation at the Cell Surface

While SEMA ligands can interact with several additional molecules (Hota and Buck, 2012), they are thought to primarily signal through type I, single-pass transmembrane proteins from the PLXN family (Perala et al., 2012). Secreted SEMA ligands also require NRP co-receptors, which form complexes with PLXNs to transduce signals downstream.

There are two NRP family members, NRP1 and NRP2, which have well-established roles in both axon guidance and angiogenesis (Kawasaki et al., 1999; Gu et al., 2003; Gelfand et al., 2014; Takashima et al., 2002; Fujisawa, 2004). While sharing only 44% overall homology, NRP1 and NRP2 have a similar domain architecture, which includes two complement binding (CUB) domains, denoted a1 and a2; two coagulation factor V/VIII homology-like domains, denoted b1 and b2, and a membrane-proximal meprin A5 (MAM) domain, denoted c (Figure 1-0-2) (Chen et al., 1997; Giger et al., 1998; He and Tessier-Lavigne, 1997; Neufeld and Kessler, 2008). NRPs bind to secreted, class 3 SEMA ligands through their extracellular a1, a2, and b1 domains, although they are unable to transduce downstream SEMA signals on their own, due to a lack of catalytic activity in their short cytoplasmic domain (Gu et al., 2002; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Neufeld and Kessler, 2008; Vander Kooi et al., 2007). In fact, studies suggest that the NRP1 cytoplasmic domain is dispensable for SEMA function, although it binds to the PDZ domain-containing protein GAIP C-terminus interacting protein 1 (GIPC1) and plays a role in regulating the spatial separation of arteries and veins (Cai and Reed, 1999; Fantin et al., 2011; Gao et al., 2000). Instead, NRPs require PLXN co-receptors to transduce signals from secreted SEMA ligands (Takahashi et al., 1999; Tamagnone et al., 1999).

While secreted SEMA ligands require NRP-PLXN complexes to signal, the majority of SEMA ligands interact directly with PLXN receptors (Neufeld and Kessler, 2008). The PLXN

family of receptors contains nine members, separated into four different subfamilies, including four type A PLXNs (PLXNA1, PLXNA2, PLXNA3, and PLXNA4), three type B PLXNs (PLXNB1, PXLNB2, and PLXNB3), and a single type C (PLXNC1) and type D (PLXND1) member (Figure 1-0-3) (Neufeld and Kessler, 2008). Common structural features across all PLXN subfamilies include the presence of an extracellular SEMA domain as well as PSI and glycine-proline (G-P) rich motifs, similar to those found in the Met receptor tyrosine kinase family (Comoglio et al., 2003; Tamagnone et al., 1999). Intracellularly, PLXNs are defined by their split GTPase-activating (GAP) domain, which is activated by SEMA ligand binding and regulated intracellularly by a series of binding and phosphorylation events, ultimately resulting in differential regulation of small intracellular G proteins and kinases to influence cell migration and morphology (Oinuma et al., 2004a). Notably, type B PLXNs are distinguished by both a C-terminal binding site for PDZ domain-containing guanine nucleotide exchange factors (PDZ-RHOGEF or ARHGEF11) and leukemia-associated RHOGEF (LARG or ARHGEF12) as well as a conserved cleavage site for furin-like pro-protein convertases (Aurandt et al., 2002; Perrot et al., 2002; Swiercz et al., 2002; Tamagnone et al., 1999). Distinctive classes of SEMA ligands exhibit specificity in their interactions with PLXN family members (Figure 1-0-3), with Class 3 secreted SEMA ligands also requiring NRP co-receptors (Neufeld and Kessler, 2008).

1.4.3 Intracellular Semaphorin Signaling Events

Downstream of PLXNs, an increasingly large number of cytosolic effectors mediate cytoskeletal dynamics and cell adhesion in various ways depending upon the SEMA ligand and PLXN subfamily member involved. Generally, PLXNs both regulate and are regulated by the collapse-response-mediator protein (CRMP) family of phosphoproteins, protein kinases,

molecule interacting with Cas ligand (MICAL) redox proteins, and small intracellular GTPases from the Rho and Ras superfamilies (Jongbloets and Pasterkamp, 2014; Puschel, 2007; Yang and Terman, 2013). Together, these effectors regulate diverse cellular processes, including axonal transport, cell death, microtubule dynamics, actin dynamics, and protein translation (Figure 1-0-4). Here, I will briefly summarize what is known about intracellular signaling downstream of PLXNs:

CRMP Family Phosphoproteins are Regulated by Semaphorin Signals

CRMP family phosphoproteins were some of the first downstream SEMA signaling components to be described, originally identified for their roles in neuronal differentiation downstream of SEMA3A ligands (Puschel, 2007). There are five different vertebrate CRMPs (CRMP1-5), which are highly expressed during vertebrate embryonic and adult neurogenesis (Doetsch and Hen, 2005; Veyrac et al., 2005; Wang and Strittmatter, 1996). While cytosolic in nature, a large pool of CRMP protein is enriched at the cell membrane, particularly at the leading edge of lamellipodia and filopodia, suggesting a role for CRMP proteins in axon guidance and growth (Minturn et al., 1995; Rosslenbroich et al., 2003). Indeed, CRMPs bind to the PLXNA1 cytoplasmic domain and are required for SEMA3A-mediated cytoskeletal collapse (Deo et al., 2004; Goshima et al., 1995). PLXNA1 binding to CRMP proteins mediates both conformational changes and C-terminal phosphorylation events that modulate CRMP activity (Brown et al., 2004; Deo et al., 2004; Goshima et al., 1995; Mitsui et al., 2002; Uchida et al., 2005). While the requirement for CRMPs downstream of SEMA3A has been clearly established, the mechanisms by which CRMPs mediate cytoskeletal collapse remain unclear. One possibility is that CRMPs regulate microtubules and actin cytoskeletal elements directly, since CRMPs are known

regulators of microtubule dynamics and co-localize with actin in growth cones and neuronal cell lines (Fukata et al., 2002; Gu and Ihara, 2000; Hotta et al., 2005; Quinn et al., 2003; Rosslenbroich et al., 2005; Yoshimura et al., 2005; Yuasa-Kawada et al., 2003). Evidence also exists for CRMP regulation of small intracellular GTPases (discussed below) that bind to and regulate PLXN cytoplasmic activity (Arimura et al., 2000; Hall et al., 2001; Jin and Strittmatter, 1997; Toyofuku et al., 2005; Turner et al., 2004; Zanata et al., 2002). In addition, CRMPs mediate receptor-mediated endocytosis (Lee et al., 2002; McDermott et al., 2004; Nishimura et al., 2003; Santolini et al., 2000; Shen et al., 2001), and bind to mammalian MICAL1 (Puschel, 2007), which has also been associated with PLXN signaling (discussed below). Together, these studies suggest that CRMP family proteins downstream of SEMA3A likely regulate multiple important cellular processes that may contribute to cytoskeletal collapse.

Plexins Regulate MICAL Redox Proteins

Another important regulatory axis downstream of SEMA-PLXN interactions involves PLXN binding to MICAL family proteins (Alto and Terman, 2017; Jongbloets and Pasterkamp, 2014; Yang and Terman, 2013). MICALs are flavoprotein monooxygenase enzymes that directly regulate the post-translational oxidation of actin subunits (Terman et al., 2002; Zhou et al., 2011). MICALs bind to PLXN cytoplasmic domains and induce F-actin disassembly directly by oxidating actin Met-44, which is critical for actin monomer contact (Hung et al., 2011; Hung and Terman, 2011; Hung et al., 2010). Interestingly, the methionine sulfoxide reductase SelR counteracts this process, providing a mechanism for actin regulation downstream of SEMA-PLXN signaling (Hung et al., 2013; Lee et al., 2013). Because of their large, multi-domain structures, MICALs can also interact with CRMPs and small GTPases (see below), suggesting a

potentially more complicated role in PLXN intracellular signaling (Deo et al., 2004; Puschel, 2007; Schmidt et al., 2008; Togashi et al., 2006). Thus, MICALs may play multiple roles in downstream SEMA signaling, regulating the actin cytoskeleton directly and/or modulating other signaling cascades involved in cell adhesion via integrins, vesicular trafficking, or other cellular processes (Puschel, 2007).

Protein Kinases Regulate and are Regulated by Plexin Receptors

Multiple kinases act downstream of SEMA ligands to regulate cell responses including actin dynamics, microtubule dynamics, axonal transport, protein translation, and cell death (Puschel, 2007). Interestingly, however, exactly how SEMA receptor activation is coupled to many of these processes remains unclear. At the cell surface, PLXNB1 complexes with receptor tyrosine kinases Met and ERBB2 in response to SEMA4D signaling, triggering invasive growth of epithelial cells (Giordano et al., 2002). In addition, Off-track receptors, which contain receptor tyrosine kinase domains but lack associated kinase activity, bind to both PLXNA3 and PLXNB1, likely recruiting other kinases to mediate downstream signaling (Winberg et al., 2001). Intracellularly, many phosphorylation events also coordinate cell behaviors in response to SEMA signaling. ADF/cofilin proteins bind to actin to regulate filament severing, and dissociation of these proteins from actin leads actin redistribution (Bamburg and Wiggan, 2002). Strikingly, activity of Lin-11, Isl-1, and Mec-3 kinase (LIMK), which phosphorylates and inactivates ADF/cofilin, is necessary for SEMA3A-mediated growth cone collapse (Aizawa et al., 2001). Several upstream regulators of LIMK and cofilin, including Rac, PAK, RhoA, ROCK, R-Ras, PI3K, and PTEN have been associated with PLXN receptor signaling and may regulate actin dynamics downstream of SEMA3A. However, LIMK1 activation is not sufficient to induce

growth cone collapse, suggesting that multiple mechanisms likely regulate this complex cellular behavior (Aizawa et al., 2001). As discussed above, another mechanism required for SEMA-mediated growth cone collapse is the alteration of microtubule dynamics downstream of CRMP and Tau (Kruger et al., 2005; Puschel, 2007). SEMA signaling regulates multiple kinases which modulate CRMP activity, including Cdk-5, GSK3β, Fyn tyrosine kinase, Fes/Fps, and Rho kinase (ROCK) (Arimura et al., 2000; Arimura et al., 2005; Brown et al., 2004; Cole et al., 2006; Mitsui et al., 2002; Sasaki et al., 2002; Uchida et al., 2005). Further investigation is necessary to determine how these inputs coordinate cytoskeletal collapse in a cell type-specific manner.

It is likely that SEMA-regulated kinases play multiple roles within the cell. For example, Cdk-5, GSK3β, and Fyn kinase regulate CRMP activity, but they also regulate axonal transport via dynein and kinesin motility (Morfini et al., 2004; Niethammer et al., 2000). SEMA3A ligand induction facilitates both anterograde and retrograde transport in DRG neurons, potentially through the involvement of these kinase complexes (Goshima et al., 1999; Goshima et al., 1997; Li et al., 2004). In addition, SEMA signaling increases localized protein synthesis in axons and growth cones, which is necessary for turning and collapse responses (Campbell and Holt, 2001; Willis and Twiss, 2006). This process is dependent upon MAPK phosphorylation (Guertin and Sabatini, 2005), which occurs in response to SEMA3A, SEMA3F, SEMA7A, and SEMA4D stimulation (Atwal et al., 2003; Aurandt et al., 2006; Bagnard et al., 2004; Campbell and Holt, 2001; Pasterkamp et al., 2003). Furthermore, SEMA3A can induce apoptosis in a manner dependent on p38 MAPK activation and JNK/c-Jun signaling (Bagnard et al., 2004; Ben-Zvi et al., 2006). PLXNs also bind to and regulate 14-3-3ε in a manner dependent upon PKA phosphorylation, which antagonizes SEMA-PLXNA signaling in *Drosophila* (Yang and Terman,

2013). A better understanding of how these complex and diverse processes are regulated by SEMA-dependent kinase activation will require additional study.

Plexins Interact with and Regulate Small Intracellular GTPases from the Rho and Ras Superfamilies

Perhaps the most widespread regulators of SEMA signaling downstream of PLXNs are small intracellular GTPases. In general, GTPases act as molecular switches that can hydrolyze GTP to GDP (Wennerberg et al., 2005). GTPases are considered to be in an active state when bound to GTP and an inactive state when bound to GDP (Vetter and Wittinghofer, 2001). These two states are catalyzed by guanine nucleotide exchange factors (GEFs), which promote the GTP-bound state to activate GTPases, and GTPase activating proteins (GAPs), which promote GTP hydrolysis to inactivate small GTPases (Wennerberg et al., 2005). GTPases, in turn, regulate cell shape and movement by binding to various downstream targets and either recruiting them to specific subcellular localizations or inducing conformational changes. Targets of GTPases include Rho kinase (ROCK), p21-activated kinase (PAK), phosphatidylinositol-4-phosphate-5-kinase (PI4P5K), neuronal Wiskott-Aldrich Syndrome protein (N-WASP), and formins (Goode and Eck, 2007; Hall, 2005; Hall and Lalli, 2010; Yang and Terman, 2013). Over 150 GTPases have been characterized within the Ras superfamily, organized into five subfamilies including Ras, Ran, Rho, Rab, and Arf (Wennerberg et al., 2005). In addition to many other important roles within the cell, Rho subfamily GTPases have been well-characterized for regulating the cytoskeleton during axon guidance (Hall, 2005; Hall and Lalli, 2010). While a plethora of evidence associates PLXN receptors with GTPase regulation, the exact mechanisms by which PLXN-GTPase signaling modulates cell behavior are varied and complex.

Interestingly, PLXNs both regulate and are regulated by small intracellular GTPases. Both sequence (Bos and Pannekoek, 2012; Hota and Buck, 2012; Negishi et al., 2005) and structural (He et al., 2009; Tong et al., 2009) homology within the PLXN cytoplasmic domain suggests that PLXNs act as GAPs for Rho and Ras subfamily GTPases. However, PLXNs are unique from other GAPs in two ways: 1) they are membrane-spanning proteins which interact with small GTPases, and 2) their GAP domain is split into two parts, with a Rho GTPase binding domain (RBD) between the two (Neufeld and Kessler, 2008; Yang and Terman, 2013). SEMA ligand binding promotes clustering of PLXN receptors while also releasing their extracellular auto-inhibition (Oinuma et al., 2004b; Takahashi and Strittmatter, 2001). To become fully active, however, some PLXNs require GTPase binding to the RBD to activate their intracellular domains (Yang and Terman, 2013). In particular, several studies suggest that PLXNs from both the A and B subfamilies require RND1 binding to the RBD to fully activate cytoplasmic GAP activity (Negishi et al., 2005). RND1 is a constitutively active Rho-family GTPase that generally antagonizes RHO signaling, and some studies suggest that RND1 might act as an on/off switch for PLXNA1, competing with RHOD, which decreases its activity (Yang and Terman, 2013). A similar switch might also exist for PLXNB1, although it may instead involve RND1 and RAC1, since their binding domains overlap within the PLXNB1 RBD (Negishi et al., 2005; Oinuma et al., 2003).

Once the PLXN GAP domain is fully activated by SEMA and RND1 binding, it can interact with and regulate additional small GTPases from both the Rho and Ras subfamilies (Bos and Pannekoek, 2012; Hota and Buck, 2012; Negishi et al., 2005; Yang and Terman, 2013). Rho family GTPases, which include RHO, RAC, and CDC42, are known to regulate formation and collapse of stress fibers, lamellipodia, and filopodia in fibroblasts and neurons, and thus have

been of great interest in the study of SEMA signaling via PLXN activation (Katoh et al., 1998; Kozma et al., 1997; Kruger et al., 2005; Nobes and Hall, 1995). While *D. melanogaster* PLXN-B interacts directly with RHOA (Hu et al., 2001), mammalian PLXNB1 regulates RHO indirectly by interacting with leukemia-associated Rho GEF (LARG) and PDZ-Rho GEF (PRG) through its C-terminal PDZ motif (Aurandt et al., 2002; Perrot et al., 2002; Swiercz et al., 2002). LARG and PRG are GEFs that activate RHOA, which leads to repulsive growth (Kruger et al., 2005). Strikingly, RHO is not required for PLXNA1-mediated cytoskeletal collapse (Turner et al., 2004), highlighting the specificity and complexity of GTPase regulation downstream of PLXN receptors. RAC1, on the other hand, directly interacts with both PLXNA1 and PLXNB1, likely within the RBD (Hu et al., 2001; Turner et al., 2004; Vikis et al., 2002). While research continues on how RAC1 regulates and is regulated by PLXNA1, PLXNB1 is thought to sequester RAC1, preventing it from interacting with PAK, and thus inhibiting actin polymerization (Hu et al., 2001; Vikis et al., 2000). Evidence also exists that RAC1 may function upstream of PLXNA1 and PLXNB2, potentially regulating their localization to the cell surface (Turner et al., 2004; Vikis et al., 2002).

While the PLXN cytoplasmic domain regulates many effectors, PLXN GAP activity seems to be directed specifically toward Ras subfamily GTPases including R-RAS and RAP1 (Kruger et al., 2005; Yang and Terman, 2013). Both PLXNA1 and PLXNB1 regulate R-RAS, which binds to highly conserved arginine fingers within the PLXN GAP domains (Oinuma et al., 2004a). While most RAS subfamily members act to regulate the extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway, R-RAS plays a minimal role in ERK/MAPK signaling, instead regulating integrin activity at the cell surface (Keely et al., 1999; Kinbara et al., 2003; Serini et al., 2003). By deactivating R-RAS through GAP activity,

PLXNs therefore disrupt cell-cell or cell-substrate interactions through cadherins and integrins, leading to cytoskeletal collapse (Kinbara et al., 2003; Oinuma et al., 2004a; Serini et al., 2003; Vitriol and Zheng, 2012). PLXNs also bind to and regulate RAP1, another Ras subfamily GTPase, whose downstream effectors RIAM and RAPL enhance integrin activity through direct binding (Bos and Pannekoek, 2012; Kinbara et al., 2003; Wang et al., 2012; Yang and Terman, 2013).

1.4.4 Semaphorins and their Receptors: Importance in Development and Disease

Originally discovered for their role in axon guidance, SEMA signaling is now considered one of four canonical families of axon guidance molecules, along with ephrins, netrins, and slits (Alto and Terman, 2017; Tessier-Lavigne and Goodman, 1996). Through one or perhaps multiple of the mechanisms described above, SEMA signaling through NRP and PLXN receptors results in proper localization of axonal growth cones to their appropriate targets (Rohm et al., 2000). Interestingly, while generally thought to mediate axonal repulsion, SEMA signals can also act to attract growth cones, depending on cellular context (Masuda et al., 2004; Moreno-Flores et al., 2003; Pasterkamp et al., 2003; Polleux et al., 2000). For example, in a process known as reverse signaling, transmembrane SEMAs can act as receptors instead of ligands to regulate cell migration through their cytoplasmic domains (Battistini and Tamagnone, 2016; Hernandez-Fleming et al., 2017; Sun et al., 2017; Toyofuku et al., 2004). Through these mechanisms, SEMAs direct the migration and segregation of neural crest cells (Ruhrberg and Schwarz, 2010), commissural axons (Zou et al., 2000), GABAergic interneurons (Marin and Rubenstein, 2003; Marin et al., 2001), cortical neurons (Chen et al., 2008), retinal neurons (Kuwajima et al., 2012), and cerebellar granule neurons (Kerjan et al., 2005; Renaud et al.,

2008), while also preventing CNS neurons from migrating out of the spinal cord (Bron et al., 2007; Mauti et al., 2007; Vermeren et al., 2003). SEMA signals additionally regulate the formation of neuroendocrine loops, influencing migration of GnRH neurons and periodic neuroglial remodeling in the hypothalamus (Cariboni et al., 2011; Cariboni et al., 2007; Giacobini et al., 2008; Messina et al., 2011; Parkash et al., 2015). Furthermore, SEMA signaling regulates synapse development, axonal branching, and dendritic pruning within the developing nervous system, potentially acting on the same neuron in multiple different ways over time (Alto and Terman, 2017; Oh and Gu, 2013; Pasterkamp and Giger, 2009; Tillo et al., 2012; Vanderhaeghen and Cheng, 2010; Yoshida, 2012).

In addition to their well-characterized roles in the nervous system, SEMA ligands and receptors regulate cell adhesion, shape, motility, survival, and differentiation in a growing list of non-neuronal tissues (Alto and Terman, 2017). For example, SEMA signaling plays many roles within the cardiovascular system, regulating vascular growth and patterning (Oh and Gu, 2013; van der Zwaag et al., 2002), heart formation and innervation (Behar et al., 1996; Feiner et al., 2001; Gitler et al., 2004; Ieda and Fukuda, 2009; Ruhrberg and Schwarz, 2010; Toyofuku and Kikutani, 2007), lymphatic vessel development (Bouvree et al., 2012; Jurisic et al., 2012), and adult vascular permeability and repair (Azzi et al., 2013; Treps et al., 2013; Wannemacher et al., 2011). Within the immune system, SEMA ligands from classes 3, 4, 6, and 7 as well as NRP and PLXN receptors play important roles, largely in immune cell migration and communication (Kumanogoh and Kikutani, 2013; Roney et al., 2013; Suzuki et al., 2008; Takamatsu and Kumanogoh, 2012). Interestingly, SEMA family proteins are expressed in both osteoblasts and osteoclasts, where they function in bone homeostasis for proper musculoskeletal function (Kang and Kumanogoh, 2013). Additionally, in the mouse reproductive system, SEMA4D is required

for proper ovarian follicle development and steroid hormone production (Dacquin et al., 2011; Regev et al., 2007). Due to their nearly ubiquitous expression (Yazdani and Terman, 2006), SEMA family proteins are being studied in a growing list of organs, including kidney, eye, muscle, and lung (Alto and Terman, 2017).

Beyond development and adult tissue homeostasis, SEMA signaling influences the balance between tumor-suppressive and tumor-promoting mechanisms in various types of cancer (Alto and Terman, 2017; Neufeld and Kessler, 2008). SEMA ligand and receptor expression is often altered in cancer, with functional impacts on tumor growth and metastasis (Gu and Giraudo, 2013; Neufeld et al., 2012; Tamagnone, 2012; Thirant et al., 2013). Notable examples include several SEMA ligands which act to restrict growth and motility in breast, prostate, and lung cancer cells (Bachelder et al., 2003; Herman and Meadows, 2007; Pan and Bachelder, 2010; Tomizawa et al., 2001; Xiang et al., 2002), while others promote growth in malignant mesothelioma (Catalano et al., 2009). Furthermore, due to their known roles in vascular regulation, SEMA family members play important roles in regulating tumor angiogenesis (Basile et al., 2004; Basile et al., 2006; Bielenberg et al., 2004; Cagnoni and Tamagnone, 2014; Maione et al., 2009; Neufeld et al., 2012; Sierra et al., 2008). SEMAs also modulate behavior of cells within the tumor microenvironment, including immune cells and fibroblasts (Cagnoni and Tamagnone, 2014; Gu and Giraudo, 2013; Squadrito and De Palma, 2011; Tamagnone, 2012). Importantly, the role that SEMAs play in cancer is highly dependent on cell type and context (Neufeld et al., 2012). Understanding how SEMA ligands and receptors function in various cellular contexts during development, homeostasis, and cancer will provide crucial insight into their activity and regulation. Moreover, characterizing how SEMA signals interact with other

signaling pathways, including HH, will provide a more global understanding of how signaling networks influence cell behavior.

1.5 Potential Cross-Regulation Between Hedgehog and Semaphorin Signaling Pathways

Both HH and SEMA play important roles in embryonic development and adult tissue homeostasis, while their disruption leads to unfavorable pathological consequences. However, little is known about how the HH and SEMA pathways might overlap and influence one another in developmental and disease contexts (Figure 1-0-5). Here, I will review the evidence for cross-regulation between HH and SEMA signaling pathways.

1.5.1 Overlapping Expression of Hedgehog and Semaphorin Pathway Members

To influence one another *in vivo*, HH and SEMA pathway components are likely to be expressed in overlapping or adjacent cell populations. Interestingly, both HH and SEMA signaling components exhibit widespread expression throughout vertebrate embryos as well as within adult tissues (Mauti et al., 2006; Perala et al., 2012; Perala et al., 2005). Of the HH ligands, DHH has a more restricted expression domain, while IHH and SHH are expressed much more broadly (Bitgood and McMahon, 1995; Varjosalo and Taipale, 2008). IHH is expressed in the yolk sac, primitive endoderm, and developing bone, while DHH is expressed mostly in the gonads (Bitgood et al., 1996; Dyer et al., 2001; St-Jacques et al., 1999; van den Brink, 2007; Vortkamp et al., 1996; Wijgerde et al., 2005; Yao et al., 2002) In contrast, SHH can be found in most epithelial tissues during organogenesis, with important roles in midline structures and the distal limb bud during early embryogenesis (Chang et al., 1994; Johnson et al., 1994; Marti et al., 1995; Meyer and Roelink, 2003; Pagan-Westphal and Tabin, 1998; Riddle et al., 1993; Sampath

et al., 1997; Schilling et al., 1999; Watanabe and Nakamura, 2000). Interestingly, SEMA ligands are also expressed in most embryonic tissues, although our understanding of their roles beyond the nervous system remains limited (Fiore and Puschel, 2003; Yazdani and Terman, 2006). Similarly, NRP and PLXN receptors can be found in tissues throughout the nervous, cardiovascular, endocrine, gastrointestinal, musculoskeletal, renal, reproductive, hepatic, immune, and respiratory systems, raising interesting questions about their functions, mechanisms, and potential involvement in HH signaling throughout those tissues (Chen et al., 1997; Kawasaki et al., 1999; Mauti et al., 2006; Perala et al., 2012; Perala et al., 2005; Yazdani and Terman, 2006).

1.5.2 Hedgehog Regulation of Semaphorin Signaling: Areas of Potential Interest

Together, several lines of evidence point to cross-regulation between SEMA and HH signaling components during embryonic development. One of the most well-studied areas of SHH-dependent embryonic patterning is the vertebrate neural tube, which is the developmental precursor to the spinal cord (Dessaud et al., 2008). SEMA ligands and receptors, including NRPs and PLXNs, are highly expressed in the neural tube during critical periods of HH-dependent development, where they play important roles in regulating axon guidance (Mauti et al., 2006). NRPs are particularly essential for the development of cranial nerves (Chen et al., 2000; Giger et al., 2000; Kitsukawa et al., 1997) and guidance of commissural axons, which must cross the midline of the embryo (Zou et al., 2000). Interestingly, commissural axons are sensitive to SHH signals emanating from the floorplate of the neural tube, and the HH receptor BOC plays important roles regulating commissural axon crossing along the midline (Charron et al., 2003; Peng et al., 2018). Furthermore, the C-terminal portion of HH ligand is targeted to axons and

growth cones in *D. melanogaster*, which could suggest additional roles in axon guidance and growth cone collapse within the cytoplasm (Chu et al., 2006). Recent evidence also suggests that HH signaling is positively regulated by small intracellular GTPase Rap1, providing a potential link between downstream HH and SEMA components (Marada et al., 2016). Together, these studies provide clues that HH signaling components may intersect with SEMA-regulated processes to coordinate cell behaviors, though significant additional research is necessary in this area.

1.5.3 Semaphorin Regulation of Hedgehog Signaling: Growing Evidence and Remaining Questions

The first direct connection between SEMA pathway components and HH signaling was discovered by Hillman et. al. in 2011, who provided several lines of evidence that NRPs positively regulate HH signaling in vitro and in vivo (Hillman et al., 2011). RNA interference against *Nrp1*, *Nrp2*, or a combination of *Nrp1* and *Nrp2* significantly reduces SHH-dependent reporter activity in NIH-3T3 fibroblasts, as well as GLI1 and PTCH1 mRNA and protein (Hillman et al., 2011). Strikingly, this reduction was equivalent to RNAi against *Smo*, a key positive regulator of HH signaling at the cell surface. Importantly, expression of *Nrp1* cDNA rescues HH target gene expression following *Nrp1* RNAi, and overexpression of *Nrp1* cDNA promotes HH signaling when the pathway is activated with SHH, SMO agonist (SAG), or co-expression of a constitutively active form of SMO, *SmoM2* (Hillman et al., 2011). Intriguingly, NRP1 (but not NRP2) mRNA and protein increased as a result of SHH stimulation, suggesting a potential positive feedback loop (Hillman et al., 2011). *In vivo*, morpholino knockdown of

NRP1a in zebrafish embryos disrupted HH-dependent patterning, phenocopying other HH loss-of-function mutations (Hillman et al., 2011).

Several additional studies further support a role for NRP receptors in HH regulation. In 2015, the same group found that deleting NRP1 and NRP2 in the developing mouse cerebellum reduced HH target gene expression and impaired HH-dependent granule neuron precursor (GNP) proliferation (Ge et al., 2015). Another study suggested that RNAi knockdown of NRP2 reduced HH target gene expression in medulloblastoma cell lines and reduced HH-driven tumorigenicity (Hayden Gephart et al., 2013). These studies support earlier findings, which suggest that overexpression NRP1 throughout embryos causes pre-axial digit duplication, similar to HH gain-of-function phenotype in the limb bud (Hui and Joyner, 1993; Kitsukawa et al., 1995). Together, these studies strongly support a role for NRPs as positive regulators of HH signal transduction.

Despite previous work establishing a role for NRPs in the regulation of HH signaling (Ge et al., 2015; Hayden Gephart et al., 2013; Hillman et al., 2011), their mechanism of action in HH signal transduction remains elusive. Unlike most cell-surface HH regulators, NRPs are thought to act downstream of HH ligands through the NRP cytoplasmic domain (Ge et al., 2015). Published data are unclear, however, as to where within the HH intracellular cascade NRPs might exert their effects. An early report suggested that NRPs modulate HH signaling between SMO and SUFU, since RNAi knockdown of *Nrp1* and *Nrp2* decreases *Gli1* expression in *Ptch1*$^{-/-}$ but not *Sufu*$^{-/-}$ mouse embryonic fibroblasts (MEFs), and since NRP1 overexpression fails to increase HH signaling in *Sufu*$^{-/-}$ MEFs (Hillman et al., 2011). More recent data, however, indicate that NRPs function downstream of SUFU, instead acting to inhibit GLI phosphorylation by PKA, which negatively regulates GLI activity (Ge et al., 2015). Contrasting evidence also exists regarding SEMA ligand involvement in HH pathway regulation. One study finds that treatment

with SEMA3A or SEMA3F has no effect on the HH response in NIH-3T3 cells (Hillman et al., 2011), while another finds exactly the opposite, claiming that SEMA3A and SEMA3F binding to NRP1 enhances HH signal transduction in NIH-3T3 cells (Ge et al., 2015). Therefore, the precise mechanism or mechanisms of NRP-mediated HH regulation remain unclear. Importantly, a role for PLXN receptors in HH signaling remains completely unexplored.

1.6 Conclusions

The widespread expression of SEMA ligands and their NRP and PLXN receptors, combined with the published data on NRP regulation of HH signaling, raise two important questions, which I sought to answer when beginning this dissertation: 1) What is the mechanism by which NRPs promote HH signaling? 2) Do PLXN co-receptors play a role in HH signal transduction? Chapter 2 will focus on the mechanisms of NRP-mediated promotion of HH signaling. Specifically, I will provide additional evidence that NRP promotes HH signaling through its cytoplasmic domain, characterizing a novel amino acid motif that mediates its function. Chapter 2 will also discuss NRP localization to the primary cilium, although we find this to be unrelated to its role in HH regulation. Instead, I will present evidence suggesting that NRP1 promotes HH signaling at the level of GLI regulation, independent of canonical PKA phosphorylation. Chapter 3 highlights a novel role for PLXN regulation of HH signal transduction. My data suggest that multiple PLXNs promote HH signaling through GAP activity within their cytoplasmic domains. Finally, in Chapter 4, I will discuss my overall interpretations of the data and describe future studies related to this work. Together, the data presented here provide strong evidence that NRPs and PLXNs modulate HH signaling, and provide preliminary clues as to their mechanisms of action. I propose that SEMA receptors, NRPs and PLXNs, are

novel HH pathway components that regulate HH signal transduction in both development and disease. These analyses will provide fundamental insight into the integration of HH and SEMA signaling inputs in a variety of cellular contexts.

1.7 Figures

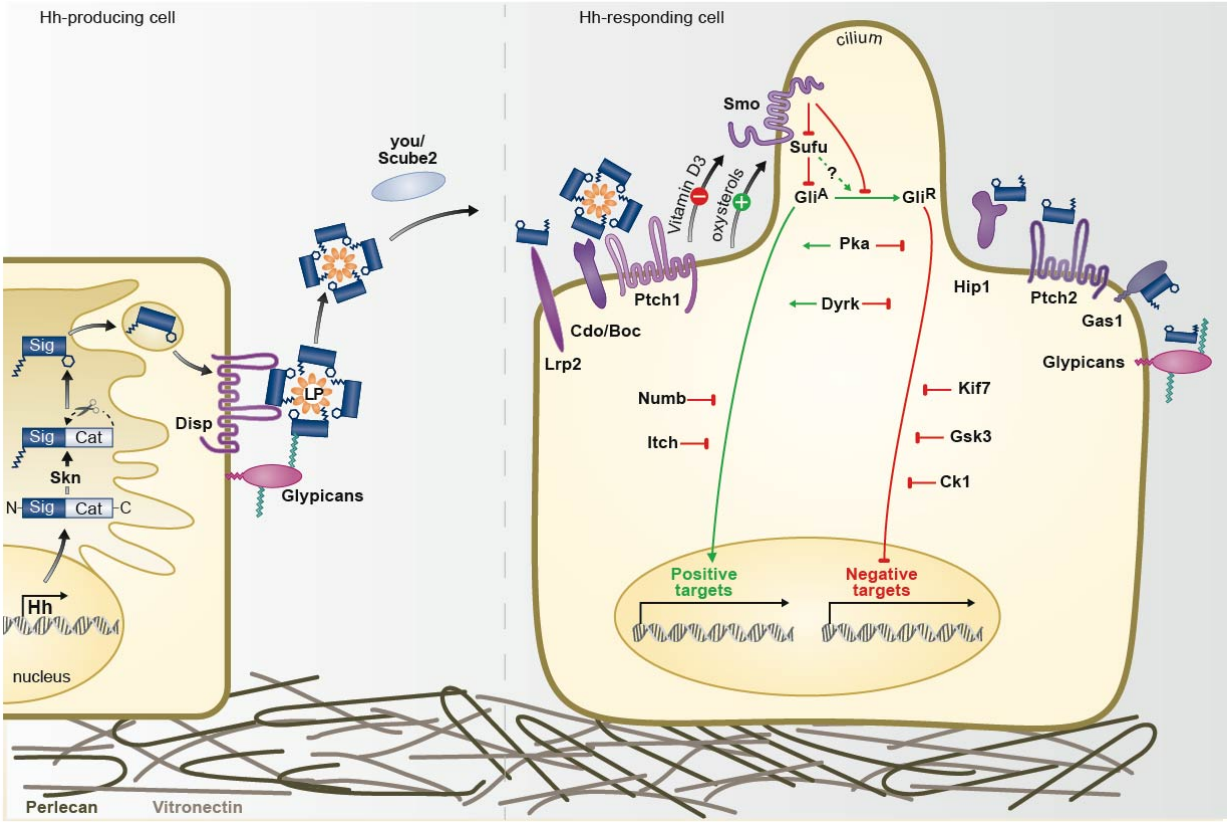

Figure 1-0-1. Overview of vertebrate Hedgehog pathway regulation.
(*Left*) In ligand-producing cells, Hedgehog (HH) proteins are synthesized as 45-kDa precursors that undergo N-terminal palmitoylation by Skinny Hedgehog (SKN) as well as cholesterol modification and autocatalytic cleavage mediated by the HH C-terminus. Lipoprotein (LP)-associated oligomers are then secreted and released from the cell surface in a process mediated by Dispatched (DISP) and Glypicans. Proteins from the SCUBE family shield the cholesterol moieties of HH ligand complexes traveling long distances. (*Right*) On the responding cell, a number of cell surface proteins bind to HH ligands to regulate pathway activity, including LRP2, CDON/BOC, PTCH1, HHIP1, PTCH2, GAS1, and Glypicans (see text for details). PTCH1 binding to HH ligand results in the activation of Smoothened (SMO), which regulates an intracellular signaling cascade that regulates the balance between GLI transcriptional activators and repressors. A number of protein kinases and other molecules regulate GLI proteins within the cell, including PKA, DYRK, NUMB, ITCH, KIF7, GSK3β, and CK1. The primary cilium (not to scale) is also important for HH pathway regulation, and several HH pathway members move into or out of this specialized compartment during signaling. Together, these and other molecules coordinate HH pathway activity. Figure adapted from (Wang et al., 2007).

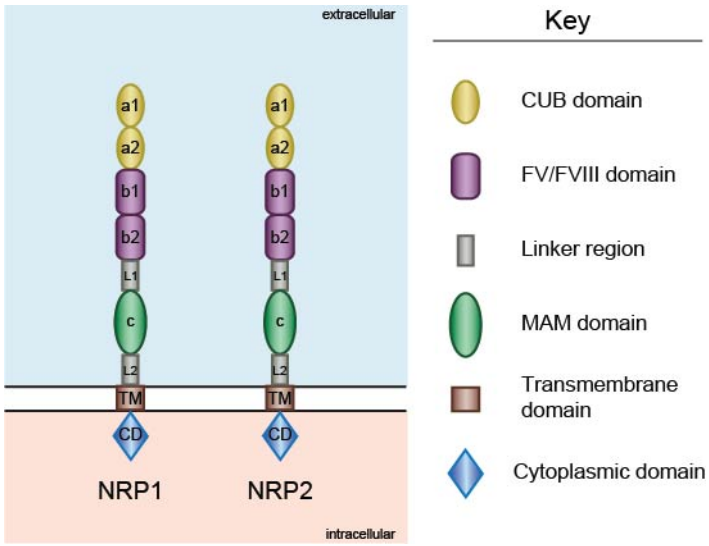

Figure 1-0-2. Neuropilins share structural homology within their extracellular domains. The Neuropilin (NRP) family of receptors includes two isoforms, NRP1 and NRP2, which share approximately 44% overall homology and conserved extracellular domains. The a1 and a2 NRP domains are complement binding (CUB) domains, which bind to secreted, class 3 Semaphorin ligands. Two coagulation factor V/VIII homology-like (FV/FVIII) domains, labeled b1 and b2, mediate interactions with VEGF ligands. The membrane-proximal region of NRP1 and NRP2 encodes a meprin A5 (MAM) domain (c), which may play a role in NRP dimerization and binding to other receptors. A single-pass transmembrane (TM) domain anchors NRPs within the plasma membrane, also mediating dimerization through a GxxxG motif. NRPs also contain a short, approximately 40 amino-acid cytoplasmic domain (CD), which is dispensable for Semaphorin signaling, but binds to PDZ-domain containing proteins through a C-terminal SEA motif.

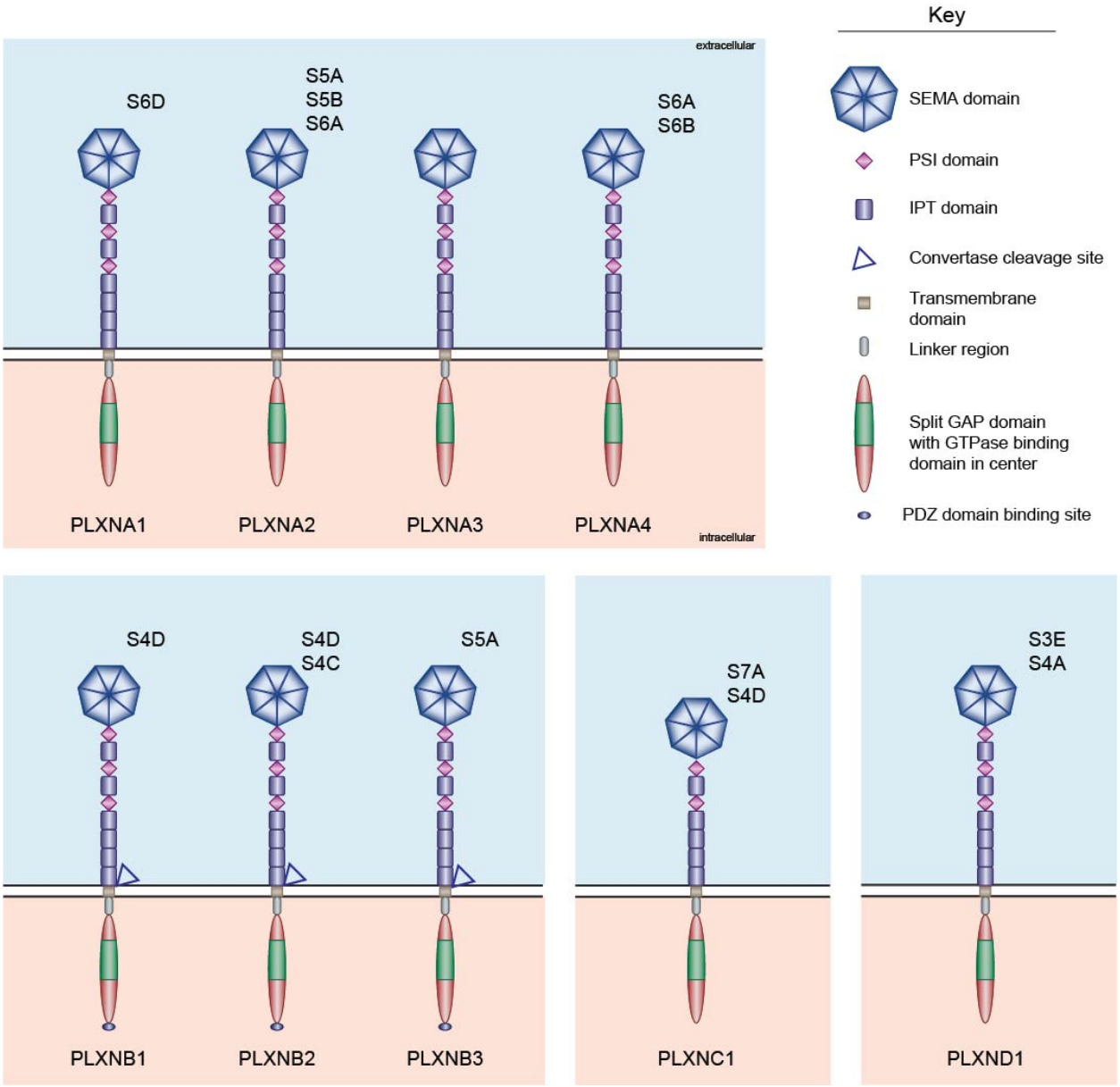

Figure 1-0-3. Plexins are organized into four subfamilies based on structural homology. The Plexin (PLXN) family of Semaphorin receptors contains nine members, organized into four subfamilies as shown. All PLXN family members contain an extracellular SEMA domain, which mediates interactions with specific Semaphorin ligands as indicated (S6D=SEMA6D, etc.), as well as an intracellular split GTPase activating (GAP) domain, which regulates a variety of signaling events. Type-B PLXNs are unique in that they contain a convertase cleavage site as well as a C-terminal PDZ-domain binding site. Type-A PLXNs as well as PLXND1 also bind to Neuropilin (NRP) receptors to mediate signaling from secreted type 3 Semaphorin ligands.

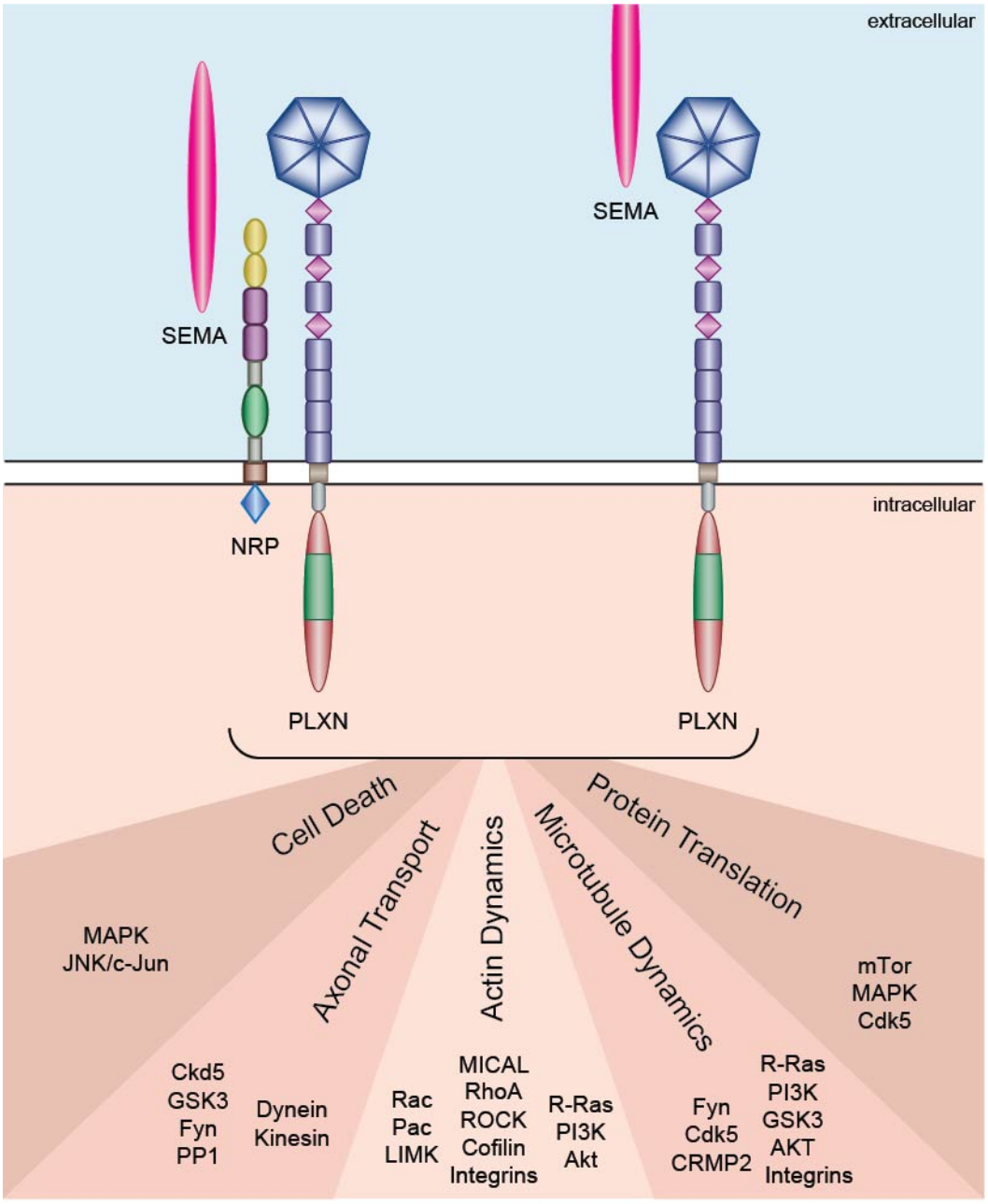

Figure 1-0-4. Semaphorin signaling regulates a wide variety of cellular processes. Semaphorin (SEMA) ligands interact with Neuropilin (NRP) – Plexin (PLXN) complexes, or bind directly to PLXNs to initiate signaling. While the NRP cytoplasmic domain is dispensable for SEMA signaling, the activated PLXN cytoplasmic domain is phosphorylated at several sites and exhibits GTPase activating (GAP) activity to regulate many intracellular processes, including cell death, axonal transport, actin dynamics, microtubule dynamics, and protein translation. The proteins listed are examples of downstream regulators within each category, and by no means represent a comprehensive list (see text for details).

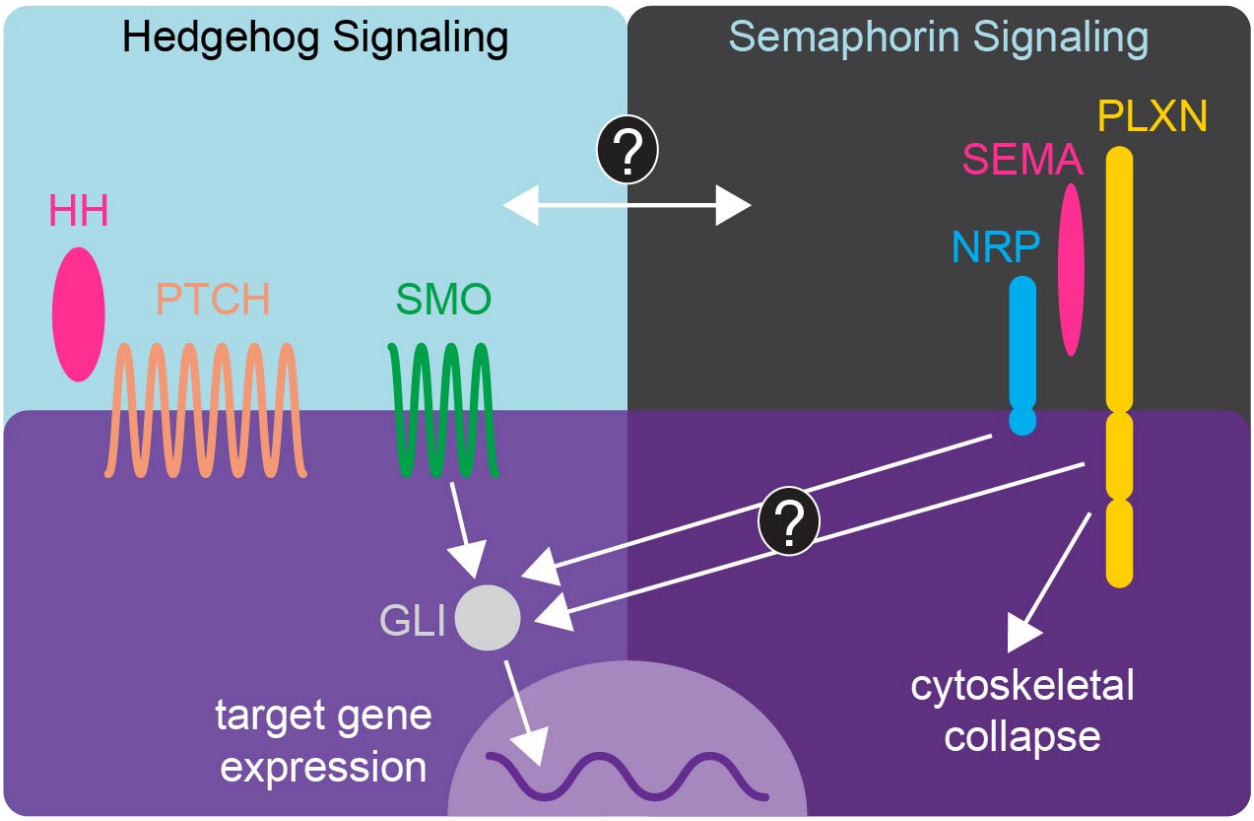

Figure 1-0-5. Hedgehog and Semaphorin signaling regulate one another through unknown mechanisms.
Shown are simplified representations of proteins from the Hedgehog (HH) signaling cascade (*Left*) and the Semaphorin (SEMA) signaling cascade (*Right*), including Patched (PTCH1), Smoothened (SMO), Gli (GLI), Neuropilin (NRP) and Plexin (PLXN). Members of these two pathways share overlapping areas of expression within the developing embryo as well as adult and diseased tissues. Published evidence suggests that HH signaling can influence cytoskeletal collapse mediated by SEMA signaling, and also that SEMA receptors influence HH target gene expression. However, the mechanisms that underlie these cross-regulatory processes remain unclear. The focus of this dissertation is to understand how SEMA receptors function within the HH signal cascade.

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Chapter 2:

Neuropilin-1 Promotes Hedgehog Signaling Through a Novel Cytoplasmic Motif

2.1 Abstract

Hedgehog (HH) signaling critically regulates embryonic and postnatal development as well as adult tissue homeostasis, and its perturbation can lead to developmental disorders, birth defects, and cancers. Neuropilins (NRPs), which have well-defined roles in Semaphorin and VEGF signaling, positively regulate HH pathway function, although their mechanism of action in HH signaling remains unclear. Here, using luciferase-based reporter assays, we provide evidence that NRP1 regulates HH signaling specifically at the level of GLI transcriptional activator function. Moreover, we show that NRP1 localization to the primary cilium, a key platform for HH signal transduction, does not correlate with HH signal promotion. Rather, a structure–function analysis suggests that the NRP1 cytoplasmic and transmembrane domains are necessary and sufficient to regulate HH pathway activity. Furthermore, we identify a previously uncharacterized, 12-amino acid region within the NRP1 cytoplasmic domain that mediates HH signal promotion. Overall, our results provide mechanistic insight into NRP1 function within and potentially beyond the HH signaling pathway. These insights have implications for the development of novel modulators of HH-driven developmental disorders and diseases.

2.2 Introduction

Hedgehog (HH) signaling is essential for tissue patterning and organ formation during embryonic and postnatal development as well as tissue homeostasis, renewal, and repair in adult animals (Briscoe and Therond, 2013; McMahon et al., 2003; Petrova and Joyner, 2014). Deregulation of the HH pathway causes a wide range of developmental abnormalities (Murdoch and Copp, 2010; Schachter and Krauss, 2008), as well as a growing number of pediatric and adult cancers (Barakat et al., 2010; Teglund and Toftgard, 2010). However, despite the widespread importance of HH signaling, our understanding of the mechanisms that regulate HH signal transduction remains incomplete.

HH signaling is tightly regulated by a number of inputs that together control the function of glioma-associated oncogene homolog (GLI) proteins, the transcriptional effectors of the mammalian HH pathway (Hui and Angers, 2011). In the absence of HH ligands, the cell surface protein Patched 1 (PTCH1) inhibits the activity of Smoothened (SMO), a putative G protein–coupled receptor that mediates intracellular HH signal transduction (Byrne et al., 2016; Luchetti et al., 2016; Ogden et al., 2008; Riobo et al., 2006). In this "off" state, GLI2 and GLI3 are phosphorylated by PKA, GSK3β, and CK1 (Hui and Angers, 2011). As a consequence of this phosphorylation, GLI2 is largely degraded, whereas GLI3 is processed into a transcriptional repressor (Pan et al., 2006; Wang et al., 2000). HH ligand binding to PTCH1 results in de-repression of SMO, which initiates a signal transduction cascade that culminates in GLI processing into transcriptional activators that modulate target gene expression in a context-specific manner (Hui and Angers, 2011; Peterson et al., 2012; Vokes et al., 2007; Vokes et al., 2008).

Multiple cohorts of cell surface proteins regulate HH pathway activity by binding to HH ligand. Dispatched1 (DISP1) and Signal sequence, CUB domain, EGF-related 2 (SCUBE2) tightly control ligand secretion (Creanga et al., 2012; D'Angelo et al., 2015; Tukachinsky et al., 2012), whereas trafficking and turnover are regulated by low-density lipoprotein receptor-related 2 (LRP2) and glypicans (GPCs) (Li et al., 2011; Ortmann et al., 2015; Willnow et al., 2012). The cell surface components growth arrest-specific 1 (GAS1), CAM-related/downregulated by oncogenes (CDON), and brother of CDON (BOC), function as essential co-receptors at the level of signal reception (Allen et al., 2011; Izzi et al., 2011). Additionally, ligand interactions with Patched 1 (PTCH1), Patched 2 (PTCH2), and Hedgehog Interacting Protein 1 (HHIP1) result in pathway antagonism (Holtz et al., 2015; Holtz et al., 2013; Jeong and McMahon, 2005). Together, these and other cell surface proteins regulate HH signaling in a multitude of tissues throughout embryonic and postnatal development.

The Neuropilins (NRPs), a small family comprised of NRP1 and NRP2, have well-established roles in axon guidance and vascular patterning (Fujisawa, 2004; Gelfand et al., 2014; Giger et al., 2000; Gu et al., 2003; Kawasaki et al., 1999; Takashima et al., 2002) and act to positively regulate HH signaling at the cell surface (Ge et al., 2015; Hillman et al., 2011). NRPs are expressed in a variety of HH-responsive tissues during critical periods of HH-related developmental patterning (Gomez et al., 2005; Mauti et al., 2006). Importantly, loss-of-function experiments demonstrated that *Nrp1a* knockdown in zebrafish disrupts HH-dependent somite development (Hillman et al., 2011), whereas genetic deletion of *Nrp1* and *Nrp2* in the mouse suppresses HH-driven cerebellar granular neuron progenitor proliferation (Ge et al., 2015). Additionally, NRPs exacerbate HH-related cancers, suggesting that they impact both HH-dependent development and HH-driven disease (Ge et al., 2015; Goel et al., 2013; Hayden

Gephart et al., 2013; Pan et al., 2007; Snuderl et al., 2013). Notably, NRPs are thought to act downstream of HH ligands (Ge et al., 2015), distinguishing their mode of action from most other cell surface regulators of the HH pathway. Previous reports suggest that NRP modulation of HH signaling occurs at the level of suppressor of fused (SUFU) (Hillman et al., 2011). More recent data, however, suggest that NRPs act downstream of SUFU, regulating GLI phosphorylation by interacting with phosphodiesterase 4D (PDE4D), which inhibits PKA (Ge et al., 2015). However, the precise mechanism of NRP function in HH signal transduction remains unclear.

Here we provide data defining a novel mechanism of NRP action in HH signaling. Specifically, we find that NRPs promote HH signaling selectively at the level of GLI activation, independent of PKA phosphorylation. We also demonstrate that NRP1, but not NRP2, traffics to the primary cilium, a highly regulated subcellular compartment required for vertebrate HH signal transduction. Strikingly, NRP1 ciliary localization does not correlate with the promotion of HH pathway activity. Instead, we find that membrane-anchored NRP1 cytoplasmic domain (CD) is both necessary and sufficient to promote HH pathway activation. Further, we map the region in the NRP1 CD that is critical for HH signal promotion to a 12-amino acid motif not previously implicated in NRP function. Overall, these data characterize NRPs as a novel class of cell surface HH pathway regulators that act downstream of ligand binding through cytoplasmic effectors to control HH pathway function.

2.3 Results

2.3.1 Neuropilin-1 and Neuropilin-2 Promote Hedgehog Signaling by Modulating GLI Activator Function

A previous study showed that NRP1 overexpression increases ligand-stimulated HH pathway activity in HH-responsive fibroblasts (Hillman et al., 2011). To confirm and extend these findings, we first tested whether NRP1 and NRP2 promote HH signaling using a luciferase-based reporter assay system in NIH-3T3 fibroblasts (Nybakken et al., 2005). Although the addition of HH ligand is sufficient to induce a transcriptional response, we found that NRP1 and NRP2 both significantly increase ligand-activated HH pathway activity, as detected by GLI-dependent luciferase output (Figure 2-1A and Figure 2-9), consistent with a previous report (Hillman et al., 2011). Notably, co-expression of *Nrp1* and *Nrp2* does not significantly change the level of NRP-mediated HH pathway promotion (Figure 2-1A). Western blot analysis confirmed that HA-tagged NRP1 and NRP2 are expressed at similar levels in NIH-3T3 cells (Figure 2-1B). Although NRP1 significantly promoted HH signaling in 90% of assays (n = 8, average-fold change 2.04), NRP2 significantly promoted HH signaling in only 40% of assays (n = 8, average-fold change 1.36, Figure 2-9). Because of this variability, we decided to focus on NRP1 for further analysis.

To determine whether HH ligand is required for NRP1-mediated HH signal promotion, we activated HH signaling by adding exogenous smoothened agonist (SAG), co-transfecting a constitutively active form of Smoothened (*SmoM2*), or co-transfecting a constitutively active form of GLI (*GLI2*Δ*N*; Figure 2-1, C –F) (Chen et al., 2002; Roessler et al., 2005; Xie et al., 1998). Strikingly, NRP1 significantly increases the HH-dependent luciferase output, regardless of the means of pathway activation (Figure 2-1, D – F). In contrast, NRP1does not alter GLI3-

mediated repression of Hedgehog signaling (Figure 2-1, G and H). Together, these data support a model in which NRP1 acts to selectively regulate GLI activator function downstream of HH ligand.

2.3.2 The Membrane-Anchored Neuropilin-1 Cytoplasmic Domain is Necessary and Sufficient to Promote Hedgehog Signaling

To determine the domain requirements for NRP-mediated promotion of HH signaling, we generated a *Nrp1* construct lacking the cytoplasmic domain, *Nrp1*$^{\Delta CD}$ (Figure 2-2A). Strikingly, NRP1$^{\Delta CD}$ does not promote HH signaling (Figure 2-2B). Western blot analyses in NIH-3T3 cells confirmed equal expression of NRP1 and NRP1$^{\Delta CD}$ (Figure 2-2C). These data are consistent with recent results suggesting that NRP1 utilizes its cytoplasmic domain to promote HH pathway activity (Ge et al., 2015). However, in contrast to previous work, a version of NRP1 that lacks all functional extracellular domains (NRP1$^{\Delta ECD}$) is sufficient to promote HH signaling (Figure 2-2, A and B). Western blot analysis confirmed NRP1$^{\Delta ECD}$ expression (Figure 2-2C). Immunofluorescent analysis of NRP1, NRP1$^{\Delta CD}$, and NRP1$^{\Delta ECD}$ under permeabilizing and non-permeabilizing conditions using dual extracellular and intracellular antibody staining confirmed the cell surface localization of NRP1 and NRP1$^{\Delta CD}$ (Figure 2-2D and Figure 2-10). To further explore the requirement for the NRP1 CD in HH signaling, we generated a cytosolic version of the CD that is not membrane-tethered (NRP1^{CD}). Notably, transfection of *Nrp1*^{CD} is not sufficient to promote HH signaling in NIH-3T3 cells (Figure 2-11), suggesting a role for the NRP1 transmembrane (TM) domain in HH signal promotion. Together, these data suggest that the membrane-anchored NRP1 CD is both necessary and sufficient to promote HH signaling.

2.3.3 *Neuropilin-1 Transmembrane Dimerization is not Required for Hedgehog Signal Promotion*

Neuropilin TM dimerization is mediated by a double G*XXX*G motif in the TM domain that stabilizes signaling complexes for both Semaphorin and VEGF ligands (Roth et al., 2008). Mutating the three glycine residues within the double G*XXX*G motif to valines completely disrupts dimerization and blocks NRP1 function in Semaphorin signaling (Roth et al., 2008). To determine whether NRP1 TM dimerization is required for HH signal promotion, we recreated these three glycine mutations in both NRP1 (Figure 2-3A) and NRP1$^{\Delta ECD}$ (Figure 2-3B). Strikingly, these mutations do not impair the ability of either construct to promote HH signaling (Figure 2-3), suggesting that NRP1 membrane attachment, but not TM dimerization, is required for its function in HH signaling.

2.3.4 *Neuorpilin1 Promotes Hedgehog Signaling Independently of GLI2 Phosphorylation by Protein Kinase A*

Previous work has suggested that NRPs regulate HH signaling by recruiting PDE4D to the cell membrane (Ge et al., 2015). PDE4D negatively regulates PKA activity by locally reducing levels of cAMP (Beavo and Brunton, 2002). PKA phosphorylates GLI transcription factors at a number of consensus and non-consensus sites to regulate their activity, including six consensus sites within the activation domain of GLI2 that are sufficient to repress GLI2 activity (Niewiadomski et al., 2014; Pan et al., 2006; Wang et al., 2000). To test whether the NRP1 CD modulates HH activity through PKA-dependent GLI phosphorylation, we generated serine-to-alanine mutations at the six consensus PKA phosphorylation sites critical for GLI2 repression (Niewiadomski et al., 2014; Pan et al., 2006; Wang et al., 2000) (GLI2^{P1-6}, Figure 2-4A). As

expected, *GLI2*$^{P1–6}$ expression results in a significant increase in HH signaling compared with WT *GLI2* (Figure 2-4B). Although GLI2 stimulates HH signaling less effectively than its constitutively active counterpart GLI2ΔN, we still observed a reliable increase in activity with *Nrp1* co-expression Figure 2-4B). This increase was not observed when we co-expressed *Nrp1*$^{\Delta CD}$, consistent with previous results (Figure 2-4B, *cf.* Figure 2-2B). Surprisingly, *Nrp1* still promotes HH signaling when co-ex pressed with *GLI2*$^{P1–6}$, suggesting that NRP1 regulates GLI activity independently of PKA phosphorylation. Importantly, *Nrp1*$^{\Delta CD}$ does not promote signaling when co-expressed with *GLI2*$^{P1–6}$, indicating that this PKA-independent promotion of HH signaling still requires the NRP1 CD (Figure 2-4B).

2.3.5 Identification of a Novel Neuropilin-1 Cytoplasmic Motif that Mediates Hedgehog Signal Promotion

To elucidate which region of the NRP1 CD promotes HH pathway activation, we initially targeted a highly conserved, C-terminal SEA motif described previously to bind PDZ-containing proteins, as this is the only region of the NRP1 CD with any previously ascribed function (Cai and Reed, 1999). Notably, adding a C-terminal HA tag to NRP1 itself could block PDZ binding at the SEA motif. However, NRP1 was able to promote HH signaling equally well, regardless of whether we placed the tag at its C terminus or N terminus (Figure 2-2; data not shown). Furthermore, deleting the NRP1 SEA (NRP1$^{\Delta 920–922}$, Figure 2-5A) did not impair NRP1-mediated promotion of HH signaling in NIH-3T3 cells (Figure 2-5B). To narrow the region of the NRP1 CD that mediates HH signaling, we deleted the N-terminal 20 amino acids of the NRP1 CD (NRP1$^{\Delta 883–902}$, Figure 2-5A) and assessed function in NIH-3T3 HH signaling assays. Strikingly, NRP1$^{\Delta 883–902}$ failed to promote HH signaling (Figure 2-5C), suggesting that the

residues required for NRP regulation of HH activity are located in the membrane-proximal half of the NRP1 CD. Western blot analyses confirmed expression of NRP1$^{\Delta 883–902}$ (Figure 2-5F), and immunofluorescent staining under non-permeabilizing conditions confirmed that NRP1$^{\Delta 883–902}$ properly localizes to the cell surface (Figure 2-5G). We then asked whether restoring part of this region would rescue NRP1 function in HH signaling; however, NRP1$^{\Delta 890–922}$ still failed to promote signaling (Figure 2-5D). Ultimately, adding back 12 additional residues from amino acid 890–902 (NRP1$^{\Delta 902–922}$) rescued NRP1-mediated promotion of HH signaling equivalently to full-length NRP1 (Figure 2-5E), confirming the importance of this region to NRP1 function in HH signaling. Although this 12-amino acid region has no previously described function, we noted the presence of two serine residues and a tyrosine residue in this motif. To investigate whether phosphorylation at these sites might regulate NRP function, we mutated these residues to alanine. Remarkably, alanine mutagenesis of these residues does not alter NRP1 promotion of HH signaling (Figure 2-12). Together, these data suggest that a conserved, 12-amino acid region of the NRP1 CD between amino acids 890 and 902 plays an essential role in HH signal promotion through selective regulation of GLI activator function.

2.3.6 Neuoropilin-1, but not Neuropilin-2, Localizes to Primary Cilia in Hedgehog-Responsive Fibroblasts

The primary cilium is a highly regulated subcellular compartment into which molecules over 40 kDa cannot freely diffuse (Kee et al., 2012), and an intact cilium is important for HH signaling to proceed normally (Goetz and Anderson, 2010). Given that NRP1 regulates HH signaling through the modulation of GLI activity, and that GLI proteins localize to cilia and

require intact cilia for their processing and function (Wong et al., 2009), we asked whether NRPs localize to the primary cilium.

To assess primary cilia localization, we expressed *Nrp1* and *Nrp2* in WT and Dynein-mutant (*Dync2h1*$^{lln/lln}$) mouse embryonic fibroblasts (MEFs) (Figure 2-6). Dynein motors mediate retrograde transport of ciliary components; thus, cilia-localized proteins accumulate within the primary cilium of Dynein-mutant MEFs, allowing for more robust detection (Ocbina et al., 2011). We found that NRP1, but not NRP2, localizes to primary cilia (identified with anti-acetylated tubulin, AcTub) in WT and Dynein-mutant MEFs (Figure 2-6). NRP1 was detected in 51% of cilia in WT MEFs and further enriched in dynein-mutant MEFs, with 68% of cilia positive for NRP1 (Figure 2-6, A, E, I, and J). NRP2, on the other hand, was only detected in primary cilia in 9% of Dynein-mutant MEFs, with no ciliary localization observed in WT MEFs (Figure 2-6, B, F, I, and J). As a positive control, SMOM2 robustly localizes to the primary cilium in both WT and Dynein-mutant MEFs (98% of cilia in each group; Figure 2-6, C, G, I, and J), consistent with previous findings (Corbit et al., 2005). In contrast, BOC, a cell surface–localized HH co-receptor (Kang et al., 2002; Tenzen et al., 2006), was detected broadly throughout the cell surface but was not observed in primary cilia (Figure 2-6, D and H–J). Importantly, no HA staining was observed in the cilia of vector-transfected cells (Figure 2-13). To further confirm these data, we stained WT and Dynein-mutant MEFs for endogenous NRP1 and detected NRP1 localization to primary cilia (Figure 2-6K). These results suggest that NRP1, but not NRP2, localizes to the primary cilium of HH-responsive fibroblasts.

2.3.7 Neuropilin-1 Localization to Primary Cilia does not Correlate with Hedgehog Signal Promotion

Although both NRP1 and NRP2 promote HH signaling, our data indicate that NRP1 functions more consistently than NRP2 in our cell signaling assays (Figure 2-9). Given that NRP1 preferentially localizes to primary cilia, we assessed the requirement for NRP1 cilia localization in HH signal promotion, taking advantage of two of the deletion constructs described previously, *Nrp1*$^{\Delta ECD}$ and *Nrp1*$^{\Delta 902–922}$. Notably, both of these constructs robustly promoted HH signaling (Figure 2-2B, Figure 2-5E, and Figure 2-7E). We performed immunofluorescent staining to examine the ciliary localization of NRP1, NRP1$^{\Delta ECD}$, or NRP$^{\Delta 1902–922}$ in NIH-3T3 cells (Figure 2-6). Although NRP1 localizes to primary cilia in roughly 40% of transfected cells (Figure 2-7, A and D), both NRP1$^{\Delta ECD}$ and NRP1$^{\Delta 902–922}$ displayed significantly reduced localization to primary cilia (Figure 2-7, B–D). Taken together, these results suggest that cilia localization does not correlate with NRP1-mediated promotion of HH signal transduction.

2.4 Discussion

Cell surface regulation of the HH signaling pathway is essential for proper tissue patterning during embryonic and postnatal development as well as adult tissue homeostasis, repair, and regeneration (Allen et al., 2011; Han et al., 2008; Hooper and Scott, 2005; Hsu et al., 2014; Izzi et al., 2011; Murdoch and Copp, 2010). Conversely, deregulation of HH cell surface components contributes to HH-driven birth defects and cancers (Lee et al., 2010; Mathew et al., 2014; Milenkovic et al., 1999; Mille et al., 2014; Mo et al., 1997; Zhang et al., 2006). NRPs are also implicated in numerous human cancers (Prud'homme and Glinka, 2012; Snuderl et al., 2013). Notably, *Nrp2* knockdown increases survival in a HH-dependent mouse model of

medulloblastoma (Hayden Gephart et al., 2013). Here we present evidence that NRPs promote HH signaling intracellularly by regulating GLI activator function. Further, we report that NRP1 localizes to the primary cilium; however, this localization does not correlate with NRP1-mediated promotion of HH signaling. Instead, we determine that the NRP1 CD and TM domains are necessary and sufficient to promote HH signal transduction. Finally, we identify a novel region of the NRP1 CD as essential for this process, a region not previously implicated in NRP1 function. Taken together, these findings identify the membrane-tethered NRP1 CD as a key positive regulator of HH signal transduction via selective regulation of GLI activator function.

2.4.1 Neuropilins as a Novel Class of Ligand-Independent Hedgehog Cell Surface Regulators

Numerous cell surface proteins promote HH pathway activity through interactions with HH ligands (Allen et al., 2011; Christ et al., 2012; Holtz et al., 2015; Holtz et al., 2013; Izzi et al., 2011; Jeong and McMahon, 2005; Kwong et al., 2014; Li et al., 2011; Milenkovic et al., 1999). Our data suggest that, unlike these proteins, cell surface–localized NRPs act downstream of ligand to regulate HH signaling. Indeed, NRP1 promotes HH pathway activity even when signaling is stimulated by GLI2ΔN, a constitutive transcriptional activator, strongly suggesting that NRPs function at the level of GLI regulation. More specifically, our data suggest that NRPs regulate GLI activator function selectively, failing to impair GLI3 repressor activity.

Although the precise mechanism of NRP-mediated regulation of GLI function remains unclear, our data are consistent with NRPs acting downstream of SUFU at the level of GLI function, since GLI2ΔN is not regulated by SUFU (Han et al., 2015). Importantly, we find that *Nrp1* still promotes HH pathway activity when co-transfected with $GLI2^{P1–6}$, a version of GLI2 that cannot be phosphorylated by PKA at six critical repressive sites. Therefore, in contrast to

previous work (Ge et al., 2015), our data suggest that NRP1 promotion of HH signaling is independent of PKA-mediated phosphorylation of GLI2. It is possible that NRP binding to PDE4D could impact PKA-dependent phosphorylation at non-consensus sites (Niewiadomski et al., 2014) or affect GSK3β activity, which is also regulated by cAMP (Khaled et al., 2002); further experiments are required to investigate these possibilities. Also worth considering is that NRP knockdown does not change the amount of GLI in the primary cilium (Hillman et al., 2011), suggesting that NRPs may regulate GLI proteins after they have been processed in the cilium, perhaps by regulating GSK3β, affecting degradation of GLI activators, or impacting endocytosis. Overall, our findings suggest that the NRP1 CD regulates GLI proteins intracellularly, independently of HH ligand binding and independently of PKA-mediated GLI phosphorylation.

Although NRPs promote signaling downstream of HH ligand, it remains unclear whether Semaphorin ligands can contribute to HH signal promotion. Class 3 Semaphorin ligands interact with the extracellular domains of NRP1 and NRP2 (Gu et al., 2002; Neufeld and Kessler, 2008). Although two previous studies present contrasting results regarding Semaphorin ligand involvement in HH signal regulation (Ge et al., 2015; Hillman et al., 2011), our data suggest that Semaphorin ligand binding is not required because the NRP1 extracellular domain is dispensable for HH signal promotion. We cannot exclude the possibility that Semaphorins or other NRP-binding ligands might still modulate HH activity. In addition to Semaphorins and VEGFs, NRPs interact with a wide variety of other proteins, including PIGF-2, heparan sulfate, TFG-β1, HGF, PDGF, FGF, L1-CAM, Plexins, and integrins (Prud'homme and Glinka, 2012). It is possible that NRP interactions with these or other binding partners also contribute to the promotion of HH signaling.

2.4.2 Identification of a Cytoplasmic Motif in Neuropilin-1 that Mediates Hedgehog Signal Transduction

Our data indicate that the membrane-attached NRP1 CD is necessary and sufficient to promote HH signaling. Notably, this contrasts with the HH co-receptors CDON and BOC, whose cytoplasmic domains are dispensable for HH signal promotion (Song et al., 2015; Tenzen et al., 2006). NRP1 and NRP2 share several areas of conservation, including a carboxyl-terminal SEA motif that binds the PDZ-containing protein GIPC1 (Cai and Reed, 1999; De Vries et al., 1998; Gao et al., 2000; Prahst et al., 2008). Strikingly, our data indicate that this motif is not required for HH signal promotion. Instead, we present evidence that a previously uncharacterized region of the NRP1 cytoplasmic domain between amino acids 890 and 902 is required for HH signal promotion (Figure 2-8). Notably, this motif is highly conserved across vertebrate species, including chicken, frog, zebrafish, mouse, rat, and human. This region is also only partially conserved between NRP1 and NRP2, suggesting potential differences in the way the two proteins function in HH signaling. Further analyses will be needed to narrow this region to the exact amino acids necessary for HH regulation and determine the degree of overlap between NRP1 and NRP2 function. Others have reported that genetic deletion of the NRP1 cytoplasmic domain in mice results in defective spatial separation between arteries and veins (Fantin et al., 2011), although no HH-dependent phenotypes have been reported. It is likely that redundancy with NRP2 functionally compensates for NRP1 loss in the promotion of HH signaling, as has been reported in both zebrafish and mice (Ge et al., 2015; Hillman et al., 2011).

It remains unclear exactly how this 12-amino acid cytoplasmic region mediates NRP1 function in HH signaling. We have mutated several conserved serine and tyrosine residues

located in this region, ruling out the possibility that phosphorylation of these residues affects downstream signaling. One possibility is that this region interacts directly or indirectly with PDE4D, which regulates PKA and could modify GLI proteins through non-consensus phosphorylation sites or through other kinases, as discussed previously. Alternatively, a recent publication identifies a suite of additional intracellular molecules that interact with the NRP cytoplasmic domain, including MYH9, MYH10, DYHC1, FLNA, EF1α1, and ENO1 (Seerapu et al., 2013). These molecules may also interact with amino acids 890–902 to mediate GLI regulation, although significant future studies will be required to analyze their potential roles in HH signal transduction. It is also possible that this motif could regulate the conformation or subcellular localization of NRP1 or perhaps play a role in regulating endocytosis of other proteins (see below).

Another aspect to consider in NRP1 function is its ability to homodimerize and heterodimerize with NRP2 (Roth et al., 2008; Sawma et al., 2014). Our data suggest that mutation of the dimerization motif in the NRP1 TM domain does not impact its ability to promote HH pathway function, in contrast to an important role for NRP TM dimerization in Semaphorin signaling (Roth et al., 2008). Similar to the involvement of Semaphorin ligands, the possibility remains that NRP TM dimerization is not required but may somehow modulate HH signaling. It is also possible that NRP interactions with other TM proteins, such as VEGF receptors, Plexins, FGF receptors, or PDGF receptors, may contribute to HH signal promotion (Muhl et al., 2017; Prud'homme and Glinka, 2012), although many of these receptors interact with NRP1 through its extracellular domain, which, as our data indicate, is dispensable for NRP1 function in HH signaling.

2.4.3 Neuropilin-1 Ciliary Localization does not Correlate with Increased Hedgehog Signal Promotion

Our data suggest that, although NRP1 can localize to primary cilia, mutant constructs with reduced cilia localization still promote HH signal transduction. Although actual entry into the highly regulated ciliary compartment does not correlate with NRP-mediated promotion of HH signaling, we cannot exclude the possibility that the low levels of cilia localization we observe may be sufficient to impact HH signal transduction. Alternatively, NRPs may play an important role elsewhere in the cell, perhaps even at the ciliary base. Accordingly, NRP1, NRP2, $NRP1^{\Delta ECD}$, and $NRP1^{\Delta 902–922}$ were all detected broadly throughout the cell membrane, including at the base of the cilium. Although cytosolic splice variants of NRP1 do exist (Cackowski et al., 2004; Gagnon et al., 2000; Rossignol et al., 2000), expression of a cytosolic version of the NRP1 cytoplasmic domain fails to promote HH signaling, suggesting that NRP1 must reach the cell surface to impact HH signaling. NRPs are commonly internalized through endocytosis (Lanahan et al., 2013; Pang et al., 2014); thus, it is possible that endocytic vesicle-associated NRPs affect PKA or GLI function.

Together, these data raise the question of why NRP1 localizes to the primary cilium. One possibility is that NRP1 (compared with NRP2) preferentially binds to another protein that mediates its localization to primary cilia. Alternatively, some parallels have been drawn between cilia and dendritic spines, including the presence of a regulated diffusion barrier (Nechipurenko et al., 2013). It is possible that NRP localization to primary cilia is a byproduct of its role in dendritic spines or other similarly regulated structures. Perhaps the same sequences or mechanisms that allow NRP entry into dendrites and axons also allow their entry into the primary cilium, with or without functional consequence. NRP1 plays well-defined roles in many

different signaling pathways (Prud'homme and Glinka, 2012), any of which might rely on cilium localization of NRP1 for proper function. Interestingly, NRP1 has several roles that differ from NRP2, including axon guidance in response to specific class 3 Semaphorins and angiogenesis (Hatanaka et al., 2009). Furthermore, NRP1 and NRP2 are expressed in overlapping and distinct cell types during development, some of which rely on primary cilia for proper function. For example, NRP1 is expressed predominantly in arterial endothelial cells, which are thought to rely on mechanosensory cilia for homeostasis (Nauli et al., 2011), whereas NRP2 is expressed predominantly in venous and lymphatic endothelial cells (Prud'homme and Glinka, 2012). These or potentially other undiscovered functions may result from differential subcellular localization of NRP1 and NRP2 relative to the cilium.

2.5 Materials and Methods

Neuropilin and GLI constructs

Nrp and *GLI* 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, chicken β actin promoter, and an internal ribosome entry site with a nuclear enhanced GFP reporter (3XNLSEGFP) (Megason and McMahon, 2002). C-terminal or N-terminal HA tags (YPYDVPDYA) were added to the constructs as indicated. Subsequent deletion and mutation variants were generated using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies 200521). To mutagenize the dimerization motif within the NRP1 TM domain, primer sequences were as follows:
gtacagcacaactacacatactgcaaccaggagtaccaccagggcactcat (forward) and
atgagtgccctggtggtactcctggttgcagtatgtgtagttgtgctgtac (reverse). To mutagenize the serines and

tyrosine within the membrane-proximal half of the NRP1 CD, primer sequences were as follows: gttctccagggcagctaggttcgcttccgccatcccattgtgcc (forward), atccacaagttcaaagttagcgttctccagggcagctagg (forward), ggcacaatgggatggcggaagcgaacctagctgccctggagaac (reverse), and cctagctgccctggagaacgctaactttgaacttgtggat (reverse). The *GLI2*$^{P1–6}$ construct was created by synthesizing a 1.4-kb portion of human *GLI2* containing serine-to-alanine mutations at residues 808, 824, 836, 867, 941, and 970 using Invitrogen GeneArt gene synthesis (Thermo Fisher Scientific) and cloning into full-length human *GLI2* using endogenous AgeI and NheI sites.

Cell Culture

Cell lines were maintained in DMEM (Life Technologies, 1965) supplemented with 10% bovine calf serum (ATCC, 30-2030) and 1 x penicillin – streptomycin-L-glutamine (Life Technologies, 10378016). Cultures were kept at 37 °C with 5% CO_2 and 95% humidity.

Cell Signaling Assays

Luciferase-based reporter assays to assess HH signaling in NIH-3T3 cells were performed as described previously using a *ptc*Δ*136-GL3* reporter construct (Nybakken et al., 2005). Briefly, cells were seeded at 2.5 X 10^4 cells/well into gelatin-coated 24-well plates. The next day, cells were transfected with empty vector (*pCIG*) or experimental constructs along with a luciferase reporter construct and β-galactosidase transfection control (*pSV-β-galactosidase*, Promega, E1081). Transfections were performed using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum medium (Invitrogen, 31985). 48 h after transfection, the culture medium was replaced with low-serum medium (0.5% bovine calf serum, 1% penicillin—

streptomycin—L-glutamine) containing either control or N-terminal SHH (NSHH)-conditioned medium. Alternatively, SAG (Sigma-Aldrich, SML1314) was added at a concentration of 300 nM to activate HH signaling. Luciferase reporter activity and β-galactosidase activity were measured 48 h later on a Spectramax M5e plate reader (Molecular Devices) using the luciferase assay system (Promega, E1501) and the Betafluor β-galactosidase assay kit (EMD Millipore, 70979), respectively. Luciferase values were divided by β-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 and averaged, with error bars representing the standard deviation between triplicate wells. 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 Analysis

Dynein-mutant (*Dyn2h1*$^{lln/lln}$) and wide-type littermate control MEFs (generously provided by Dr. Kathryn V. Anderson, Memorial Sloan Kettering (Ocbina et al., 2011)), were plated at 1.5 X 10^5 cells/well in a 6-well dish with a coverslip at the bottom of each well. Cells were transfected 24 h after plating using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum medium (Invitrogen, 31985). Approximately 6 h after transfection, cells were placed in low-serum medium (0.5% bovine calf serum, 1% penicillin—streptomycin–L-glutamine) for 48 h. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, washed with PBS, and permeabilized with 0.2% Triton X-100 in PBS for 5 min before antibodies were added. Primary antibodies included mouse IgG1 anti-HA.11 (1:1000, Biolegend, 901502), goat IgG anti-NRP1 (1:100, R&D Systems, AF566), and mouse IgG2b anti-

acetylated tubulin (1:2500, Sigma-Aldrich, T7451). Coverslips were incubated with primary antibodies overnight, followed by a 10-min DAPI stain (1:30,000 at room temperature, Invitrogen, D1306) and 1 h incubation with secondary antibodies, including Alexa Fluor 546 goat anti-mouse IgG1 (Y1), Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 goat anti-mouse IgG2b, and Alexa Fluor 555 goat anti-mouse IgG2b (1:500, Invitrogen, A21123, A21202, A21141, and A21147, respectively). Coverslips were mounted to glass slides using Shandon ImmuMount mounting medium (Fisher, 9990412). Immunofluorescent analysis and imaging were performed on a Leica SP5X upright two-photon confocal microscope using LAS AF software (Leica) and a Leica X63 (type, HC Plan Apochromat CS2; NA1.2) water immersion objective. Cilium counts were performed in a single-blind fashion. Control constructs included *Boc* and *SmoM2*.

Western Blot Analysis

COS-7 or NIH-3T3 cells were transfected using Lipofectamine 2000 (Invitrogen, 11668) and Opti-MEM reduced serum medium (Invitrogen, 31985). Cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA) 48 h after transfection, sonicated using a Fisher Scientific sonic dismembrator, model 500 (four pulses at 20%), and centrifuged at 14,000 X *g* for 25 min at 4 °C to remove the insoluble fraction. Protein concentrations were determined using a BCA protein assay kit (Fisher, PI23225). After boiling for 10 min, 50 μg of protein from each sample was 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 TBS with 0.5% OmniPur Tween 20 (TBST, EMD Millipore, 9480) and blocked in Western blocking buffer (30 g/liter

bovine serum albumin with 0.2% NaN_3 in TBST) for 1 h to overnight. Blots were probed with the following antibodies: mouse IgG1 anti-HA.11 (1:1000, Covance, MMS-101P-200), goat IgG anti-Neuropilin-1 (1:100, R&D Systems, AF566), and mouse IgG1 anti-β-tubulin (1:10,000, generously provided by Dr. Kristen J. Verhey, University of Michigan). Secondary antibodies were diluted 1:10,000 and included peroxidase-conjugated AffiniPure goat anti-mouse IgG, light chain–specific (Jackson ImmunoResearch, 115-035-174), and peroxidase-conjugated AffiniPure donkey anti-goat IgG, light chain–specific (Jackson ImmunoResearch, 705-035-147). Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, WBKLS0500) was added for 10 min before membranes were exposed to HyBlot CL autoradiography film (Denville, E3018) and developed using a Konica Minolta SRX-101A medical film processor.

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2.7 Author contributions

The experiments were conceived and designed by J. M. P., B. L. A., and R. J. G. J. M. P., A. N. M., and N. E. F. performed the experiments, and J. M. P. compiled the data. J. M. P. and B. L. A. analyzed the data and wrote and edited the manuscript. R. J. G. provided reagents, technical assistance, and assistance with manuscript preparation and editing.

Neuropilin-1 promotes Hedgehog signaling through a novel cytoplasmic motif

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2.8 Figures

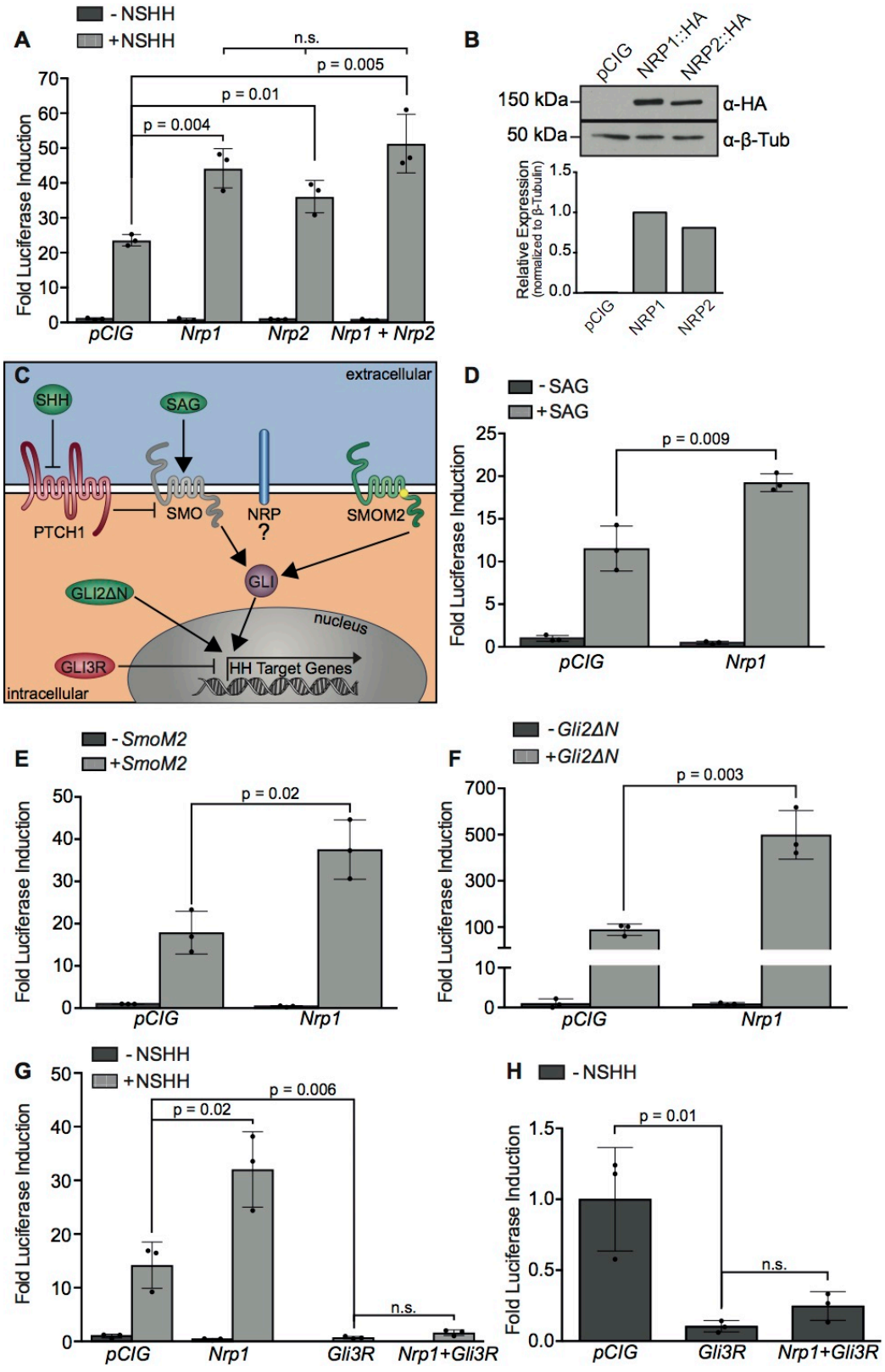

A
- NSHH
+NSHH
n.s.
p = 0.005
p = 0.01
p = 0.004
Fold Luciferase Induction
pCIG
Nrp1
Nrp2
Nrp1 + Nrp2
B
pCIG
NRP1::HA
NRP2::HA
150 kDa
50 kDa
α-HA
α-β-Tub
Relative Expression (normalized to β-Tubulin)
C
extracellular
SHH
SAG
PTCH1
SMO
NRP ?
SMOM2
GLI
GLI2ΔN
nucleus
GLI3R
HH Target Genes
intracellular
D
- SAG
+SAG
p = 0.009
E
- SmoM2
+SmoM2
p = 0.02
F
- Gli2ΔN
+Gli2ΔN
p = 0.003
G
p = 0.006
Gli3R
Nrp1+Gli3R
H
p = 0.01

Figure 2-1. Neuropilin-1 promotes Hedgehog signaling at the level of GLI activation.
A. HH-dependent luciferase reporter activity measured in NIH-3T3 cells transfected with the indicated constructs and stimulated with NSHH-conditioned medium (*NSHH*). Data are reported as mean -fold induction ± S.D., with *p* values calculated using two-tailed Student's *t* test. *n.s.*, not significant. B. *top*, Western blot analysis of NIH-3T3 lysates collected from cells expressing the indicated HA-tagged proteins. *Bottom*, quantitation of NRP levels relative to β-tubulin. *a-β-Tub*, anti-β-tubulin. C. schematic of various modes of HH pathway activation at the level of ligand (*HH*), small-molecule SMO agonist (SAG), oncogenic SMO mutations (*SMOM2*), and constitutive GLI activator (*GLI2*Δ*N*). D–F. luciferase reporter activity similar to A in NIH-3T3 cells stimulated with SAG (D) or co-transfected with *SmoM2* (E) or *GLI2*Δ*N* (F). G and H. luciferase reporter activity similar to A in NIH-3T3 cells transfected with the *Gli3* repressor.

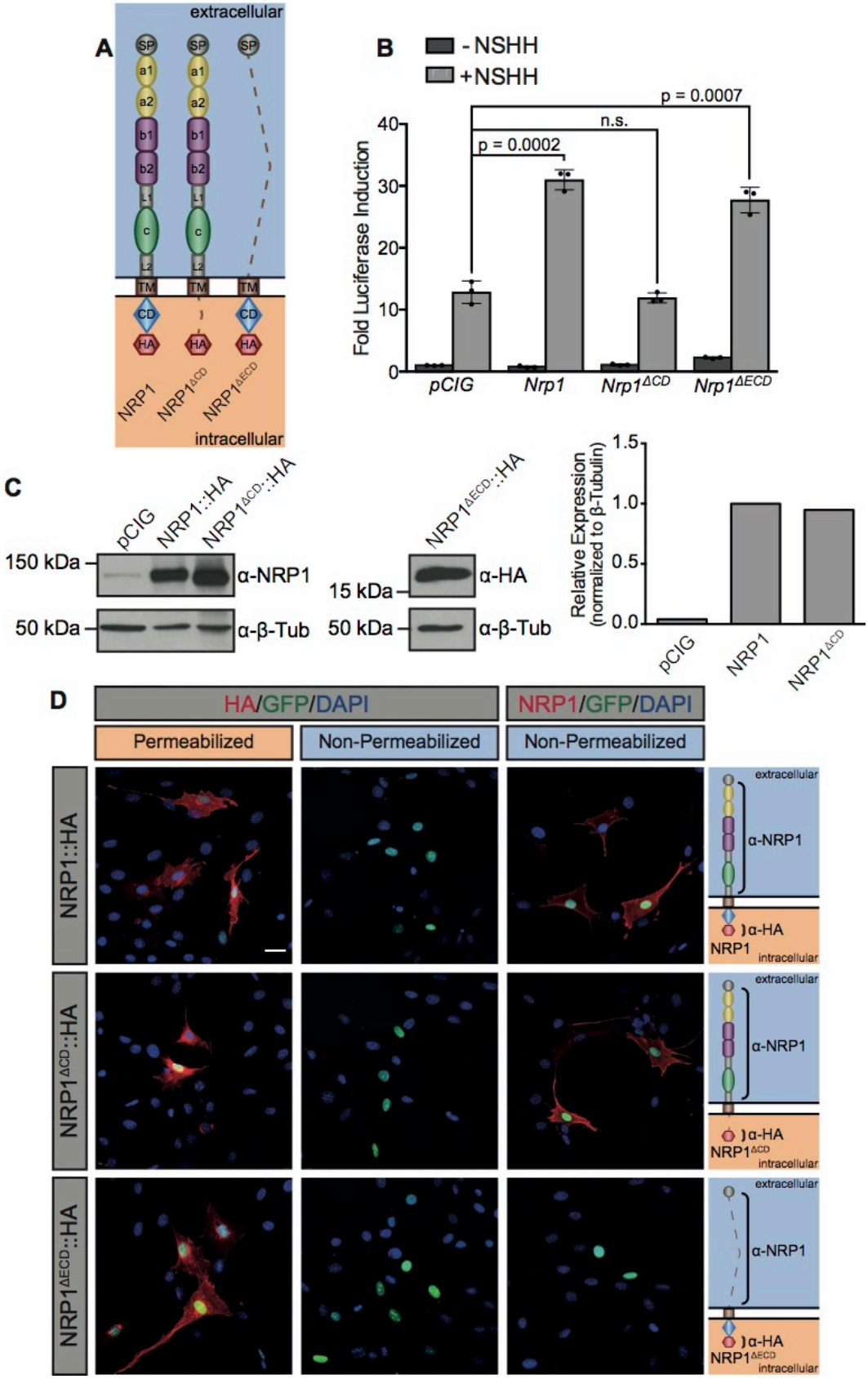

A
extracellular
SP
a1
a2
b1
b2
L1
c
L2
TM
CD
HA
NRP1
NRP1ΔCD
NRP1ΔECD
intracellular
B
- NSHH
+NSHH
p = 0.0007
n.s.
p = 0.0002
Fold Luciferase Induction
40
30
20
10
0
pCIG
Nrp1
Nrp1ΔCD
Nrp1ΔECD
C
pCIG
NRP1::HA
NRP1ΔCD::HA
NRP1ΔECD::HA
150 kDa
α-NRP1
50 kDa
α-β-Tub
15 kDa
α-HA
Relative Expression (normalized to β-Tubulin)
1.5
1.0
0.5
0.0
pCIG
NRP1
NRP1ΔCD
D
HA/GFP/DAPI
NRP1/GFP/DAPI
Permeabilized
Non-Permeabilized
Non-Permeabilized
NRP1::HA
NRP1ΔCD::HA
NRP1ΔECD::HA
extracellular
α-NRP1
α-HA
NRP1
intracellular
NRP1ΔCD
NRP1ΔECD

Figure 2-2. Neuropilin-1 cytoplasmic and transmembrane domains are necessary and sufficient to promote Hedgehog signaling.
A. Schematic of full-length NRP1, NRP1$^{\Delta CD}$, and NRP1$^{\Delta ECD}$. *Dotted lines* indicate regions that were deleted in each construct. B. HH-dependent luciferase reporter activity measured in NIH-3T3 cells transfected with the indicated constructs and stimulated with NSHH-conditioned medium (+*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. C. Western blot analysis of HA-tagged protein expression in NIH-3T3 cells. Anti-β-tubulin (a-*β-Tub*) was used as loading control. *Right*, quantitation of NRP1 and NRP1$^{\Delta CD}$ levels relative to β-tubulin. D. antibody detection of an extracellular NRP1 epitope (α-*NRP1*, *red*) and an intracellular HA tag (α-*HA*, *red*) in permeabilized (*left panels*) and non-permeabilized (*center* and *right panels*) conditions to assess cell surface localization of NRP1, NRP1$^{\Delta CD}$, and NRP1$^{\Delta ECD}$. Nuclear GFP (*green*) indicates transfected cells, whereas DAPI (*blue*) marks all nuclei. Shown are diagrams of each construct, with *brackets* indicating antibody-binding sites. *Scale bar* = 10 μm.

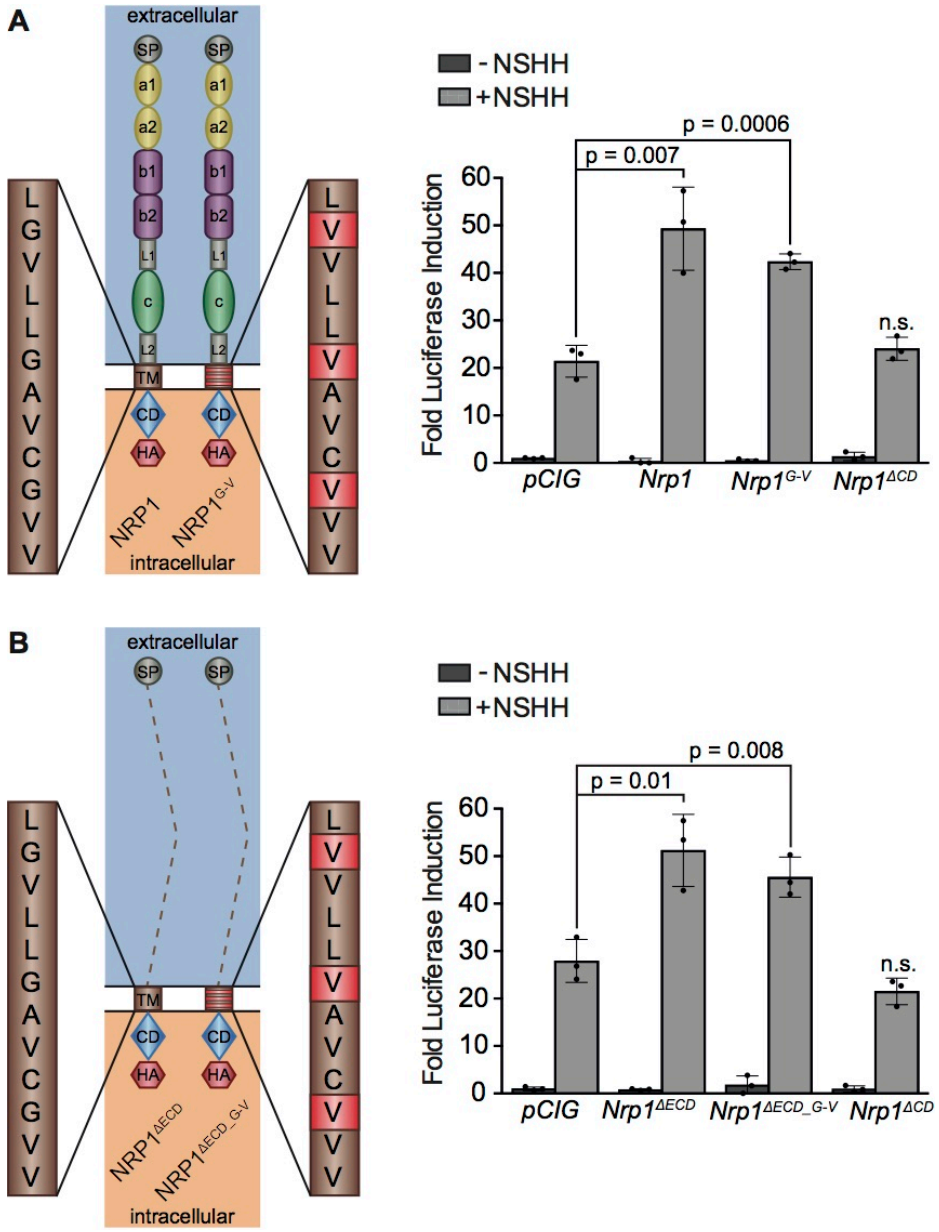


Figure 2-3. Neuropilin-1 transmembrane dimerization is not required to promote Hedgehog signaling.

A. *Left*, schematic of full-length NRP1 and NRP1$^{G\text{-}V}$, in which three glycine residues are mutated to valines. *Right*, HH-dependent luciferase reporter activity measured in NIH-3T3 cells transfected with the indicated constructs and stimulated with NSHH-conditioned medium (+ *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. B. *left*, schematic of NRP1$^{\Delta ECD}$ and NRP1ECD$^{\Delta ECD_G\text{-}V}$. *Right*, HH-dependent luciferase reporter activity measured in NIH-3T3 cells transfected with the indicated constructs and stimulated with NSHH-conditioned medium. Data are reported as mean -fold induction ± S.D., with *p* values calculated using two-tailed Student's *t* tests.

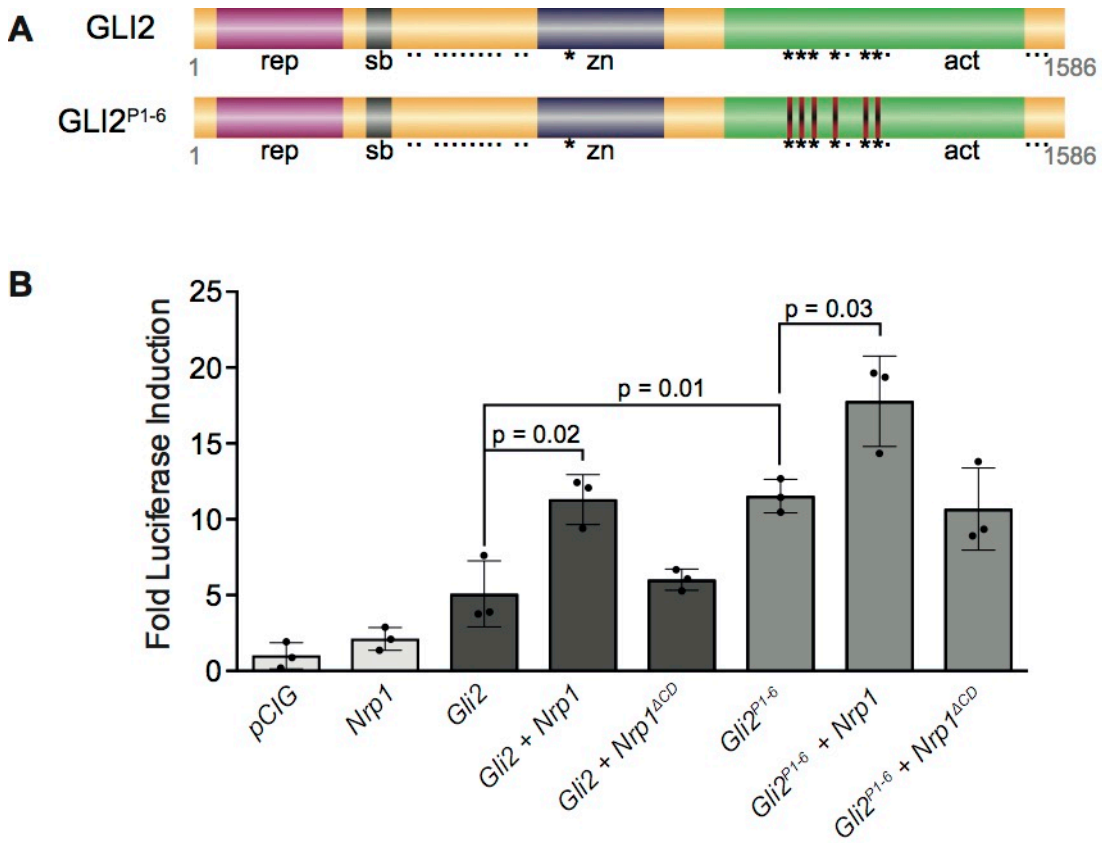


Figure 2-4. GLI2 phosphorylation by Protein Kinase A is not required for Neuropilin-1-mediated Hedgehog promotion.
A. Diagram of GLI2 indicating the repressor domain (*rep*), SUFU binding region (*sb*), zinc finger binding domain (*zn*), and activation domain (*act*). *Asterisks* mark consensus PKA phosphorylation sites, whereas *dots* mark non-consensus sites. *Red bars* indicate locations of serine-to-alanine mutations to disrupt PKA phosphorylation at six key repressive sites. B. luciferase reporter activity in NIH-3T3 cells transfected with *Nrp* and *GLI2* constructs as indicated. Data are reported as mean -fold induction ± S.D., with *p* values calculated using two-tailed Student's *t* tests.

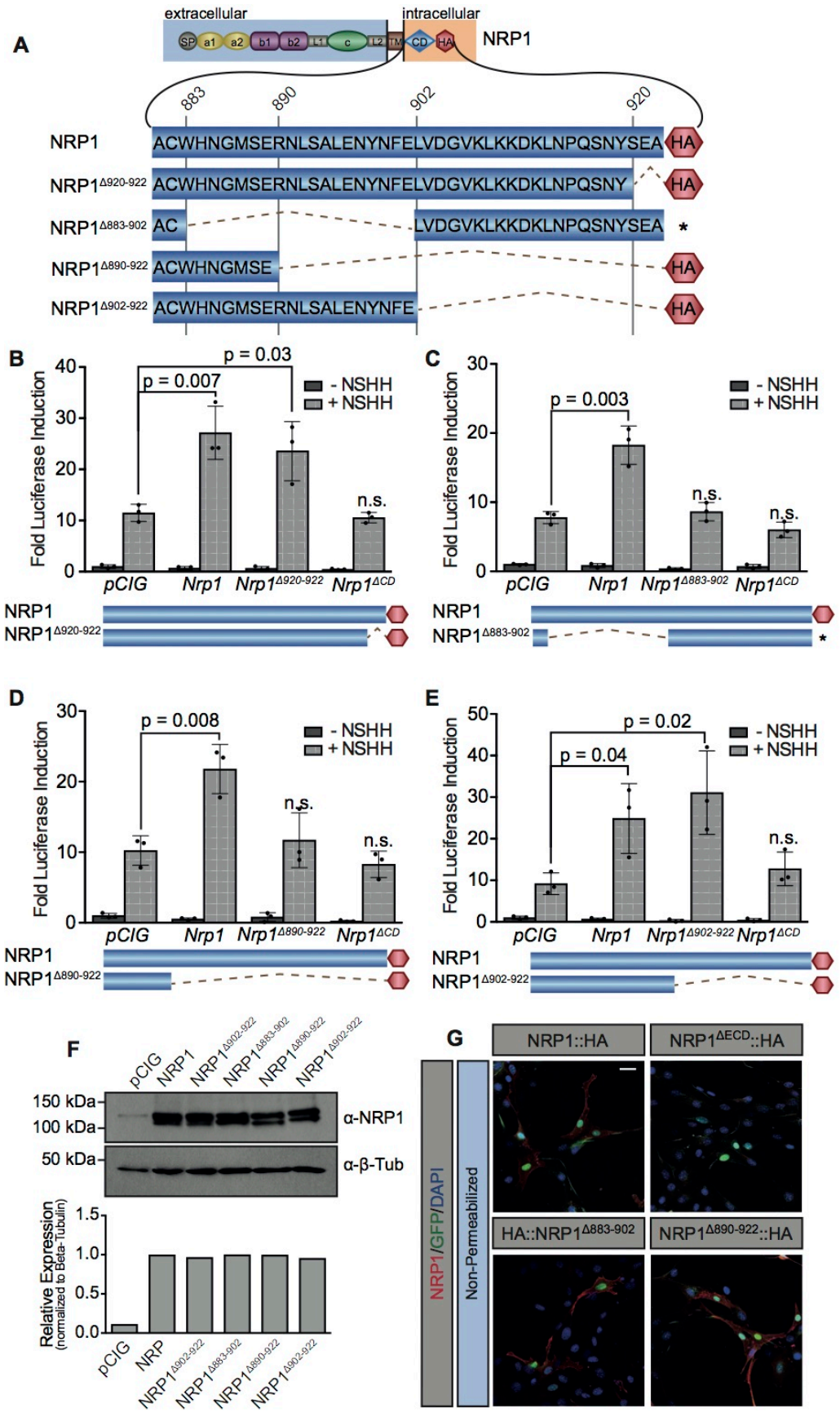

A
extracellular
intracellular
SP
a1
a2
b1
b2
L1
c
L2
TM
CD
HA
NRP1
883
890
902
920
NRP1 ACWHNGMSERNLSALENYNFELVDGVKLKKDKLNPQSNYSEA HA
NRP1Δ920-922 ACWHNGMSERNLSALENYNFELVDGVKLKKDKLNPQSNY HA
NRP1Δ883-902 AC LVDGVKLKKDKLNPQSNYSEA *
NRP1Δ890-922 ACWHNGMSE HA
NRP1Δ902-922 ACWHNGMSERNLSALENYNFE HA
B
Fold Luciferase Induction
p = 0.03
p = 0.007
- NSHH
+ NSHH
n.s.
pCIG
Nrp1
Nrp1Δ920-922
Nrp1ΔCD
NRP1
NRP1Δ920-922
C
Fold Luciferase Induction
p = 0.003
- NSHH
+ NSHH
n.s.
n.s.
pCIG
Nrp1
Nrp1Δ883-902
Nrp1ΔCD
NRP1
NRP1Δ883-902
*
D
Fold Luciferase Induction
p = 0.008
- NSHH
+ NSHH
n.s.
n.s.
pCIG
Nrp1
Nrp1Δ890-922
Nrp1ΔCD
NRP1
NRP1Δ890-922
E
Fold Luciferase Induction
p = 0.02
p = 0.04
- NSHH
+ NSHH
n.s.
pCIG
Nrp1
Nrp1Δ902-922
Nrp1ΔCD
NRP1
NRP1Δ902-922
F
pCIG
NRP1
NRP1Δ902-922
NRP1Δ883-902
NRP1Δ890-922
NRP1Δ902-922
150 kDa
100 kDa
50 kDa
α-NRP1
α-β-Tub
Relative Expression
(normalized to Beta-Tubulin)
pCIG
NRP
NRP1Δ902-922
NRP1Δ883-902
NRP1Δ890-922
NRP1Δ902-922
G
NRP1::HA
NRP1ΔECD::HA
HA::NRP1Δ883-902
NRP1Δ890-922::HA
NRP1/GFP/DAPI
Non-Permeabilized

Figure 2-5. Identification of a 12-amino acid motif in the Neuropilin-1 cytoplasmic domain required for Hedgehog signal promotion.
A. Diagram of the NRP cytoplasmic domain, with amino acid number indicated (*top*) and deletions indicated by *dotted lines*. B–E. luciferase reporter activity in NIH-3T3 cells transfected with NRP constructs as indicated and stimulated with NSHH-conditioned medium (+ *NSHH*). Data are reported as mean -fold induction ± S.D., with *p* values calculated using two-tailed Student's *t* tests. F, *top*, Western blot analysis of HA-tagged protein levels in NIH-3T3 cell lysates with detection of β-tubulin (β-*Tub*) as a loading control. *Bottom*, quantitation of NRP levels relative to β-tubulin. G, antibody detection of an extracellular NRP1 epitope (α-NRP1, *red*) in non-permeabilized NIH-3T3 cells to assess cell surface localization of NRP1, $NRP1^{\Delta ECD}$, $NRP1^{\Delta 883–902}$, and $NRP1^{\Delta 890–922}$. Nuclear GFP (*green*) indicates transfected cells, whereas DAPI (*blue*) marks all nuclei. Note that $NRP1^{\Delta ECD}$ lacks the NRP1 antibody epitope and served as a negative control. *Scale bar* = 10 μm.

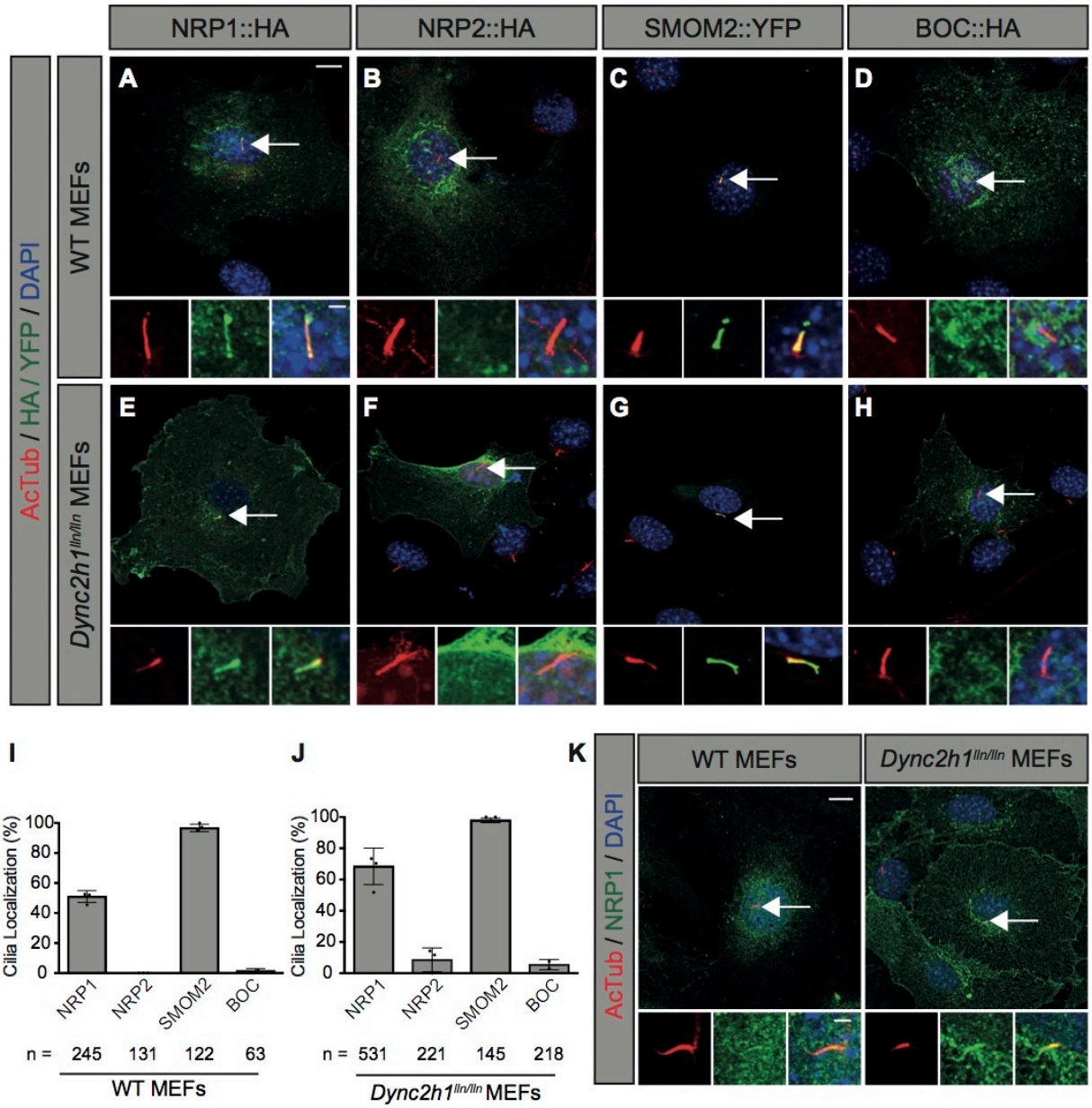


Figure 2-6. Neuropilin-1, but not Neuropilin-2, localizes to primary cilia in Hedgehog-responsive fibroblasts.

A–H. Antibody detection of HA/YFP (*green*) and primary cilia (*red*, AcTub) in WT (*top*) and Dynein-mutant (*Dync2h1*$^{lln/lln}$) MEFs (*bottom*). *Dync2h1*$^{lln/lln}$ MEFs exhibit impaired retrograde transport out of primary cilia. *Arrows* indicate the location of primary cilia. *Insets* show higher-magnification views of primary cilia in individual (*left* and *center*) and merged (*right*) channels. DAPI indicates nuclei (*blue*). I and J. quantitation of data represented in images from WT (*left*) and *Dync2h1*$^{lln/lln}$ (*right*) MEFs, reported as mean ± S.D., with scatterplot values indicating averages of three independent experiments and the total number of cells analyzed listed below each bar. Data are reported from at least three independent experiments. K. Antibody detection of endogenous NRP1 in WT (*left panel*) and *Dync2h1*$^{lln/lln}$ (*right panel*) MEFs. *Scale bar* = 10 μm, *inset scale bar* = 1 μm.

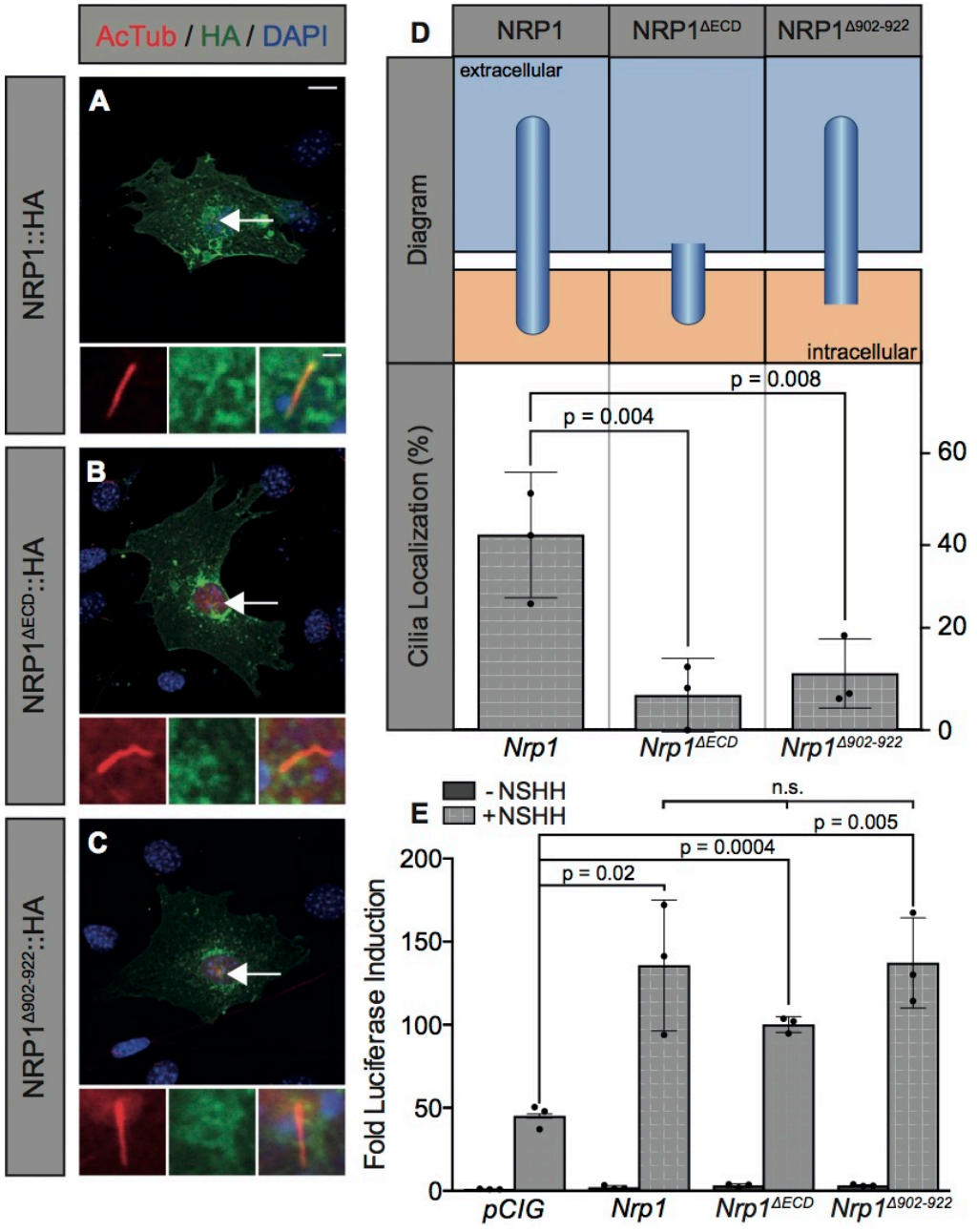


Figure 2-7. Ciliary localization of Neuropilin-1 does not correlate with Hedgehog signal promotion.

A–C. antibody detection of NRP1 (A), NRP1$^{\Delta ECD}$ (B), and NRP1$^{\Delta 902–922}$ (C) in NIH-3T3 cells (HA, *green*; AcTub, *red*; DAPI, *blue*). D. chart summarizing the structure (*top*) and cilium localization (*bottom*) of each construct imaged on the *left*. *n* = 165, 166, and 162 cells for *Nrp1*, *Nrp1*$^{\Delta ECD}$, and *Nrp1*$^{\Delta 902–922}$, respectively. Scatterplot values indicate averages of three independent experiments, with column height indicating the overall average ± S.D. E. luciferase reporter activity in NIH-3T3 cells transfected with constructs as indicated and stimulated with NSHH-conditioned medium (+ *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. *Scale bar* = 10 μm, *inset scale bar* = 1 μm.

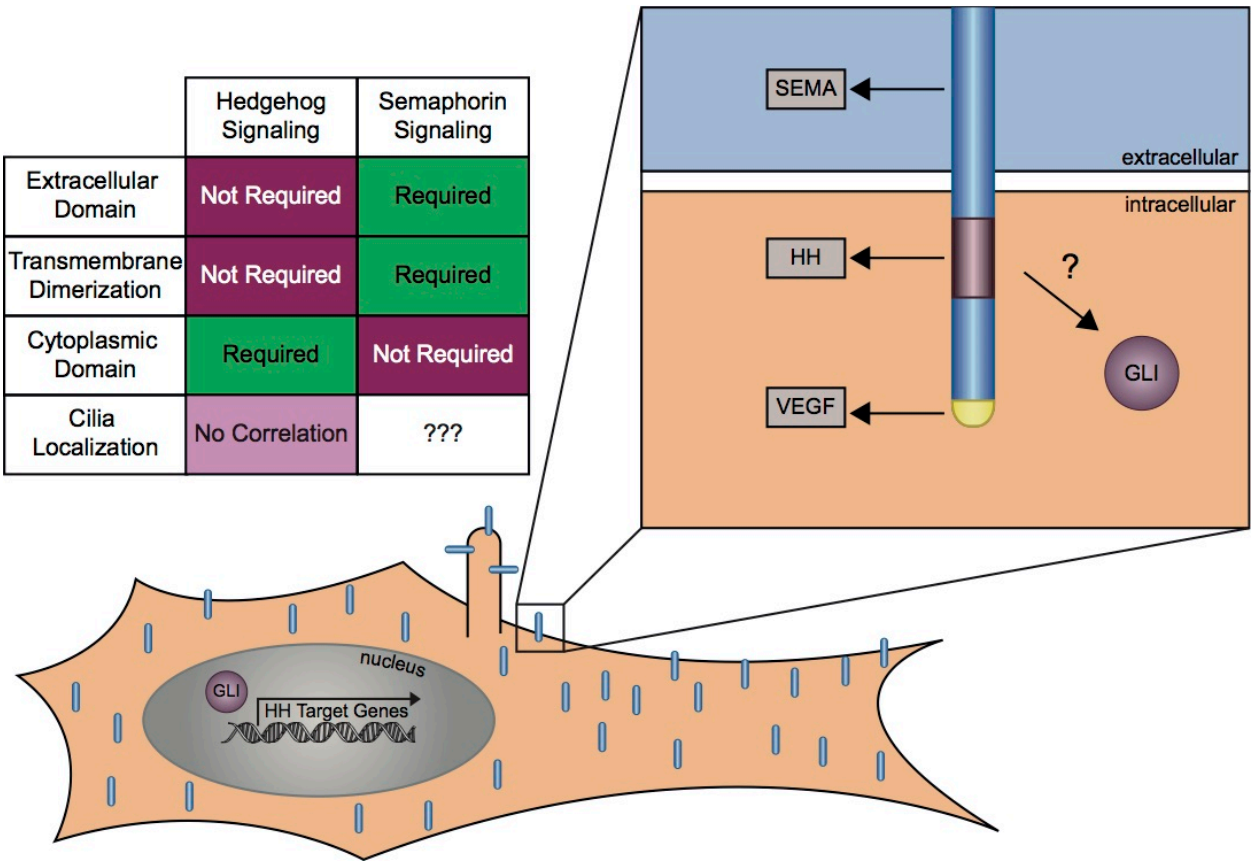
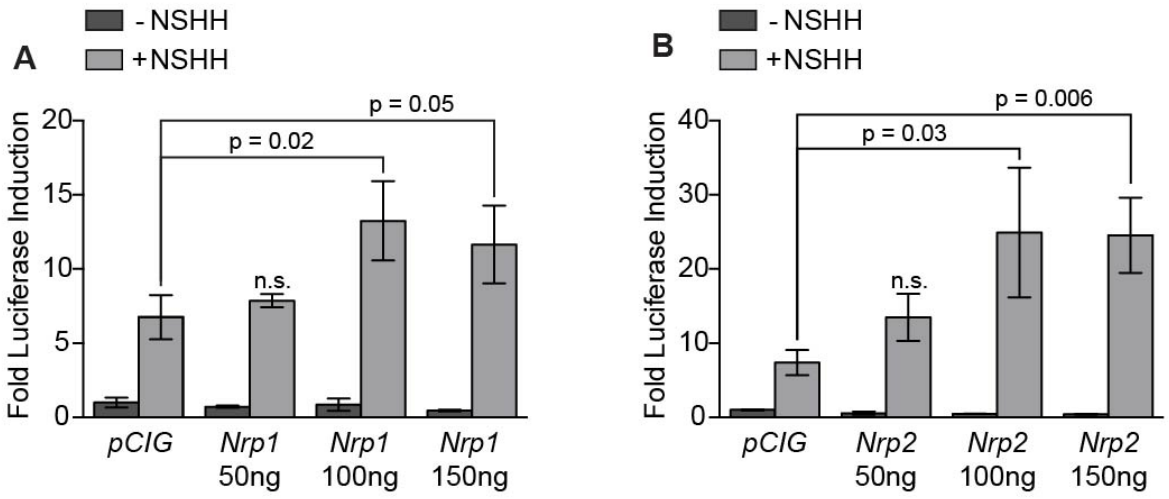


Figure 2-8. Summary and model of Neuropilin function in Hedgehog signal transduction. *Top left panel*, chart summarizing requirements for the NRP1 extracellular domain, dimerization domain, cytoplasmic domain, and cilia localization in HH and Semaphorin signaling. *Bottom panel*, schematic of NRP expression throughout the cell surface, including in primary cilia. *Top right panel*, cell surface–localized NRP1 mediates HH signaling through its cytoplasmic domain, specifically requiring amino acids 890 – 902. This contrasts with the extracellular domain, which is required for Semaphorin signaling, and a conserved SEA motif that is required for VEGF signaling. The NRP1 cytoplasmic domain regulates HH signaling at the level of GLI activity, increasing GLI transcriptional activation through an unknown mechanism.



C

	NRP1		NRP2	
assay	fold change	p-value	fold change	p-value
1	2.63	0.008	1.34	0.095
2	2.74	0.005	1.29	0.284
3	2.40	0.027	1.52	0.055
4	1.55	0.115	1.39	0.025
5	2.11	0.007	1.82	0.027
6	1.40	0.007	0.83	0.197
7	1.87	0.004	1.53	0.012
8	1.66	0.008	1.16	0.103
avg.	2.04	0.022	1.36	0.100

Figure 2-9. Optimization of NIH-3T3 transfection conditions.
NIH-3T3 cells were transfected with 50, 100, and 200ng of *Nrp1* (A) or *Nrp2* (B), respectively. HH-dependent luciferase reporter activity was measured in ligand stimulated (+NSHH) and unstimulated (-NSHH) cells. Data reported as mean fold induction ± SD, with p-values calculated using a two-tailed Student's t test. 100 ng transfection amount was chosen for subsequent assays. (C) Summary of luciferase assay data in which *Nrp1* and *Nrp2* were directly compared in eight independent assays. Fold change reported between ligand-stimulated vector only (*pCIG*) triplicate wells and ligand-stimulated *Nrp1*- or *Nrp2*-transfected triplicate wells. Yellow highlight denotes significance ($p<0.05$).

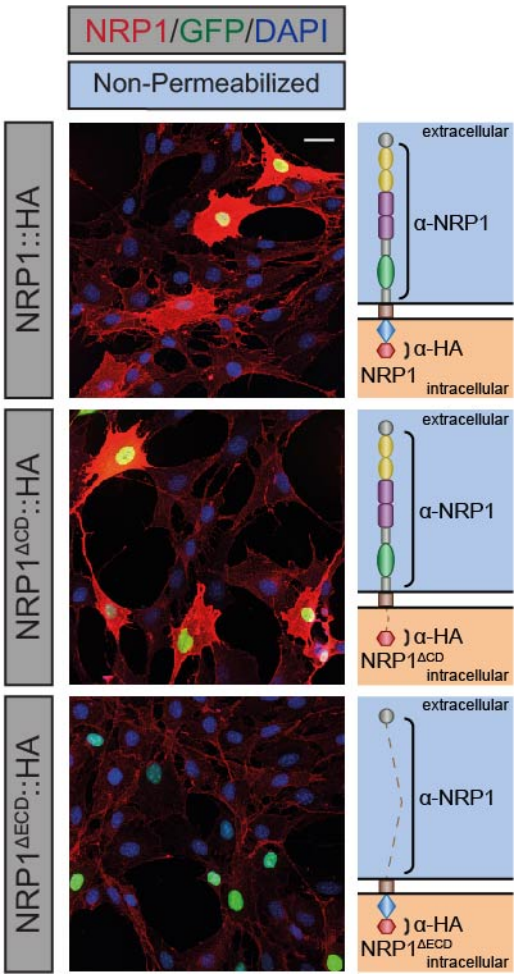


Figure 2-10. Neuropilin-1 antibody detects endogenous as well as overexpressed Neuropilin-1 protein.
Antibody detection of endogenous NRP1 (red) in NIH-3T3 cells transfected with NRP1, $NRP1^{\Delta CD}$, and $NRP1^{\Delta ECD}$ as indicated. Exposure adjusted for endogenous rather than overexpressed protein (cf. Figure 2-2). DAPI staining indicates nuclei (blue). GFP expression identifies transfected cells (green). Diagrams of each construct to right indicate antibody-binding sites. Scale bar = 10μm.

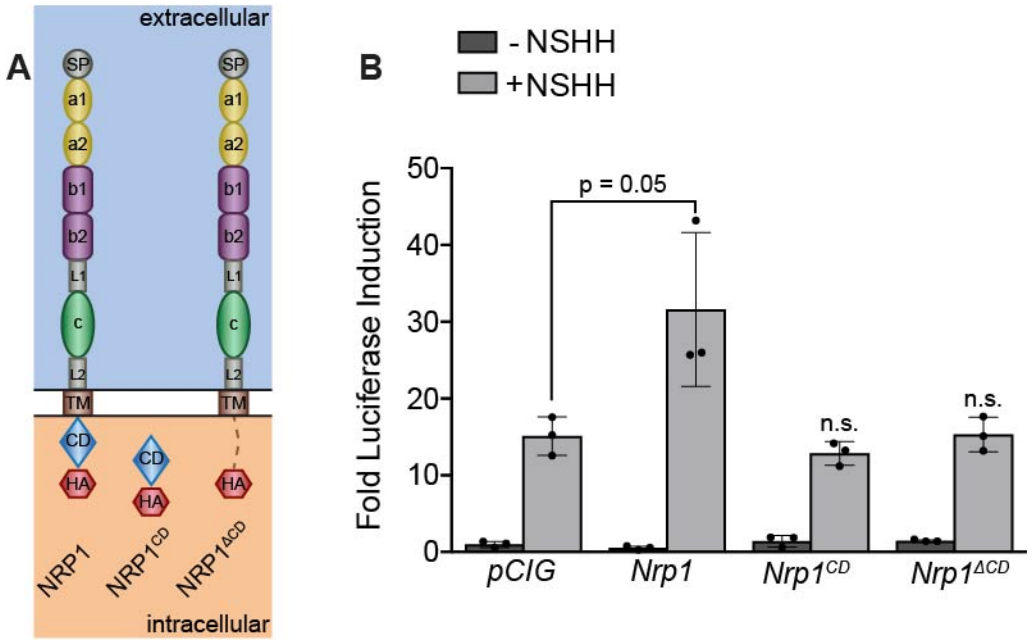


Figure 2-11. Cytosolic Neuropilin-1 cytoplasmic domain does not promote Hedgehog signaling.

A. Diagram of full-length NRP1, a cytosolic version lacking the extracellular and transmembrane domains ($NRP1^{CD}$), and a version lacking the cytoplasmic domain ($NRP1^{\Delta CD}$). B. HH-dependent luciferase reporter activity measured in NIH-3T3 cells. Data reported as mean fold induction ± SD, with p-values calculated using a two-tailed Student's t test.

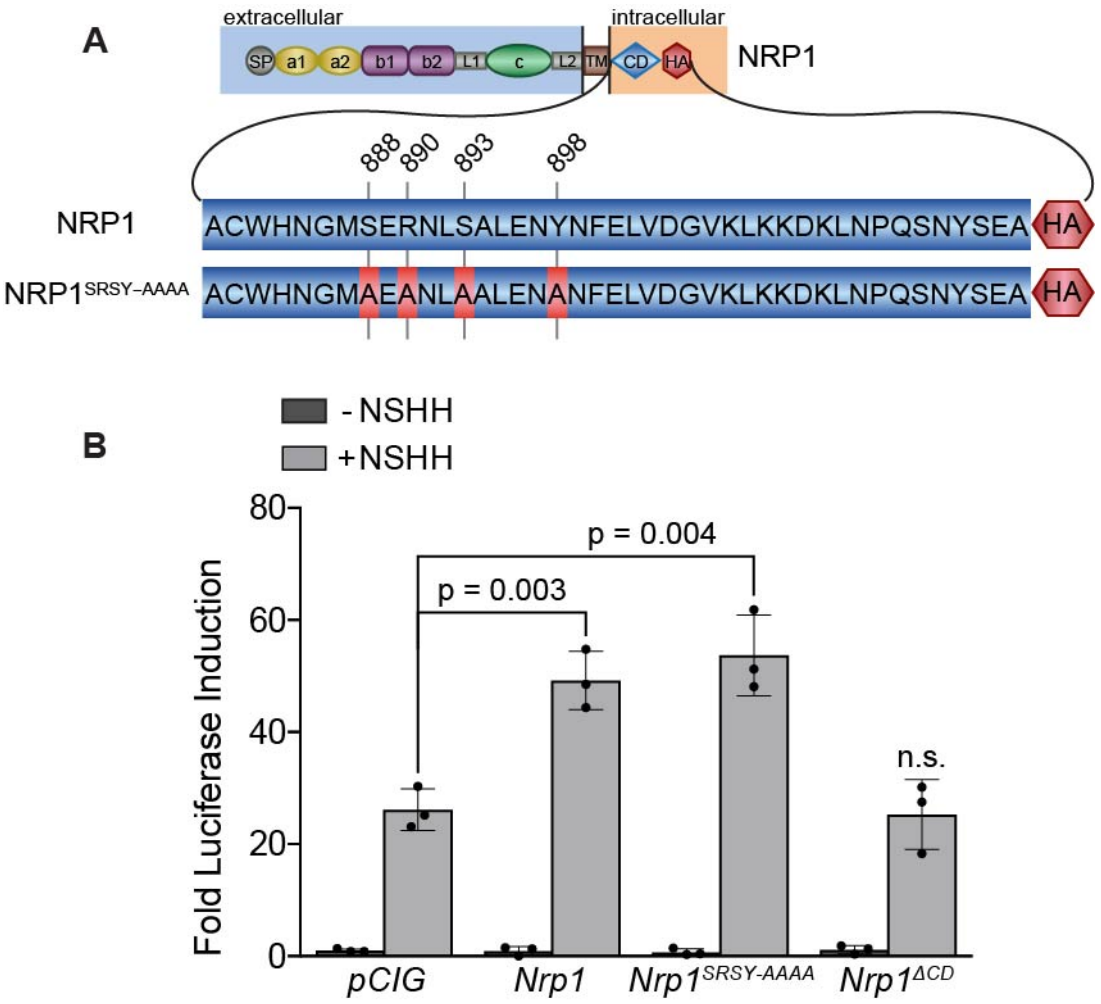


Figure 2-12. Phosphorylation of key residues is dispensable for Neuropilin-1 promotion of Hedgehog signaling.
(A) Diagram of NRP1 cytoplasmic domain and NRP1$^{SRSY-AAAA}$ indicating mutations of key residues to Alanine (B) HH-dependent luciferase reporter activity measured in NIH-3T3 cells. Data reported as mean ± SD, with p-values calculated using a two-tailed Student's t test.

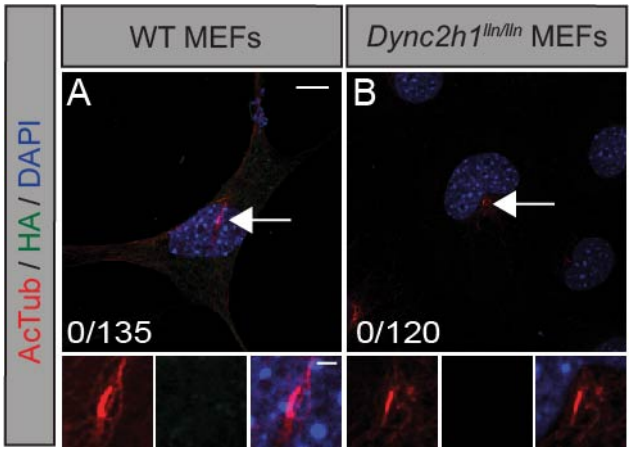


Figure 2-13. HA antibody staining in vector-transfected cells.
Antibody detection of primary cilia (*red*, AcTub) and HA (*green*) in *pCIG*-transfected WT (*left*) and Dynein mutant (*Dync2h1*$^{lln/lln}$, *right*) MEFs. DAPI indicates nuclei (*blue*). Exposure adjusted to match Figure 5 to demonstrate the background level of HA staining. Scale bar = 10μm. Inset scale bar = 1μm.

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Chapter 3:

Identification of Plexins as Novel Regulators of Hedgehog Signaling

3.1 Abstract

Hedgehog signaling directs the formation of a wide variety of tissues, and plays important roles in embryonic and postnatal development as well as adult tissue homeostasis. Characterizing the cell-surface proteins that control Hedgehog pathway activity is critical to understanding Hedgehog function in both development and disease. Neuropilins, established receptors for class 3 Semaphorins, have recently been identified as positive regulators of the Hedgehog pathway. Neuropilins function as co-receptors for Plexin-mediated Semaphorin signaling, but a role for Plexins in Hedgehog signaling has not been explored. Here, I provide evidence that multiple Plexin family members promote Hedgehog signaling in NIH-3T3 fibroblasts. I show that the Plexin cytoplasmic domain is required for this promotion. Plexins regulate small intracellular GTPases through a split GAP domain within their cytoplasmic portion. Strikingly, I find that GAP function is required for Plexin-mediated Hedgehog promotion. Furthermore, I show that constitutive activation of the Plexin GAP domain amplifies promotion of Hedgehog signal transduction. Together, these data identify Plexin receptors as novel components of the Hedgehog pathway, providing new insight into the regulation of Hedgehog signal transduction.

3.2 Introduction

The Hedgehog (HH) signaling pathway critically coordinates many cellular processes to inform embryonic development and tissue homeostasis, while deregulation of this pathway can drive a number of diseases, including cancer (Barakat et al., 2010; Briscoe and Therond, 2013; Hooper and Scott, 2005; McMahon et al., 2003; Petrova and Joyner, 2014; Teglund and Toftgard, 2010) (Ng and Curran, 2011; Sharpe et al., 2015). Current models of HH signal transduction invoke a set of core components, including the twelve-pass transmembrane protein Patched 1 (PTCH1), which inhibits pathway activity in the absence of HH ligand by repressing a second cell-surface protein Smoothened (SMO) (Marigo and Tabin, 1996; Stone et al., 1996). SMO is a seven-pass transmembrane protein exhibiting GPCR-like activity (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), which orchestrates the processing of intracellular GLI transcription factors (GLIs) into transcriptional repressors. HH ligand binding to PTCH1 leads to de-repression of SMO, which causes a shift in GLI processing to transcriptional activators, thus altering the balance of HH target gene expression (Hui and Angers, 2011).

Beyond these core pathway components, many additional proteins regulate HH signaling at the cell surface in a tissue- and stage-specific manner (Allen et al., 2011; Allen et al., 2007; Briscoe and Therond, 2013; Holtz et al., 2015; Holtz et al., 2013). In vertebrates, many of these components are essential for HH transduction, but act redundantly to achieve HH regulation. For example, the HH co-receptors growth arrest-specific 1 (GAS1), CAM-related/downregulated by oncogenes (CDON), and brother of CDON (BOC), cooperate to regulate HH signaling, but deleting any one of these receptors yields relatively mild embryonic phenotypes (Allen et al., 2011; Allen et al., 2007). When all three co-receptors are deleted, however, HH signaling is significantly disrupted, and severe phenotypes result that resemble the loss of multiple HH

ligands or of SMO (Allen et al., 2011; Zhang et al., 2001). Similarly, the PTCH1 homolog Patched 2 (PTCH2) and Hedgehog interacting protein 1 (HHIP1) act semi-redundantly with PTCH1 to antagonize HH signaling in vertebrates (Holtz et al., 2013; Jeong and McMahon, 2005). A plethora of additional proteins regulate HH ligand distribution and reception, including Dispatched (DISP), Signal sequence, CUB domain, EGF-related (SCUBE), glypicans (GPCs) and low-density lipoprotein receptor-related 2 (LRP2) (Bandari et al., 2015; Burke et al., 1999; Caspary et al., 2002; Christ et al., 2015; Christ et al., 2012; Creanga et al., 2012; Hollway et al., 2006; Kawakami et al., 2005; Kawakami et al., 2002; Ma et al., 2002; Tukachinsky et al., 2012; Vyas et al., 2008; Woods and Talbot, 2005; Yan and Lin, 2008). The mechanisms by which many of these HH regulators function have been difficult to elucidate due to their redundant nature. Furthermore, additional HH regulators may have been missed by previous genetic screens due to their collective requirements. In addition, gene duplication events and increased complexity within vertebrate HH signaling, including a requirement for the primary cilium, make it even more difficult to study HH regulators which lack invertebrate homology, such as SCUBE, GAS1, Neuropilin1 (NRP1), and Neuropilin 2 (NRP2). As a result, our overall understanding of HH regulation remains incomplete.

Recently, Neuropilins (NRPs) were identified as positive regulators of HH signaling, acting through their cytoplasmic domains to promote HH signal transduction (Ge et al., 2015; Hillman et al., 2011; Pinskey et al., 2017). NRPs were originally characterized as receptors for secreted Semaphorin (SEMA) ligands, which regulate cell migration and growth cone collapse during axon guidance (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). NRPs directly interact with class 3 SEMA ligands, but require PLXN co-receptors to transduce

SEMA signals intracellularly (Takahashi et al., 1999). Despite functioning together with NRPs in SEMA signaling, a role for PLXNs in HH signaling has not been explored.

PLXNs are a family of conserved, single-pass transmembrane proteins. There are nine different PLXN family members, which fall into four subfamilies based on homology (A, B, C, and D) (Tamagnone et al., 1999). Secreted SEMA ligands bind to Neuropilins, which form receptor complexes with PLXNs from the A and D subfamilies (Neufeld and Kessler, 2008; Takahashi et al., 1999). Many other SEMA ligands, however, interact directly with PLXN extracellular domains to activate downstream signaling events (Neufeld and Kessler, 2008). In the absence of SEMA ligands, the PLXN extracellular domain acquires an auto-inhibitory state (Takahashi and Strittmatter, 2001). Ligand binding to NRP co-receptors or directly to the SEMA domain of PLXNs releases this autoinhibition, resulting in receptor dimerization, rotation of the PLXN cytoplasmic domain, and activation of an intracellular GTPase activating protein (GAP) domain (Neufeld and Kessler, 2008; Takahashi and Strittmatter, 2001). While our understanding of signaling downstream of the PLXN GAP domain remains incomplete, GTPases from the Rac, Ras, Rho, and Rap subfamilies are inactivated by PLXN GAP activity, which results in the disassembly of focal adhesions and reduced clustering of integrins, thereby disrupting interactions between the cell and the extracellular matrix (Barberis et al., 2004; Jongbloets and Pasterkamp, 2014; Puschel, 2007; Rohm et al., 2000b; Yang and Terman, 2013). As a result, PLXN activation by SEMA ligands causes cytoskeletal collapse and defects in cellular migration (Barberis et al., 2004; Jongbloets and Pasterkamp, 2014).

Here, we investigated a role for PLXNs in HH pathway regulation. Our data suggest that multiple PLXNs, including representatives from the A and B subfamilies, positively regulate HH signaling. Similar to NRPs, we find that the PLXN cytoplasmic domain (CD) 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 find that PLXNs function very similarly in SEMA and HH cascades. Mutating key residues within the cytoplasmic PLXN GAP domain or FYN kinase phosphorylation sites prevents PLXN from promoting HH signaling. Furthermore, deleting the PLXN extracellular domain to create a constitutively active receptor augments HH promotion, suggesting that PLXNs positively regulate HH signaling through their intracellular activity. 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.

3.3 Results

3.3.1 Multiple Plexins Promote Hedgehog Signaling in NIH-3T3 Fibroblasts

While multiple studies have demonstrated a role for NRPs in HH signal promotion (Ge et al., 2015; Hillman et al., 2011; Pinskey et al., 2017), a role for PLXNs in HH signaling remains unexplored. PLXNs consist of nine members that can be classified into four different subfamilies based on homology (PLXNA1-4, PLXNB1-3, PLXNC1, and PLXND1) (Neufeld and Kessler, 2008; Tamagnone et al., 1999). Because PLXNs from the A subfamily interact with NRPs (Takahashi et al., 1999), we initially investigated whether PLXNA1 expression in NIH-3T3 fibroblasts could promote HH-responsive luciferase reporter activity compared with vector-transfected control cells. Strikingly, PLXNA1 expression drives significantly increased levels of HH reporter activity in NIH-3T3 cells (Figure 3-1 A), similar to NRP1 (Pinskey et al., 2017). Of note, PLXNA1 does not promote HH signaling in the absence of pathway activation with HH ligand (Figure 3-1 A). To address whether HH promotion was specific to PLXNA1, we also

examined PLXNA2, PLXNA3, and PLXNA4 in similar assays. Our data suggest that all members of the PLXN A subfamily promote HH signaling following pathway activation with HH ligand (Figure 3-1, B-D). Overall, PLXN-transfected cells exhibit approximately double the level of HH signaling as compared to the vector control within each experiment (Figure 3-1 B-D). Surprisingly, PLXNB2, which is not known to interact with NRPs, also promotes HH signaling to a similar extent as A-subfamily PLXNs, suggesting that PLXN-mediated HH promotion may be independent of NRP interaction (Figure 3-1, E-F) (Neufeld and Kessler, 2008). Importantly, GFRα1, an unrelated cell-surface protein within the glial cell line-derived neurotrophic factor receptor (GFR) family, does not promote HH signaling (Figure 3-1 F). Taken together, these data suggest that multiple PLXN family members promote HH signaling in NIH-3T3 cells.

3.3.2 The PlexinA1 Transmembrane and Cytoplasmic Domains are Necessary for the Promotion of Hedgehog Signal Transduction

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 (Capurro et al., 2008; Chang et al., 2011; Christ et al., 2012; Izzi et al., 2011; Lee et al., 2001; Tenzen et al., 2006; 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$^{\Delta TMCD}$) or the CD alone

(PLXNA1$^{\Delta CD}$) prevent PXLNA1 from promoting HH signaling in NIH-3T3 cells (Figure 3-2 A-B). Western blot analyses confirmed PLXNA1, PLXNA1$^{\Delta TMCD}$, and PLXNA1$^{\Delta CD}$ expression and PLXNA1$^{\Delta TMCD}$ secretion (Figure 3-2 C). Further, immunofluorescence staining for an extracellular MYC epitope under permeabilizing and non-permeabilizing conditions confirmed the cell surface localization of PLXNA1 and PLXNA1$^{\Delta CD}$ as well as the secretion of PLXNA1$^{\Delta TMCD}$, as compared to a control BOC construct with a C-terminal MYC tag (Figure 3-2 D). These results suggest that the PLXNA1 CD is required for promotion of HH signaling.

3.3.3 GAP Activity Within the Plexin Cytoplasmic Domain is Required for the Promotion of Hedgehog Pathway Activity

The PLXN CD is essential for intracellular SEMA signal transduction, acting through a split GAP domain to induce cytoskeletal collapse (Neufeld and Kessler, 2008; Puschel, 2007). To investigate the contribution of cytoplasmic GAP activity to PLXN promotion of HH signaling, we made arginine to alanine mutations in residues 1429 and 1430 of mouse PLXNA1 that disrupt GAP activity, rendering PLXNA1 a nonfunctional SEMA receptor in the Cos7 cell collapse assay (Rohm et al., 2000a). Strikingly, mutating these key conserved arginine residues within the PLXNA1 GAP domain (PLXNA1R1) also resulted in an inability of PLXNA1 to promote HH signaling (Figure 3-3 A-B). Similarly, mutating the GAP domain in PLXNB2 (PLXNB2R1) also prevented promotion of HH signaling (Figure 3-3 C-D). Importantly, western blot analyses confirmed expression of PLXNA1R1 and PLXNB2R1 at similar levels to their wild type counterparts (Figure 3-4). In addition, immunofluorescence staining for an extracellular MYC epitope in permeabilized and non-permeabilized conditions confirmed the cell surface localization of PLXNA1R1 and PLXNB2R1 compared to a C-terminally tagged

BOC control (Figure 3-3 E). Together, these results suggest that GAP activity is necessary for PLXN-mediated promotion of HH signaling.

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). As a result, deleting the autoinhibitory PLXN ECD results in a constitutively active GAP motif and induces robust cytoskeletal collapse through downstream signaling events (Takahashi and Strittmatter, 2001). To further test whether PLXN GAP function regulates HH signaling, we deleted the PLXNA1 ECD (PLXNA1$^{\Delta ECD}$) and measured HH-dependent luciferase reporter activity in NIH-3T3 cells. Not only is PLXNA1$^{\Delta ECD}$ still able to promote HH signaling, this promotion is significantly augmented in the presence of the constitutively active PLXN GAP domain (Figure 3-5 A-B). While full length PLXN roughly doubles the level of HH signaling on average, PLXNA1$^{\Delta ECD}$ consistently increases the level of HH signaling by 5-7 fold (Figure 3-17). Importantly, mutating the same two arginines to alanines within the GAP domain (1429 and 1430 in full length PLXNA1, 323 and 324 in PLXNA1$^{\Delta ECD}$) in the context of the ECD deletion significantly reduces the level of HH promotion, but does not completely abrogate PLXN-mediated HH pathway induction when compared with PLXN lacking the entire CD (Figure 3-5 C-D). Immunofluorescence analyses indicated appropriate localization of these constructs to the cell surface as well as cytoskeletal collapse in PLXNA1$^{\Delta ECD}$ and to some extent PLXNA1, or lack thereof in PLXNA1R1$^{\Delta ECD}$ and PLXNA1$^{\Delta CD}$ (Figure 3-5 E). Notably, deletion of the PLXNA2 ECD similarly drives high levels of HH pathway activity (Figure 3-6). These data further support the notion that PLXN intracellular GAP activity regulates HH signal promotion.

3.3.4 Tyrosine Phosphorylation by Fyn Kinases is Required for Hedgehog Promotion

A recent publication identified two highly conserved Fyn non-receptor tyrosine kinase phosphorylation sites within the PLXN cytoplasmic domain that are essential for SEMA signaling in the zebrafish eye (St Clair et al., 2018). To ask whether Fyn-dependent phosphorylation is necessary for PLXN-mediated HH promotion, we mutated PLXNA1 tyrosines 1605 and 1677 to alanines and measured HH-dependent luciferase activity in NIH-3T3 cells (Figure 3-7). Strikingly, mutating these tyrosines prevented PLXN promotion of HH signaling, suggesting that Fyn-dependent phosphorylation is required for PLXN-mediated HH promotion.

3.3.5 Plexins are not Enriched in the Primary Cilium

The primary cilium is an important platform for HH signaling molecules, and many HH pathway components, including NRP1 (Pinskey et al., 2017), are enriched in this subcellular compartment. To test whether PLXNs localize to the primary cilium, I overexpressed MYC-tagged PLXNs in NIH-3T3 cells and performed immunofluorescent staining for MYC and Acetylated Tubulin (AcTub), which marks the primary cilium. Interestingly, PLXNs were broadly localized throughout the cell, but largely excluded from the nucleus (Figure 3-8). Unlike NRP1, PLXN staining was not enriched within the primary cilium for any of the constructs tested (Figure 3-8). These data suggest that PLXN regulation of HH signaling does not require cilia localization.

3.3.6 Plexin-A1 Promotes Hedgehog Signaling at the Level of GLI Regulation

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). Deleting the N terminal portion of GLI2 (Gli2ΔN), the main HH pathway activator, circumvents GLI processing and results in constitutive activation of GLI2 (Hui and Angers, 2011). As a result, transfecting *Gli2ΔN* into our luciferase reporter assay in NIH-3T3 cells results in hundreds or even thousands of fold activation of HH reporter activity (Pinskey et al., 2017). Incredibly, co-transfecting *Gli2ΔN* with *Plxna1*$^{\Delta ECD}$ still results in an elevated HH response (Figure 3-9). Interestingly, *Plxna1* does not promote HH signaling with *Gli2ΔN* pathway activation, similar to its GAP mutant form (*Plxna1r1)* (Figure 3-9). These data suggest that PLXNs may function downstream of HH ligand at the level of GLI regulation, although strong PLXN GAP activation may be necessary for HH promotion with *Gli2ΔN*.

3.3.7 Using a CRISPR Approach to Delete Plexin-A1 and Plexin-B2 from Hedgehog-Responsive NIH-3T3 Cells

Previous studies have shown that reducing the levels of NRP1, NRP2, or a combination of NRP1 and NRP2 significantly reduces HH signaling (Ge et al., 2015; Hillman et al., 2011). Therefore, it is important to examine the effects that PLXN loss-of-function has on HH signaling in cells and tissues. Unfortunately, there are nine members within the PLXN family of receptors, making loss-of-function studies quite challenging. Further, it is likely that functional redundancy

will complicate these experiments, given that multiple PLXNs from different subfamilies promote HH signaling (Figure 3-1).

One approach to address PLXN loss-of-function is to use CRISPR-Cas9 to delete *Plxns* in HH-responsive immortalized cell lines. The advantage of this approach is both speed and feasibility of complex, multiple-gene knockouts. Within the NIH-3T3 line, *Plxn* family members are expressed to varying degrees (Figure 3-10). *Plxna1* and *Plxnb2* are most strongly expressed, followed by *Plxnd1, Plxna3*, and *Plxna2*, while *Plxnb3, Plxnc1, Plxna4,* and *Plxnb1* are expressed at very low levels if detectable at all (Figure 3-10). I have designed CRISPR guide RNAs and begun experiments targeting exons 3 and 4 of *Plxna1* (Figure 3-11) and exons 19-24 of *Plxnb2* (Figure 3-14) in NIH-3T3 cells. While these experiments are ongoing, initial results look promising. In over a hundred cell lines isolated from single cell-derived colonies within the first round of CRISPR, several lines look promising for *Plxna1* deletion via genotyping, including lines 5, 87, 91, 112, and 126 (Figure 3-11). Furthermore, these same cell lines exhibit reduced PLXNA1 protein, as detected by western blot (Figure 3-12) and immunofluorescent staining (Figure 3-13) with an endogenous α-PLXNA1 antibody. Additional work is needed to further validate these lines as well as functionally examine how loss of *Plxna1* in NIH-3T3 cells impacts HH responsiveness. Moreover, ongoing experiments also seek to delete the GAP domains of *Plxnb2* alone and in combination with the *Plxna1* deletion to more comprehensively reduce PLXN levels within these cells. RNA-seq data from the ENCODE project suggests that *Plxna3* and *Plxnd1* may also need to be targeted to observe a functional outcome (Figure 3-10). Overall, a significant amount of work remains to complete these analyses.

3.3.8 Analyzing Plexin-A1 and Plexin-A2 Loss-of-Function in the Developing Mouse Embryo

Genetic studies in mice may also prove useful for understanding how PLXN loss-of-function impacts *in vivo* embryonic development. Interestingly, while *Plxna1*$^{-/-}$ and *Plxna2*$^{-/-}$ mice are viable and fertile, *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ animals die during embryogenesis, although precisely when and why these embryos perish remains unclear. To analyze potential effects of *Plxna1* and *Plxna2* deletion on HH-dependent tissues, I first collected embryos at E10.5, collected sections at the forelimb level, and examined neural tube patterning using immunofluorescent staining for FoxA2, Nkx2.2, Olig2, Nkx6.1, Dbx1, and Pax3. In a single *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ embryo that has been collected, HH-dependent patterning of the neural tube is unaffected compared to littermate controls (Figure 3-15). Because a significant number of PLXN family members are highly expressed in the developing nervous system (Perala et al., 2005), I decided to change my approach to examine later-stage embryos with an additional *Gli1*^{lacZ} allele, using X-gal staining to readout HH pathway activity in a wide variety of tissues simultaneously. While these experiments are in progress, initial analysis of a *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ embryo at E14.5 revealed several intriguing phenotypes (Figure 3-16). Compared to a *Plxna1*$^{+/-}$*;Plxna2*$^{+/-}$ littermate control, the overall body size of the *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ embryo was reduced, with a crown-rump length approximately 91% of the control (Figure 3-16). While slightly damaged during dissection, lungs from the *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ embryo were also reduced in size, approximately 73% of the length of the *Plxna1*$^{+/-}$*;Plxna2*$^{+/-}$ control (Figure 3-16). The *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ embryo also displayed a slight tail defect, perhaps indicating a mild deficiency in somitogenesis (Figure 3-16). Importantly, X-gal staining revealed an overall reduction in *Gli1* expression in a number of areas, including the midbrain-hindbrain junction and in hair follicles on the head and face, particularly around the eye (Figure 3-16). These data suggest that loss of

Plxna1$^{-/-}$ and *Plxna2*$^{-/-}$ may reduce HH signaling *in vivo*, although more embryos will need to be collected and analyzed to make any conclusions.

3.4 Discussion

HH signaling plays important roles in tissue homeostasis and embryonic development, coordinating a number of cellular processes including proliferation, fate specification, and survival (Briscoe and Therond, 2013). Receptors for SEMA ligands, the NRPs and PLXNs, are expressed in a wide variety of tissues during active HH regulation (Kawasaki et al., 1999; Mauti et al., 2006; Perala et al., 2012; Perala et al., 2005). Here, we present evidence that PLXNs positively regulate HH signaling. Unlike many previously described cell surface HH regulators, PLXNs promote HH signaling through their cytoplasmic domains at the level of GLI regulation. More specifically, we find that GAP activity within the PLXN cytoplasmic domain is required for HH promotion. Furthermore, increasing GAP activity strongly upregulates the HH response, suggesting that SEMA ligand activation of PLXNs may also impact HH regulation. Taken together, these findings identify a novel role for the PLXN family of receptors in HH pathway regulation.

3.4.1 Semaphorin Receptors Act Promiscuously in Multiple Signaling Pathways

While NRPs and PLXNs were first discovered as receptors for SEMA ligands (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Takahashi et al., 1999), their broader function in other signaling pathways have also been described. NRPs play known roles in VEGF signaling to regulate angiogenesis, and they interact with a wide variety of proteins, including PIGF-2, heparan sulfate, TGF-β1, HGF, PDGF, FGF, L1-CAM, and integrins (Muhl et al., 2017;

Prud'homme and Glinka, 2012; Roth et al., 2008). PLXNs form complexes with off-track, MET, Ron, scatter factor, and VEGFR2 in various cellular contexts (Conrotto et al., 2004; Giordano et al., 2002; Toyofuku et al., 2004; Winberg et al., 2001). This raises many questions, however, about the nature of these receptors' activities within individual and overlapping signaling contexts. For example, what factors determine whether a NRP receptor binds to a PLXN co-receptor and transduces a SEMA signal or acts through its CD to regulate HH signaling? Can these processes happen simultaneously, and if so, how do they influence one another?

An important outstanding question is how SEMA ligand impacts HH signaling. SEMA ligand's role in HH promotion remains unclear, and several conflicting pieces of evidence exist in the literature. In one study, addition of SEMA ligands (SEMA3A, 3B, 3C, 3E, and 3F) in combination with HH ligand or SAG increase HH signaling in NIH-3T3 cells (Ge et al., 2015). Conversely, blocking NRP interaction with SEMA ligands using an antibody reduces GLI expression (Ge et al., 2015). It has therefore been suggested 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, NIH-3T3 cells, in which these studies were performed, express endogenous PLXNs, particularly PLXNA1 and PLXNB2 [ENCODE; GEO:GSM970853) (Consortium, 2012)]. Given the results presented here, an alternate explanation of SEMA-mediated HH promotion may be that SEMA ligands act through endogenous PLXNs to increase HH reporter activity. This may explain why other studies have shown 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 (Pinskey et al., 2017). Another discrepancy in the literature is whether the NRP ECD is required for HH promotion (Ge et al., 2015; Pinskey 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.

3.4.2 Neuropilin and Plexin Cooperation in Hedgehog Signaling

We previously reported that NRPs promote HH signaling through a novel cytoplasmic motif—a region of the protein that is dispensable for SEMA signaling (Fantin et al., 2011; Pinskey et al., 2017). This suggests that NRPs may act very differently within SEMA and HH signaling contexts. PLXNs on the other hand, seem to function very similarly in HH and SEMA signaling, through Fyn-dependent tyrosine phosphorylation and 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 ligands, does not disrupt HH promotion (Pinskey 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 also find that PLXN A subfamily members that do interact with NRPs also promote HH signaling, and we cannot exclude the possibility that these PLXNs complex with endogenous NRPs to mediate HH promotion. Therefore, the ideas that NRPs and PLXNs function independently and together in HH signaling are not mutually exclusive, and additional studies in *Plxn* and *Nrp* mutant cell lines will be key to elucidate each proteins' role.

3.4.3 Potential Hedgehog Connections Downstream of GAP Activity in the Plexin Cytoplasmic Domain

We find that HH pathway activity is regulated by the PLXN cytoplasmic GAP domain. However, it remains unclear how signaling downstream of the GAP 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 (Jongbloets and Pasterkamp, 2014; Puschel, 2007; Yang and Terman, 2013). This makes it somewhat difficult to narrow possible candidates that might mediate HH signaling. Furthermore, our understanding of the cellular mechanisms downstream of the PLXN GAP domain, including which GTPases are regulated, remains incomplete, and it is possible that the protein connecting PLXNs to HH has not been discovered. However, we find that PLXNs from both the A and B subfamilies can promote HH signaling, which may be an important clue in answering this difficult 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. Therefore, primary candidates for future study should have demonstrated roles downstream of both A and B subfamily PLXNs.

Another point to consider is that mutating key tyrosines phosphorylated by Fyn kinase (St Clair et al., 2018) also prevents PLXN promotion of HH signaling. While one of these sites lies within the PLXN GAP domain, it is unclear if these mutations generally impact GAP function or interactions with other proteins outside the realm of GAP regulation. Therefore, it is possible that PLXNs regulate HH signaling through a protein regulated by GAP domains (i.e. small GTPases), through a protein regulated by Fyn-phosphorylated tyrosines, through a protein regulated by both

of these cytoplasmic region, or through multiple proteins that interact with both of these regions. Further studies are necessary to investigate these broad possibilities.

More globally, it is important to keep in mind that PLXN GAP activation induces cytoskeletal collapse through the disassembly of focal adhesions and integrins (Bos and Pannekoek, 2012; Kinbara et al., 2003; Oinuma et al., 2004; Serini et al., 2003; Wang et al., 2012). Therefore, another possibility is that cytoskeletal changes mediate PLXN-dependent HH promotion. GLI proteins downstream of the HH signaling cascade localize to a microtubule-based structure called the primary cilium for processing and activation (Hui and Angers, 2011). While we do not observe PLXN localization to the primary cilium, it is likely that PLXN-induced cytoskeletal collapse disrupts the integrity of the primary cilium. Interestingly, while primary cilia are required for SMO-mediated activation of HH signaling, loss of the primary cilium can result in higher levels of HH signaling due to the inability of GLI3 to be processed into its repressor form (Wong et al., 2009). This is consistent with our finding that PLXNA1$^{\Delta ECD}$ promotes HH signaling even when the pathway is activated by GLI2ΔN. Additional experiments are needed to explore these possibilities.

3.4.4 Plexin Redundancy and Loss of Function

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. This raises an important challenge in studying PLXN function in HH signaling in that our findings are complicated by the presence of endogenous

PLXN proteins, particularly given that PLXNs from multiple subfamilies promote HH signaling. The PLXN TM mediates dimerization of PLXN receptors, so it is possible and likely that PLXN constructs in this study containing a TM domain interact with endogenous PLXNs. One solution, therefore, would be to replace the PLXN TM with an artificial TM which does not dimerize. To truly study the function of a single PLXN family member in HH signaling, however, a PLXN null background would be necessary. Furthermore, several studies suggest that PLXNs exhibit largely overlapping expression patterns *in vivo*, complicating loss of function studies (Mauti et al., 2006; Perala et al., 2005). Additional and more extensive characterization of PLXN expression at different developmental stages within the mouse embryo will be necessary to identify suitable tissues for *in vivo* study. Alternatively, CRISPR/Cas9 technology could be used to delete multiple PLXNs in cell lines to create a cleaner background for study.

3.5 Materials and Methods

Plexin Constructs

Plexin 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 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

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 kept at 37 °C with 5% CO_2 and 95% humidity.

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 10^4 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 (data points) and averaged (bar height), with error bars representing the standard deviation between triplicate wells. 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 Analysis

NIH-3T3 fibroblasts were plated at 1.5 X 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). Coverslips were incubated with primary antibodies overnight, followed by a 10min DAPI stain (1:30,000 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, 150mM NaCl, 0.1% Triton X-100, 1% sodium deoxycholate, and 5mM 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% NaN_3 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-PlexinA1 (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 Audoradiography Film (Denville, E3018) and developed using a Konica Minolta SRX-101A Medical Film Processor.

CRISPR

Guide RNAs were designed to delete exons 3-4 of PlexinA1 and exons 19-24 of Plexin B2 using the GRCm38/mm10 mouse genome on the UCSC genome browser (https://genome.ucsc.edu). Off-target effects were predicted using http://crispr.mit.edu/ and http://crispor.tefor.net. Selected guides were cloned into espCas9(1.1) using BbsI (Bauer et. al., 2014). Guides used in this manuscript include: *Plxna1* 5' sense: caccGGGTCTAGGATAGTGTGTCG; *Plxna1* 5' antisense: aaacCGACACACTATCCTAGACCC; *Plxna1* 3' sense: caccgACATTTGGCCTAGTGGCCCC; *Plxna1* 3' antisense: aaacGGGGCCACTAGGCCAAATGTc; *Plxnb2* 5' sense: caccGGGACCCCATGCCAGCCGTA; *Plxnb2* 5' antisense: aaacTACGGCTGGCATGGGGTCCC; *Plxnb2* 3' sense: caccGCGCACATGTGCATTGCCGT; and *Plxnb2* 3' antisense: aaacACGGCAATGCACATGTGCGC. Guides were transfected into NIH-3T3 cells at approximately 50% confluency, using 2-5 μg of each guide and 0.5-1 μg of PGK-Puromycin selection plasmid (generously provided by Dr. Kristen J. Verhey, University of Michigan). After 48hrs, cells were split 1:5 into selection media containing 10-50 μg/mL puromycin (Sigma, P8833). Cells were maintained in selection media for 48 hours or until all cells on untransfected control plates had died. Cells were then plated into 96-well plates at 0.5 cells/well to isolate single cell-derived colonies. Colonies were genotyped (*Plxna1* primers: GCAGCTTGATGGTAGGCACTGGAGC, GAGGCAAAACGCTGGGGCTCTTCTG; *Plxnb2* primers: CGAAGCCGGAGTCTTTGAGT, ATCGCAGAAACCCTTCCCAG) and *Plxn* deletion was confirmed using Western blot, Immunofluorescent microscopy, and qRT-PCR analyses.

Mouse Models

Plxna1 (Yoshida et al., 2006) and *Plxna2* (Duan et al., 2014; Suto et al., 2007) mice, both on mixed genetic backgrounds, were generously provided by Dr. Alex Kolodkin and Dr. Roman Giger. $Gli1^{lacZ}$ animals were 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.

For timing of embryonic stages, 12:00 PM on the day a vaginal plug was detected was considered E0.5. X-gal staining was performed as previously described (Holtz et al., 2015).

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3.7 Figures

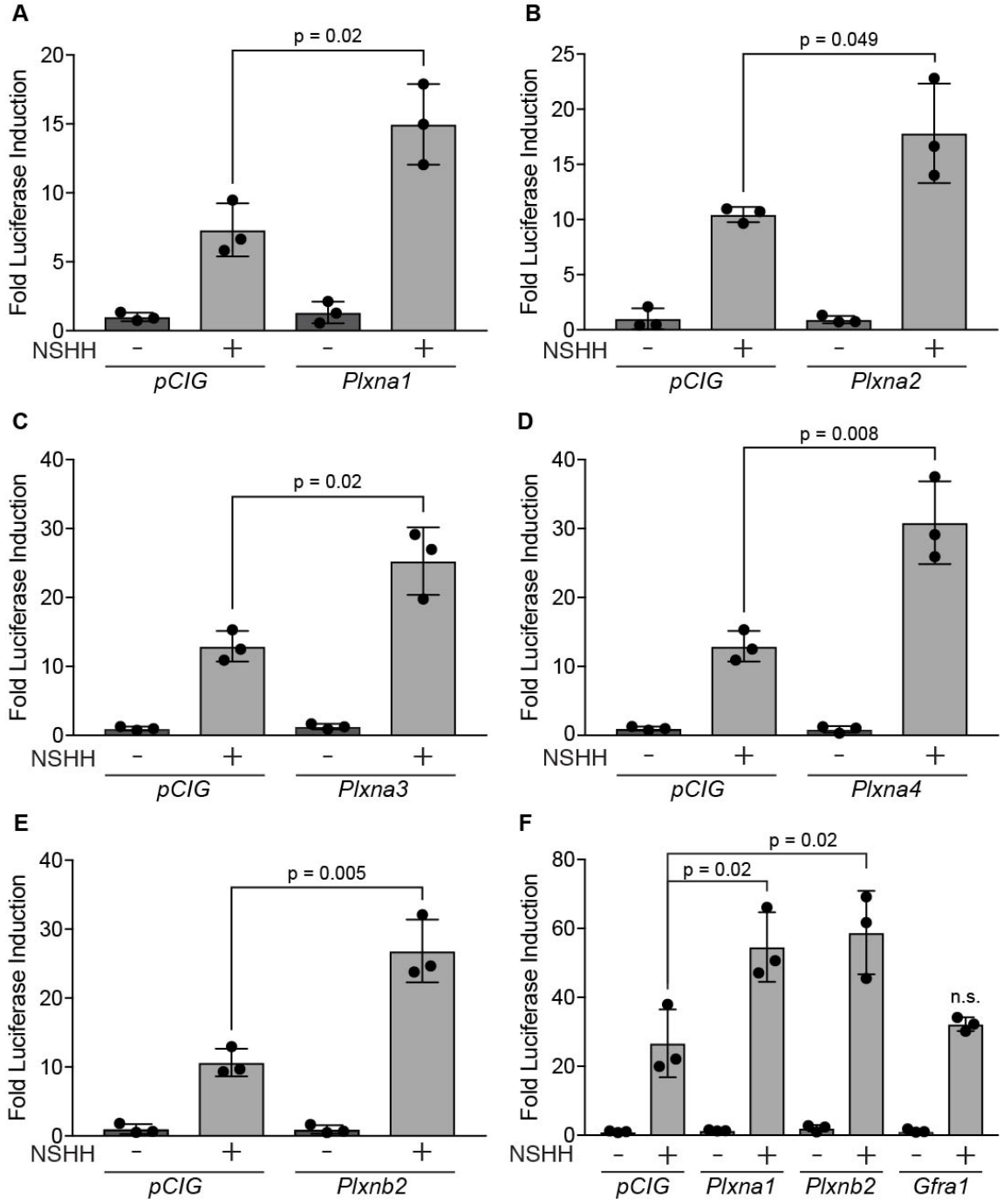

Figure 3-1. Multiple Plexins promote Hedgehog signaling
HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned media (+NSHH). Data are reported as mean fold induction ± S.D., with p-values calculated using two-tailed Student's t tests. n.s. = not significant.

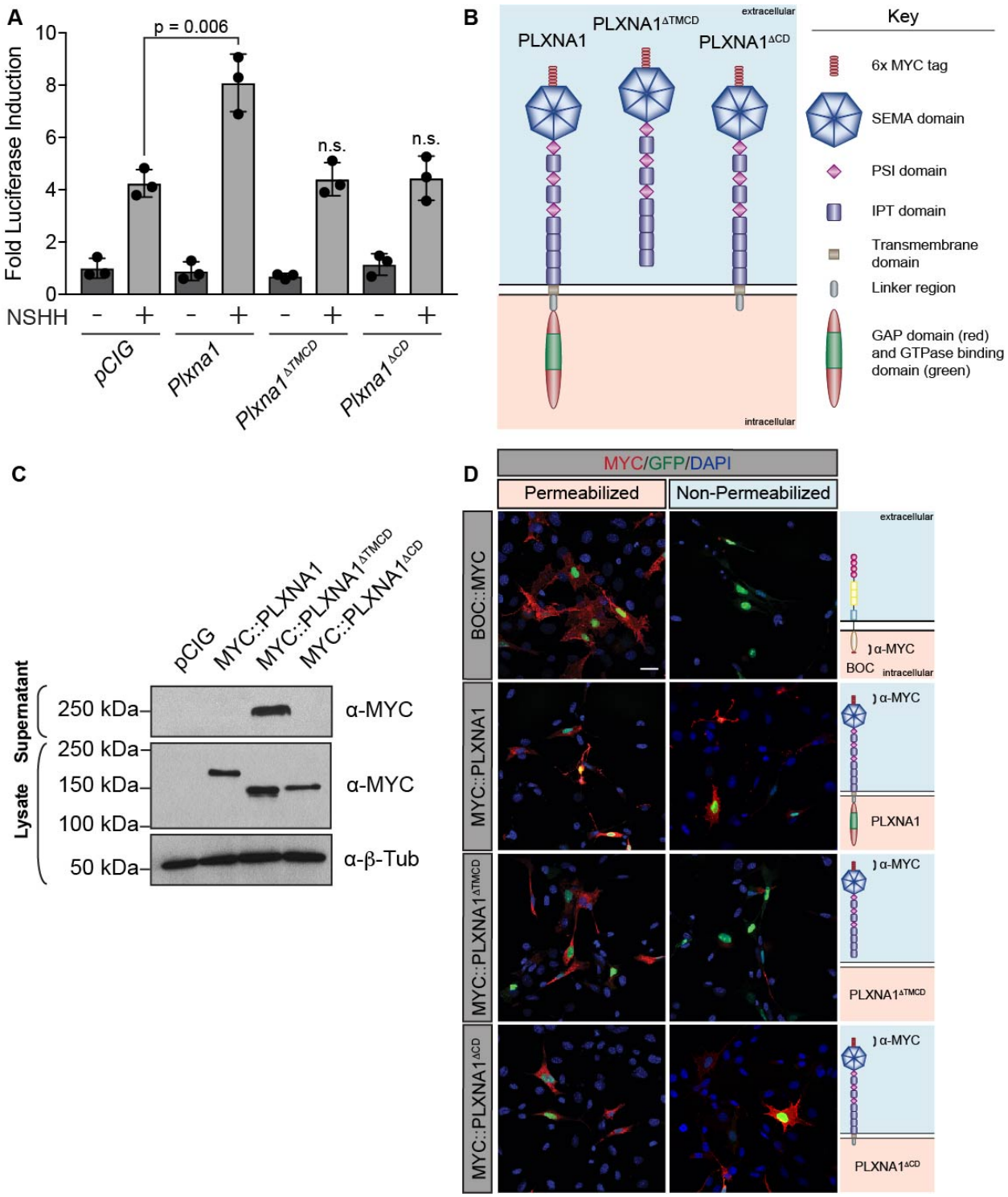

Figure 3-2 The Plexin-A1 cytoplasmic and transmembrane domains are required for the promotion of Hedgehog pathway activity.

A. HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of 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. B. Schematics of full-length and truncated versions of PLXNA1. C. Western blot confirming expression of MYC-tagged PLXNA1 constructs in NIH-3T3 cells. Anti-Beta-tubulin (α-β-Tub) was used as a loading control. Normalized relative expression for both the supernatants and lysates are shown below the blot. D. Antibody detection of MYC tagged constructs (red) in permeabilized (left panels) and non-permeabilized (right 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. Diagrams (right) describe each construct, with brackets indicating antibody binding sites. Scale bar = 10 μm.

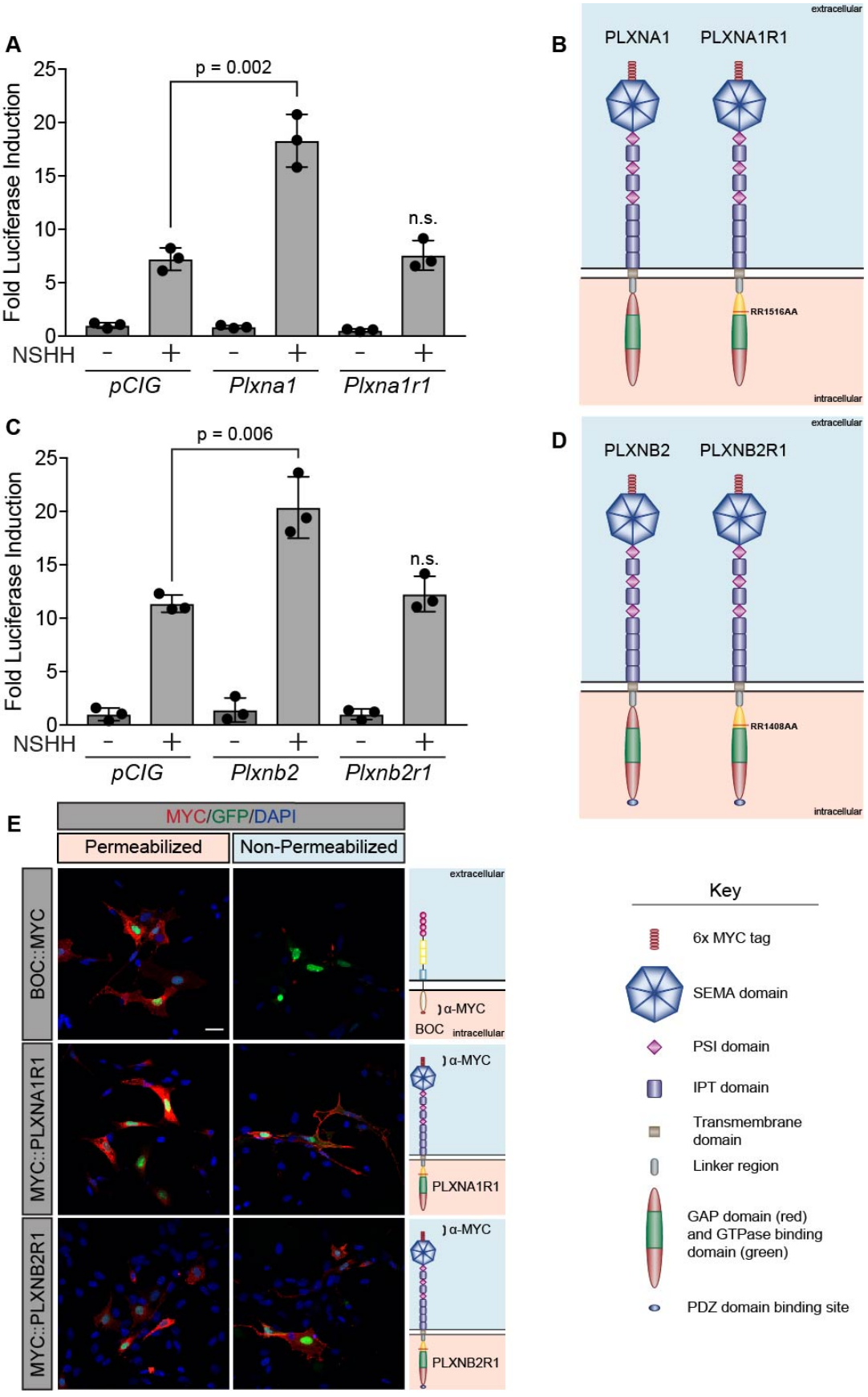
A
Fold Luciferase Induction
p = 0.002
n.s.
NSHH
pCIG
Plxna1
Plxna1r1
B
PLXNA1
PLXNA1R1
extracellular
RR1516AA
intracellular
C
Fold Luciferase Induction
p = 0.006
n.s.
NSHH
pCIG
Plxnb2
Plxnb2r1
D
PLXNB2
PLXNB2R1
extracellular
RR1408AA
intracellular
E
MYC/GFP/DAPI
Permeabilized
Non-Permeabilized
BOC::MYC
MYC::PLXNA1R1
MYC::PLXNB2R1
α-MYC
BOC
PLXNA1R1
PLXNB2R1
Key
6x MYC tag
SEMA domain
PSI domain
IPT domain
Transmembrane domain
Linker region
GAP domain (red) and GTPase binding domain (green)
PDZ domain binding site

Figure 3-3. Mutation of the Plexin GAP domains abrogates promotion of Hedgehog signaling.
A and C. HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of 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. B and D. Schematics of full length and mutated versions of PLXNA1 and PLXNB2. E. Antibody detection of MYC tagged constructs (red) in permeabilized (left panels) and non-permeabilized (right 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. Diagrams (right) describe each construct, with brackets indicating antibody binding sites. Scale bar = 10 μm.

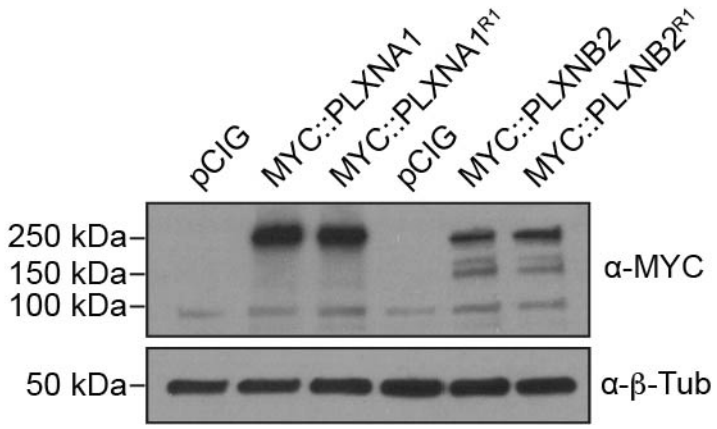

Figure 3-4. Plexin GAP domain mutants are expressed at equal levels in NIH-3T3 cells.
Western blot confirming expression of MYC-tagged PLXNA1 and PLXNB2 constructs in NIH-3T3 cells. Anti-Beta-tubulin (α-β-Tub) was used as a loading control.

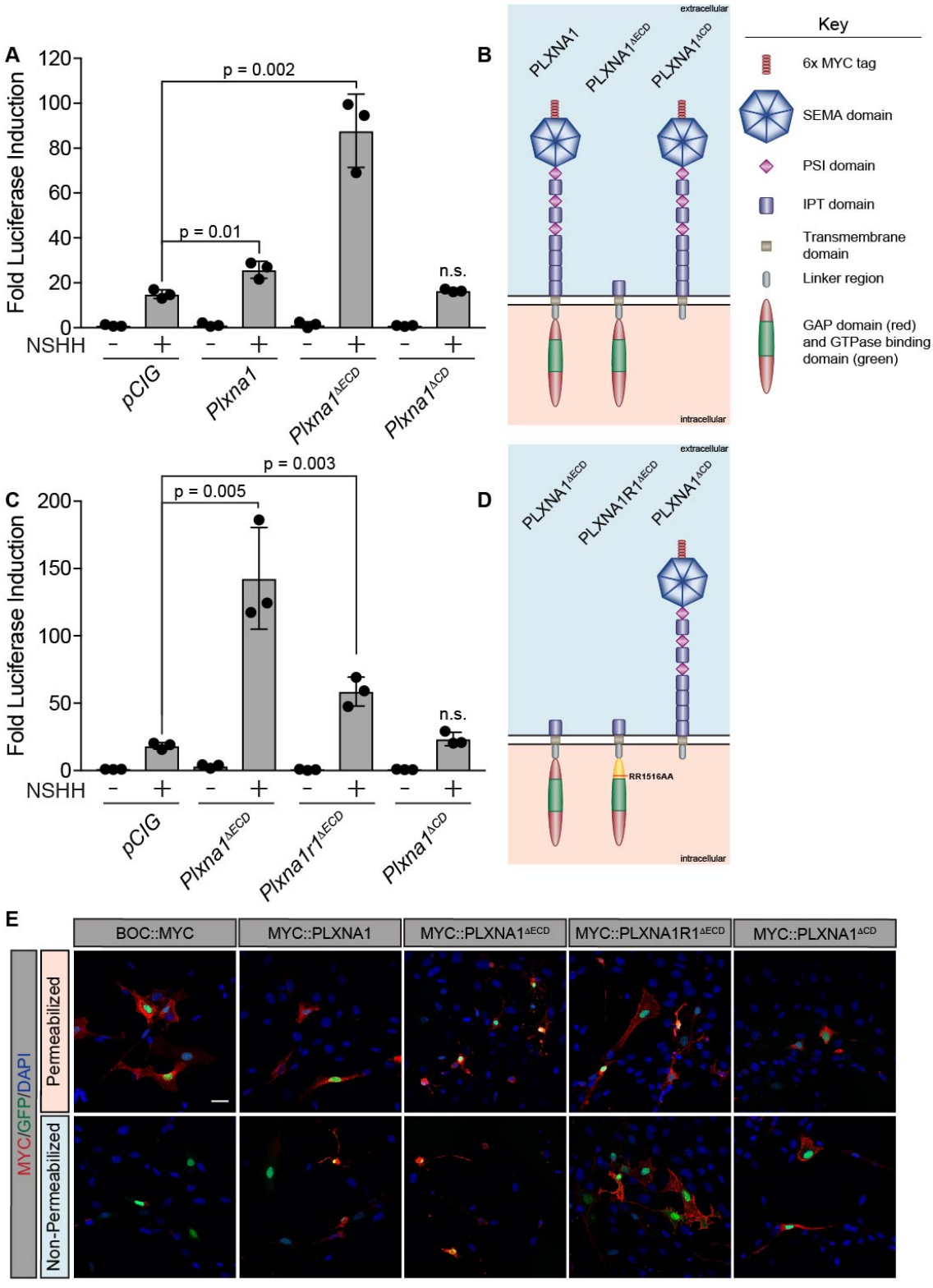
A
Fold Luciferase Induction
120
100
80
60
40
20
0
p = 0.002
p = 0.01
n.s.
NSHH
pCIG
Plxna1
Plxna1ΔECD
Plxna1ΔCD
B
extracellular
PLXNA1
PLXNA1ΔECD
PLXNA1ΔCD
intracellular
Key
6x MYC tag
SEMA domain
PSI domain
IPT domain
Transmembrane domain
Linker region
GAP domain (red) and GTPase binding domain (green)
C
Fold Luciferase Induction
200
150
100
50
0
p = 0.003
p = 0.005
n.s.
NSHH
pCIG
Plxna1ΔECD
Plxna1r1ΔECD
Plxna1ΔCD
D
extracellular
PLXNA1ΔECD
PLXNA1R1ΔECD
PLXNA1ΔCD
RR1516AA
intracellular
E
BOC::MYC
MYC::PLXNA1
MYC::PLXNA1ΔECD
MYC::PLXNA1R1ΔECD
MYC::PLXNA1ΔCD
MYC/GFP/DAPI
Permeabilized
Non-Permeabilized

Figure 3-5 Deletion of the Plexin-A1 extracellular domain drives high level, ligand-dependent Hedgehog pathway activation.
A and C. HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of 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. B and D. Schematics of full length and truncated or mutated versions of PLXNA1. E. Antibody detection of MYC tagged constructs (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. Scale bar = 10 μm.

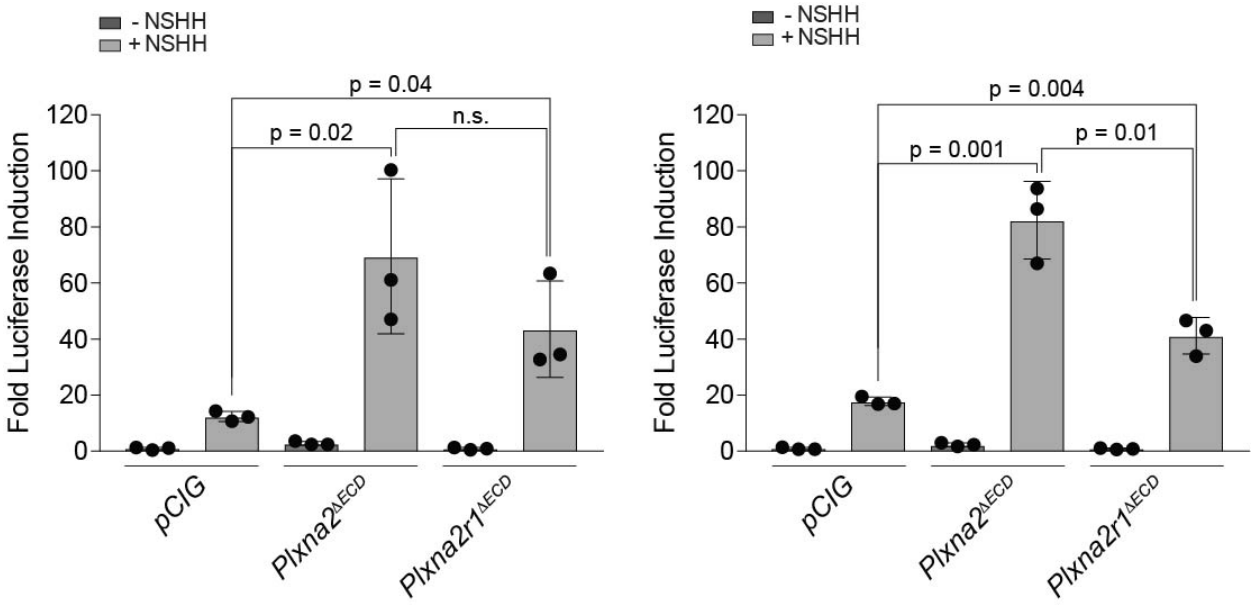

Figure 3-6. Deletion of the Plexin-A2 extracellular domain drives high level, ligand-dependent Hedgehog pathway activation.
HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of the indicated constructs and stimulated with control (-NSHH) or NSHH-conditioned 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. Shown are two experiments using the same constructs and conditions to highlight the variable result with PLXNA2R1$^{\Delta ECD}$.

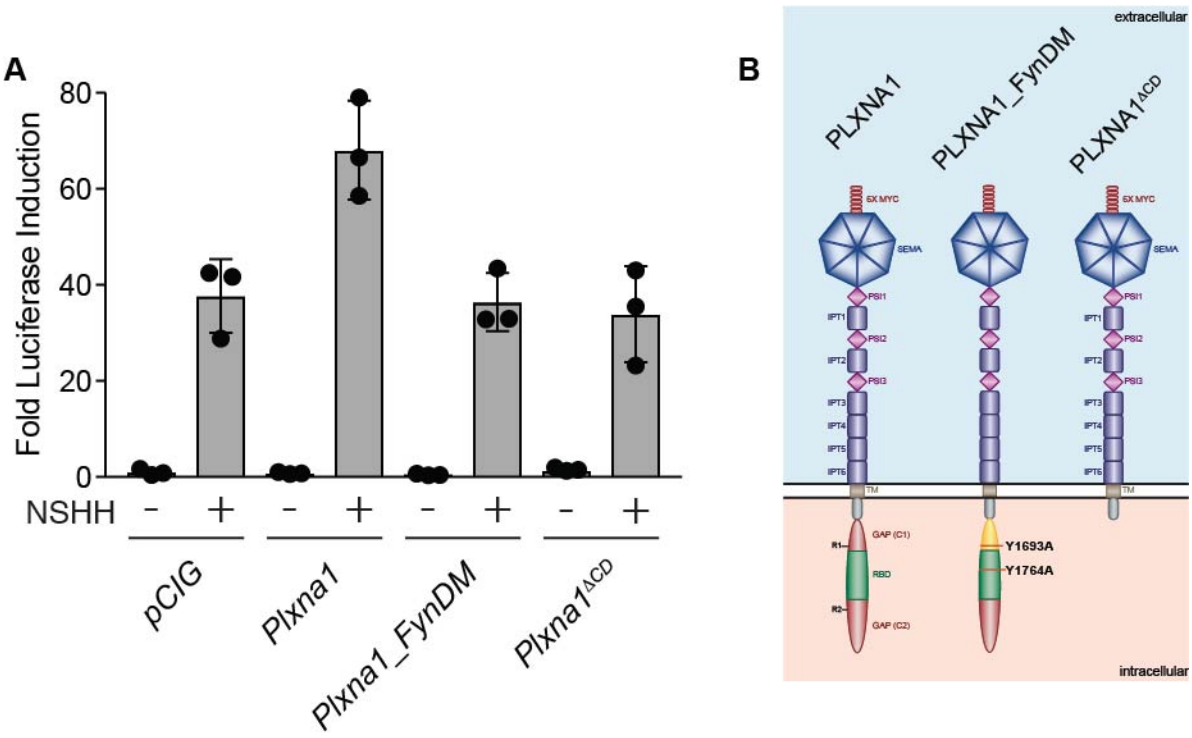

Figure 3-7. Mutation of Fyn kinase phosphorylation sites abrogates Plexin-A1-mediated Hedgehog pathway activation.

A. HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of 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. B. Schematics of full length, mutated (FynDM), and truncated (ΔCD) versions of PLXNA1.

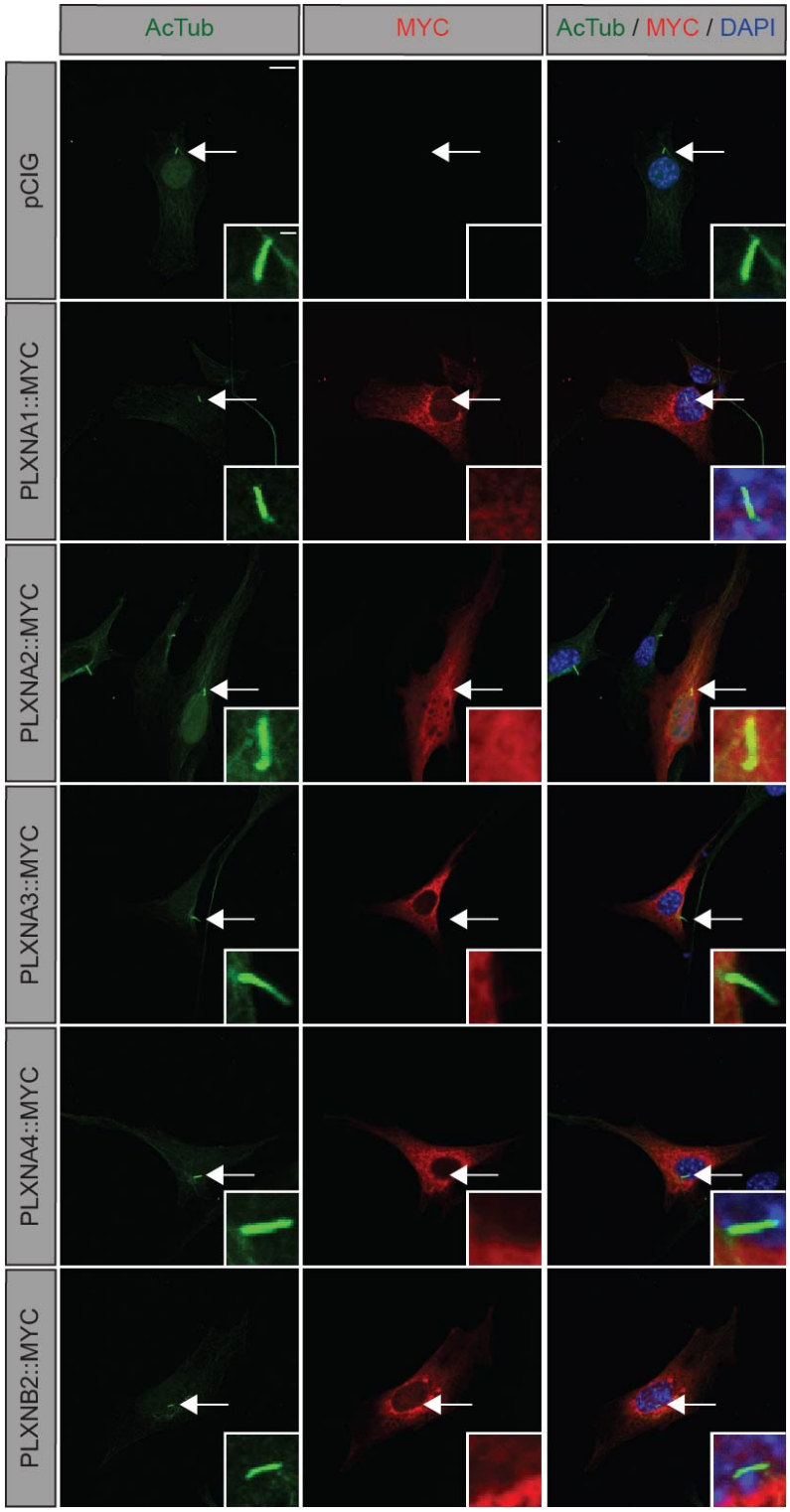

Figure 3-8. Plexins are not enriched in primary cilia of NIH-3T3 cells.
Antibody detection of MYC tagged constructs (red) and cilia marker Acetylated tubulin (AcTub) in NIH-3T3 cells. DAPI (blue) stains nuclei. Scale bar = 10 μm. Inset scale bar = 1 μm. *Note: PLXNA2 construct may be incorrect and should be repeated to validate result.

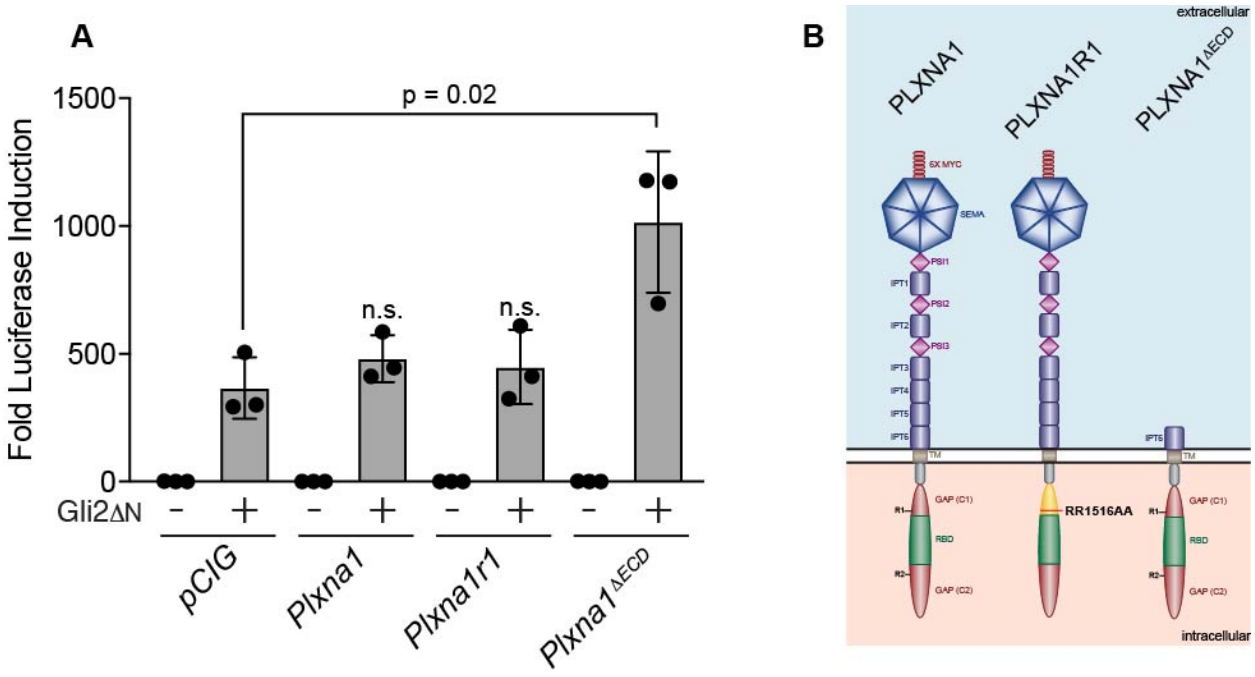

Figure 3-9. Plexin-A1 lacking its extracellular domain, but not full-length Plexin-A1, promotes Hedgehog signaling with GLI2ΔN pathway activation.
A. HH-dependent luciferase reporter activity was measured in NIH-3T3 cells transfected with 150 ng of the indicated constructs and stimulated with vector (-Gli2ΔN) or Gli2ΔN (+Gli2ΔN) co-transfection. Data are reported as mean fold induction ± S.D. with p-values calculated using two-tailed Student's t tests. n.s. = not significant. B. Schematics of full length and truncated or mutated versions of PLXNA1.

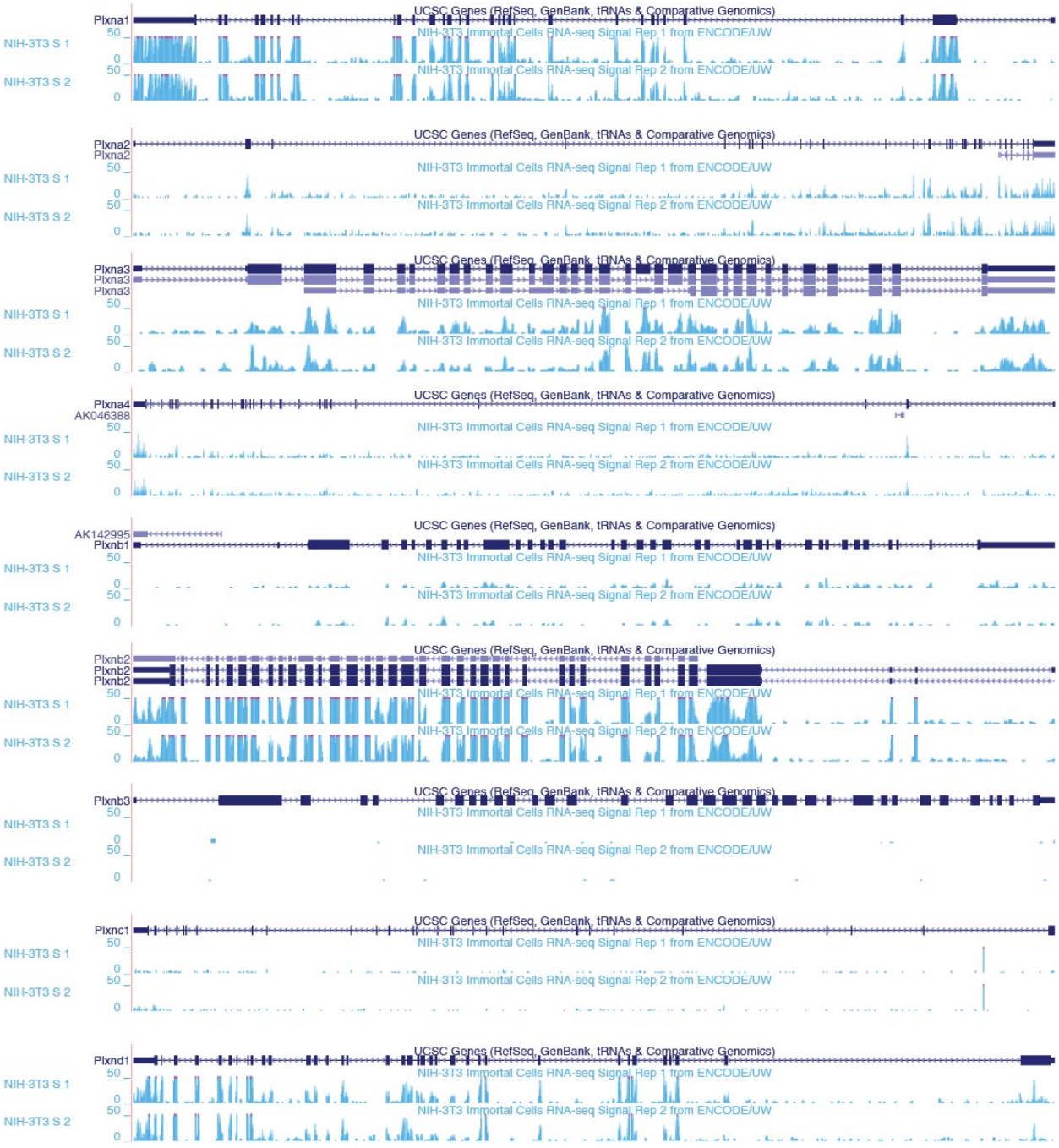

Figure 3-10. A subset of Plexins are expressed in NIH-3T3 cells.
RNA-seq data from the ENCODE project indicating expression of *Plxn* family members as indicated in NIH-3T3 cells. Data were aligned to the mouse GRCm38/mm10 assembly using the UCSC Genome Browser (https://genome.ucsc.edu).

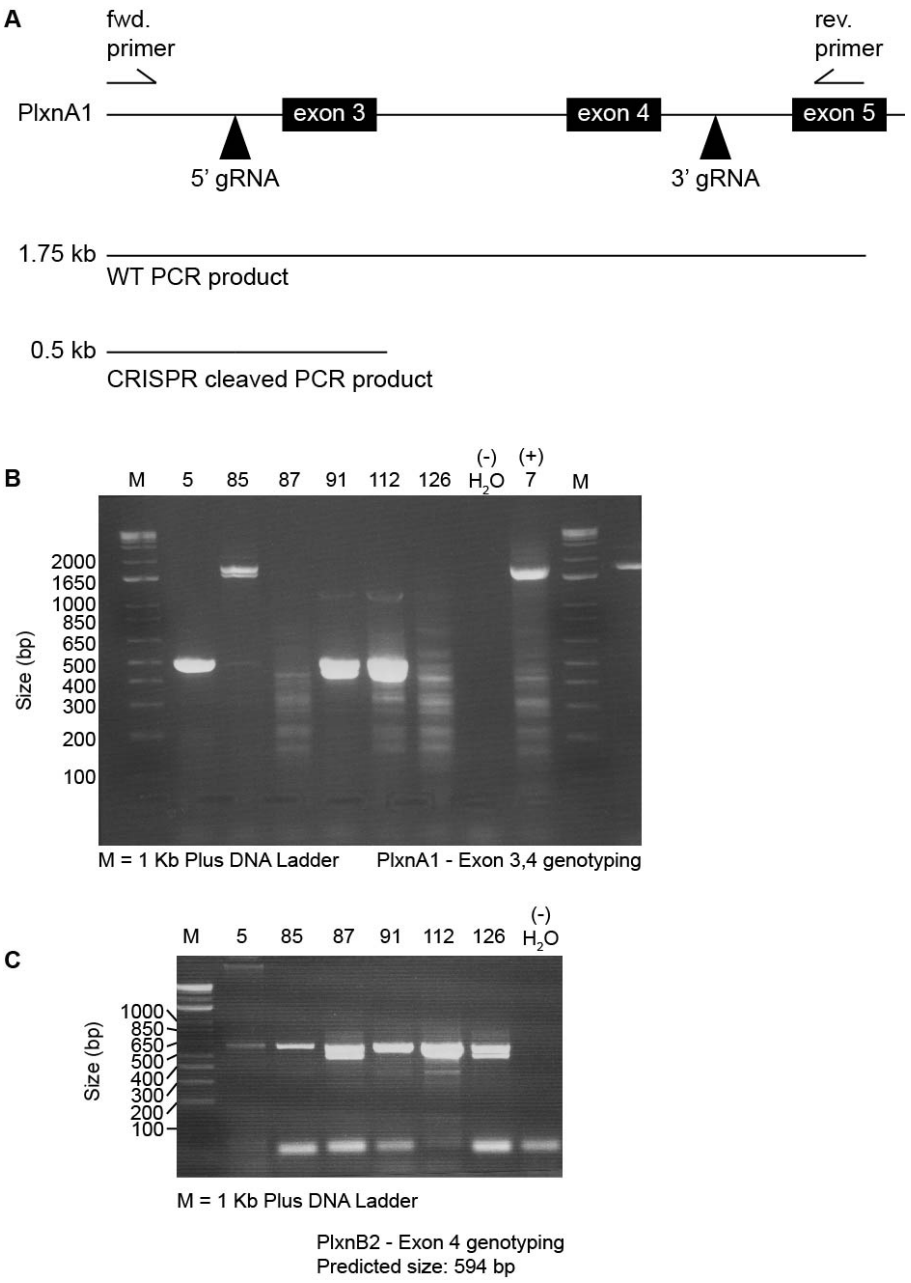

Figure 3-11. Plexin-A1 CRISPR approach and preliminary results.
A. Diagram indicating CRISPR approach to delete exons 3 and 4 of *Plxna1* in NIH-3T3 cells. Arrows indicate binding locations of the forward (fwd.) and reverse (rev) genotyping primers. Arrowheads denote binding regions for 5' and 3' guide RNAs (gRNA), with black boxes indicating exon positions within the locus. Lines below indicate sizes of wild type (WT) and CRISPR cleaved PCR products. B. Preliminary genotyping results from CRISPR lines isolated from single cells. Ladder sizes (M) indicated to left of image in base pairs (bp). A water control (H_2O) and positive cell line control (7) are also included. C. Genotyping results using a set of control primers targeting exon 4 of *Plxnb2*.

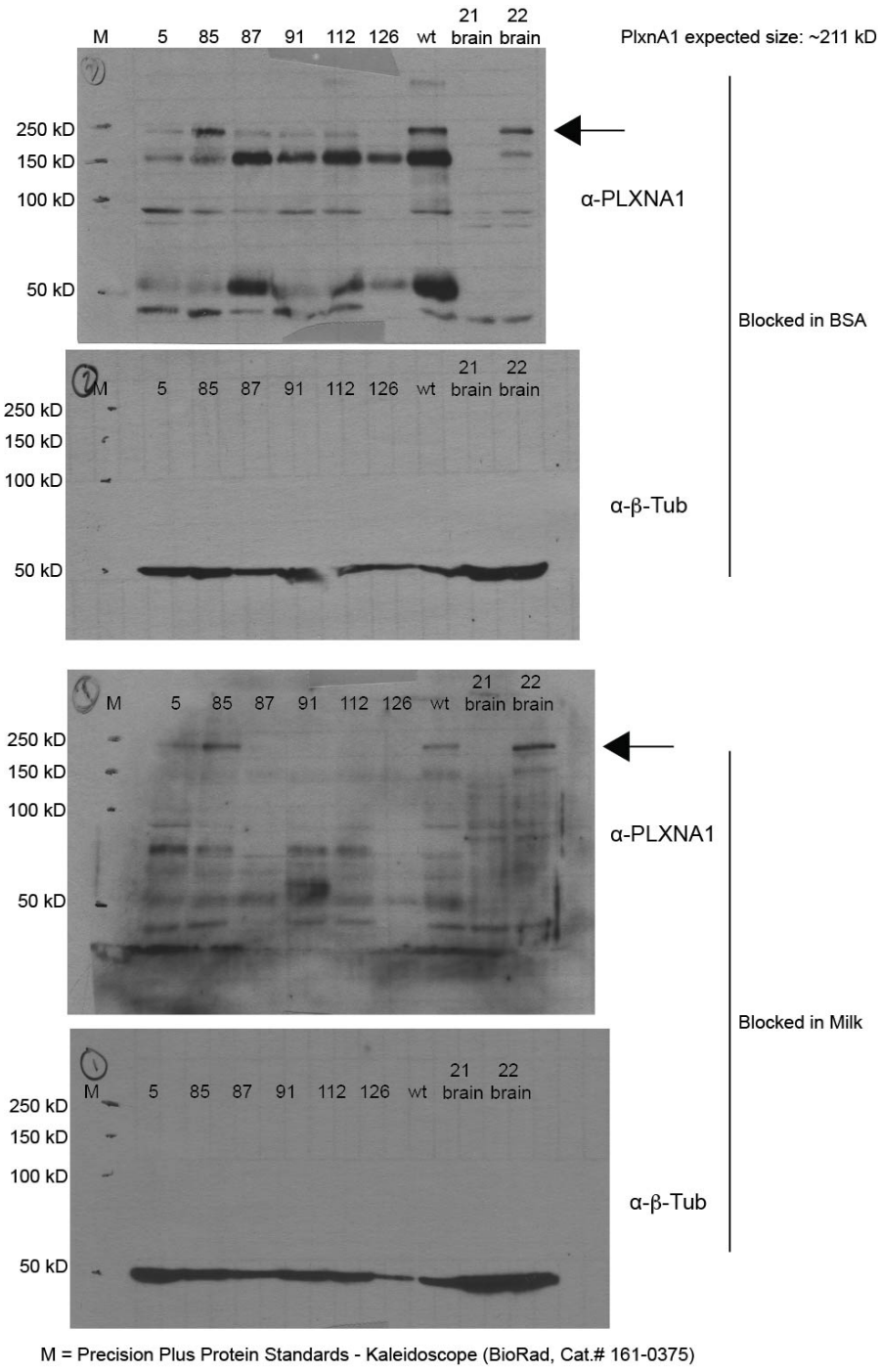

Figure 3-12. Plexin-A1 levels are reduced following CRISPR in NIH-3T3 cells.
Western blot confirming reduction of endogenous PLXNA1 in NIH-3T3 cells following CRISPR deletion and selection. Lysates from *Plxna1*$^{+/-}$*;Plxna2*$^{+/-}$ (22) and *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ (21) E14.5 mouse brains were used as controls, in addition to untransfected NIH-3T3 cell lysate (wt). The top two blots were blocked in BSA, while the bottom two were blocked in 0.5% milk. Anti-Beta-tubulin (α-β-Tub) was used as a loading control.

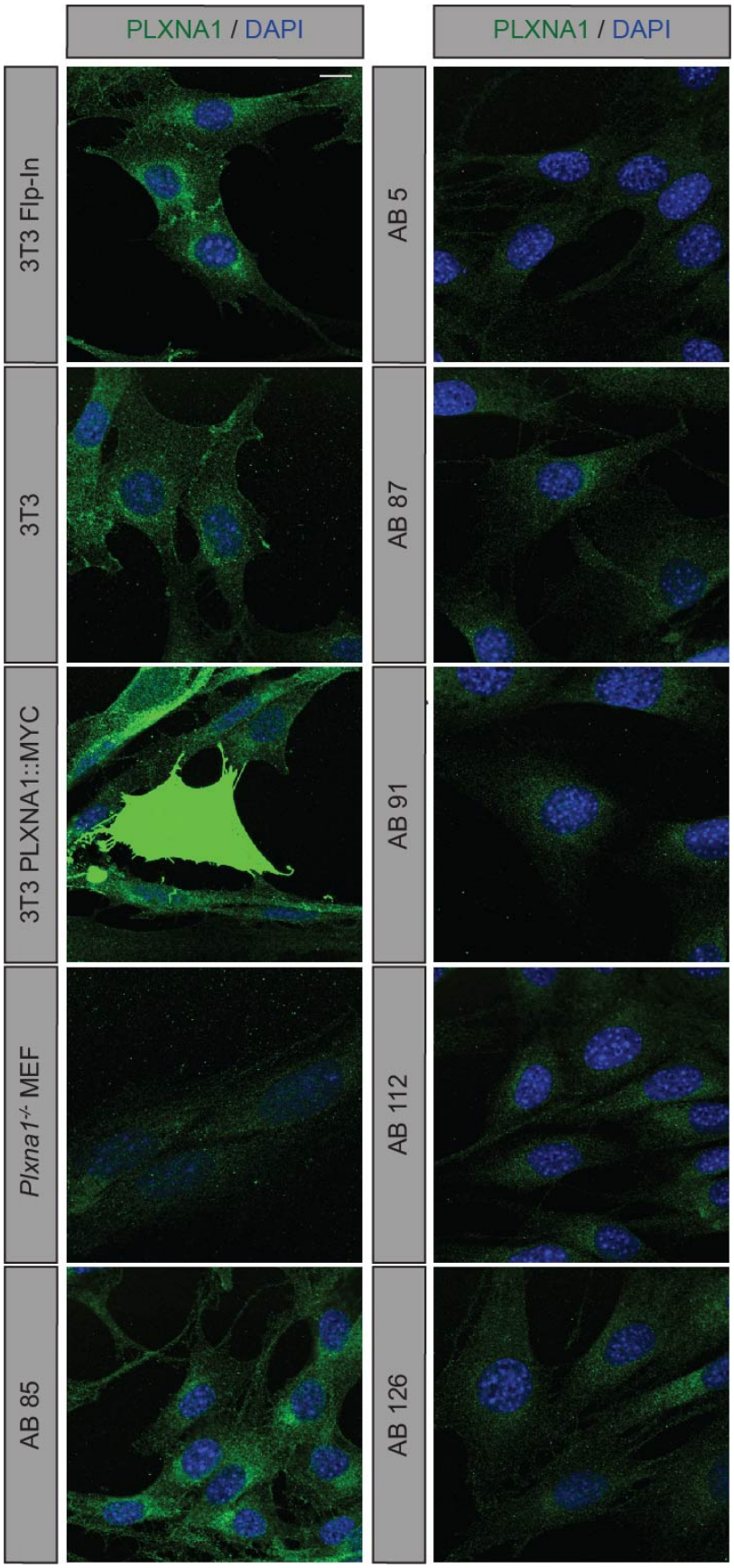
PLXNA1 / DAPI
PLXNA1 / DAPI
3T3 Flp-In
3T3
3T3 PLXNA1::MYC
Plxna1-/- MEF
AB 85
AB 5
AB 87
AB 91
AB 112
AB 126

Figure 3-13. Immunofluorescent staining for Plexin-A1 in CRISPR NIH-3T3 cell lines.
Antibody detection of Endogenous PLXNA1 (green) and DAPI (blue) antibody staining in indicated cell lines. Note: Imaging settings, including gain, were kept consistent from image to image with the exception of the DAPI channel in *Plxna1*$^{-/-}$ MEFs, which was increased to detect faint signal. Scale bar = 10 μm.

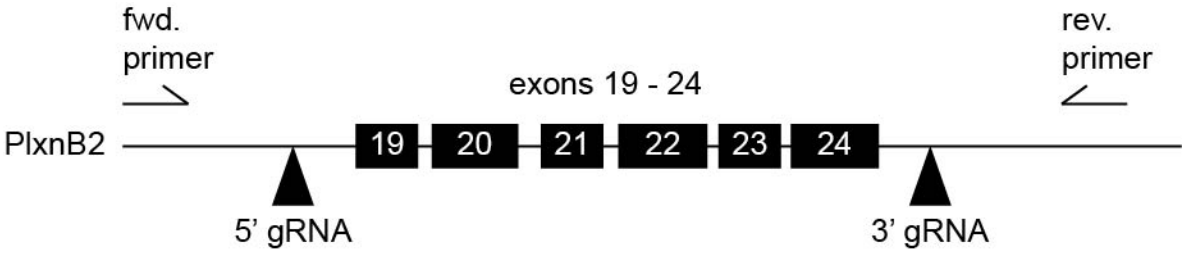

Figure 3-14. Plexin-B2 CRISPR approach.
Diagram indicating CRISPR approach to delete exons 19-24 of *Plxnb2* in NIH-3T3 cells. Arrows indicate binding locations of the forward (fwd.) and reverse (rev) genotyping primers. Arrowheads denote binding regions for 5' and 3' guide RNAs (gRNA), with black boxes indicating exon positions within the locus.

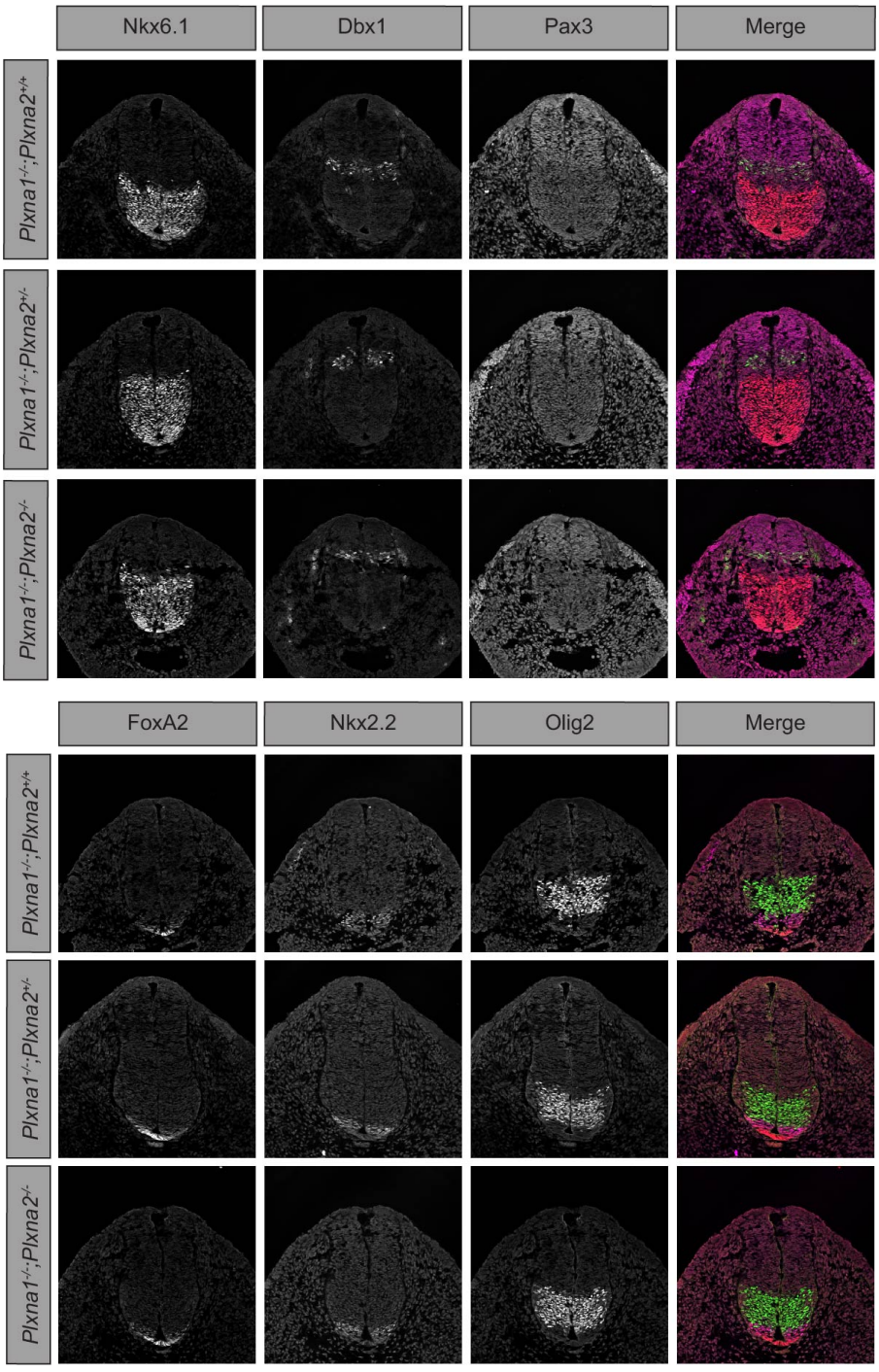

Figure 3-15. Stage 10.5 embryos lacking Plexin-A1 and Plexin-A2 display overtly normal neural patterning.
Cross-sectional images of E10.5 *Plxna1*$^{-/-}$*;Plxna2*$^{+/+}$ (top row), *Plxna1*$^{-/-}$*;Plxna2*$^{+/-}$ (middle row), and *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$ (bottom row) mouse embryos. Antibody staining for neural progenitor populations is indicated by column headings.

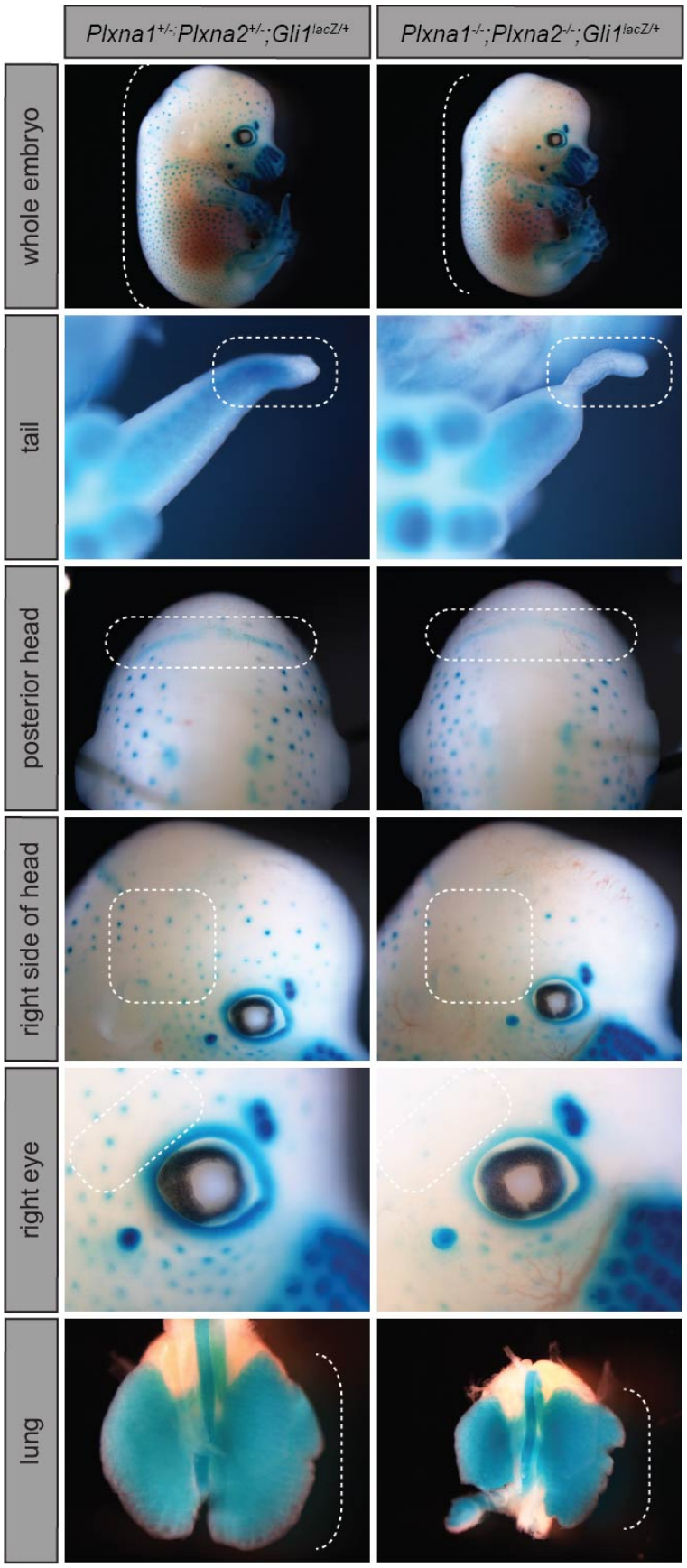
Plxna1+/-;Plxna2+/-;Gli1lacZ/+
Plxna1-/-;Plxna2-/-;Gli1lacZ/+
whole embryo
tail
posterior head
right side of head
right eye
lung

Figure 3-16. Stage 14.5 embryos lacking Plexin-A1 and Plexin-A2 exhibit reduced Gli1^{lacZ} reporter expression and several morphological defects.
Images of X-Gal stained *Plxna1*$^{+/-}$*;Plxna2*$^{+/-}$*;Gli1*$^{lacZ/+}$ (*left*) and *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$*;Gli1*$^{lacZ/+}$ (*right*) E14.5 mouse embryos. Various views of different tissues, including the whole embryo, tail, posterior and right side of head, right eye, and lung are included as indicated, with white dotted lines highlighting regions of interest. Images highlight reduced crown-rump length (91% of control) and reduced lung size (73% of control) within the *Plxna1*$^{-/-}$*;Plxna2*$^{-/-}$*;Gli1*$^{lacZ/+}$ embryo, as well as a possible tail somite defect and several areas of reduced X-Gal staining.

	PLXNA1		PLXNA1$^{\Delta ECD}$	
assay	fold change	p-value	fold change	p-value
1	1.63	0.024	6.85	0.0004
2	1.50	0.047	3.69	0.00001
3	1.33	0.036	4.80	0.007
4	1.77	0.056	10.42	0.00005
5	1.72	0.011	5.85	0.002
avg.	1.59	0.035	6.32	0.001

Figure 3-17. Deletion of the Plexin extracellular domain reproducibly increases Plexin-A1-mediated Hedgehog pathway activity.
Summary of luciferase assay data in which *Plxna1* and *Plxna1*$^{\Delta ECD}$ were directly compared in five independent assays. Fold change reported between ligand-stimulated vector only (*pCIG*) triplicate wells and ligand-stimulated *Plxna1*- or *Plxna1*$^{\Delta ECD}$ - transfected triplicate wells. Yellow highlight denotes significance ($p<0.05$).

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Chapter 4:
Discussion and Future Directions

4.1 Neuropilins and Hedgehog Signaling: Summary of Key Findings

A host of cell surface-associated and secreted proteins bind HH ligands to either antagonize or enhance HH pathway activity. For example, the transmembrane proteins PTCH1 and PTCH2 as well as the secreted antagonist HHIP1 each sequester HH ligand through binding to specific extracellular domains (Chuang et al., 2003; Chuang and McMahon, 1999; Holtz et al., 2015; Holtz et al., 2013; Jeong and McMahon, 2005). Conversely, the HH co-receptors GAS1, CDON, and BOC also interact with HH ligands through extracellular domains, and are required for HH signal transduction during early embryogenesis (Allen et al., 2011; Allen et al., 2007; Del Sal et al., 1992; Kang et al., 1997; Kang et al., 2002; Kavran et al., 2010; Lee et al., 2001; Song et al., 2015; Tenzen et al., 2006). In contrast to these ligand-binding cell surface HH regulators, data presented in Chapter 2 suggest that cell surface-associated NRP receptors, in particular NRP1, promote HH signaling intracellularly through the cytoplasmic domain. Unlike PTCH1, PTCH2, and HHIP1 (Holtz et al., 2013), NRP1 modulates HH signaling even when the pathway is activated downstream of ligand by SMO activation or co-transfection of GLI activator, suggesting that NRP1 functions at the level of GLI transcriptional regulation. This unique mechanism of action distinguishes NRPs from other HH cell-surface regulators and raises important questions about their roles in HH regulation *in vivo*.

One of the most surprising and unexpected results from my analyses is that NRP1 localizes to the primary cilium, a subcellular compartment necessary for HH signal transduction

(Goetz and Anderson, 2010). Many molecules within the HH signaling cascade move into or out of the primary cilium during HH signaling, including SMO, PTCH1, PTCH2, and GLI proteins (Corbit et al., 2005; Haycraft et al., 2005; Holtz et al., 2013; Huangfu et al., 2003; Liu et al., 2005; Rohatgi et al., 2007). This is a highly-regulated process, as the base of the cilium forms a size-exclusion barrier, requiring active transport of large cytosolic proteins and import of membrane proteins through unclear mechanisms (Kee et al., 2012; Takao et al., 2014). Strikingly, I observed both overexpressed and endogenous NRP1 enriched in the primary cilium of NIH-3T3 and dynein mutant (*Dync2h1*$^{lln/lln}$) cells. Interestingly, however, NRP2 is not enriched in the primary cilium, nor is NRP1$^{\Delta ECD}$, although both proteins robustly promote HH signaling. Together, these data suggest that NRP1 cilia localization does not correlate with its ability to regulate HH, and leads to questions about other potential roles that NRP1 might play within the primary cilium.

Instead of cilia localization, my structure-function analyses suggest that the NRP1 cytoplasmic and transmembrane domains are both necessary and sufficient to regulate HH signaling. This finding both supports and contrasts with published literature. One previous study suggests that the NRP1 CD is required, but claims that the extracellular domains are also required for HH promotion (Ge et al., 2015). In addition, this study suggests that SEMA ligand binding enhances NRP-mediated HH promotion, in contrast to previous work (Hillman et al., 2011). My findings support the idea that the NRP1 CD is required for HH promotion, but suggest that SEMA ligand interaction is dispensable, since the extracellular domains which mediate NRP-SEMA interactions can be deleted with no effect on HH promotion. Furthermore, I find that NRP dimerization, which is required for SEMA signal transduction, can be disrupted with no effect on its ability to promote HH signaling. Interestingly, I identified a previously

undescribed 12-amino acid motif within the NRP1 cytoplasmic domain that mediates HH promotion. Future experiments outlined below will help elucidate a role for SEMA ligand binding in NRP and PLXN promotion of HH signaling and improve our understanding of how NRP activity downstream of its novel cytoplasmic motif impacts the HH signaling cascade.

Taken together, the data presented in Chapter 2 provide compelling evidence that NRPs regulate HH signaling and offer mechanistic insight into how NRP1 functions in HH signal transduction. It is clear that NRPs play important roles within and beyond HH signaling, and our understanding of how these molecules behave within different cellular contexts is only beginning to unfold.

4.2 Neuropilins and Hedgehog Signaling: Future Directions

4.2.1 How do Neuropilins Regulate GLI Proteins?

While the data presented in Chapter 2 provide important information about how NRPs function in the HH signaling cascade, the precise mechanism of NRP-mediated regulation of GLI activator function remains unclear. I have narrowed down the HH promoting motif to a region between amino acids 890 and 902 in the cytoplasmic domain of rat NRP1. Notably, this region is highly conserved between vertebrate species and is partially conserved between NRP1 and NRP2. I have ruled out the possibility that phosphorylation of residues within this motif affects downstream signaling by mutating conserved serine and tyrosine residues within this region. However, one could invoke several possible alternative mechanisms:

One possibility is that NRP interacts with an intracellular binding partner to regulate GLI activity. While I cannot exclude the possibility that NRPs interact directly with GLI proteins, NRPs may also act through some known or unknown intermediate to indirectly regulate GLI

activation. Recent evidence suggests that the NRP CD can bind to a number of intracellular molecules, including MYH9, MYH10, DYHC1, FLNA, EF1α1, and ENO1 (Seerapu et al., 2013). One could imagine taking a candidate approach using immunoprecipitation assay to test whether these interactions are maintained in the absence of the 12-amino acid HH promoting motif. Further experiments are required to determine whether these molecules interact with the NRP1 amino acids 890-902, and also whether they intersect with HH signal transduction in some way.

One study has been published proposing that phosphodiesterase 4D (PDE4D) mediates a connection between NRPs and GLI proteins (Ge et al., 2015). PDE4D interacts with the NRP CD and antagonizes PKA, a negative regulator of GLI activity (Ge et al., 2015). However, I present data in Chapter 2 which suggests that NRP can still promote HH signaling when the pathway is activated using a version of GLI2 with the seven main PKA phosphorylation sites mutated to alanine, suggesting that additional mechanisms may be involved. It remains possible that NRP binding to PDE4D could impact PKA-dependent GLI phosphorylation at non-consensus sites found throughout the length of the GLI sequence (Niewiadomski et al., 2014). Alternatively, NRP may affect GSK3β activity, which is also regulated by cAMP downstream of PDE4D (Khaled et al., 2002). Additional mutagenesis experiments are necessary to explore these possibilities.

Perhaps a better strategy to identify HH mediators downstream of NRPs would be to perform an unbiased screen for proteins which interact with the HH promoting motif within the NRP cytoplasmic domain. One way to do this might be to use BioID tagged NRP constructs to distinguish proteins which interact with the full-length version and the version lacking amino acids 890-902 (Roux et al., 2013). Mass spectrometry could then be used to identify possible

candidates, which could be further validated with immunoprecipitation assays and then tested as HH regulators. If binding to a downstream partner indeed mediates HH regulation downstream of NRPs, one might predict that adding the twelve-amino-acid HH promoting motif to a heterologous sequence would be sufficient to confer HH promotion. It is likely that membrane attachment will be required for HH promotion, since a cytosolic version of the NRP1 CD fails to promote HH signaling in NIH-3T3 cells. Therefore, possible candidates for this type of experiment might be proteins which contain a single-pass transmembrane motif but do not regulate HH signaling on their own. This line of investigation could also provide information about whether other regions of the NRP transmembrane domain or CD are important for HH promotion, a possibility we cannot formally exclude with my current results.

Although my data suggest that the NRP1 ECD is not required for HH promotion, the fact remains that NRPs interact with a wide variety of transmembrane and secreted proteins that could modulate NRP function in HH signaling. In addition to SEMA and VEGF ligands, NRPs bind to PIGF-2, heparan sulfate, TGF-β1, HGF, PDGF, FGF, L1-CAM, Plexins, and integrins at the cell surface (Prud'homme and Glinka, 2012). While the assumption is that most of these interactions are mediated through NRP's extracellular domains, the HH-regulating motif within the NRP1 CD could contribute to these interactions, or its activity could be modulated when NRP1 forms complexes with any number of these partners. It is therefore possible that these or other interactions could also contribute to NRP-mediated promotion of HH signaling.

Because NRPs play known roles in SEMA and VEGF signaling (Gu et al., 2002; Gu et al., 2003; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), it is also conceivable that intracellular mechanisms or genomic targets regulated by these pathways might influence GLI activity. I find that the NRP extracellular domains which mediate interactions with SEMA and

VEGF ligands are dispensable for HH promotion. However, another group has shown that the addition of SEMA ligands can enhance NRP-mediated HH promotion (Ge et al., 2015), and I have not directly tested the impact of addition of SEMA or VEGF ligands in our experimental system. I cannot exclude the possibility that specific target genes of these pathways or even a more global cellular response like cytoskeletal disassembly or chromatin regulation could contribute to NRP-mediated HH regulation. NRPs also play important roles in regulating endocytosis (Pang et al., 2014), which could relate to their mechanism in HH signaling. Further experiments involving transcriptional inhibitors or inhibitors of endocytosis are required to investigate these possibilities.

4.2.2 Is Neuropilin Subcellular Localization Important for Hedgehog Regulation?

Another interesting and potentially important aspect of NRP function in HH signal transduction is its subcellular localization. Our findings suggest that NRP1 must be localized to the cell membrane in order to function in HH regulation, since a cytosolic version does not promote HH signaling. It is logical to speculate why NRP membrane localization might be important for signaling at all: Do NRP1 proteins form some sort of cluster within a membrane region that is important for their activity? Does NRP1 form a complex at the cell surface with other proteins through its TM or CD domains? NRP1 homodimerizes and heterodimerizes with NRP2 (Roth et al., 2008; Sawma et al., 2014). However, my data suggest that mutating the dimerization motif within the NRP1 TM domain does not impact its ability to promote HH signaling, contrasting with the important role of NRP dimerization in Semaphorin signaling (Roth et al., 2008). It is possible that the NRP TM dimerization is not required but somehow modulates HH signal transduction. Another possibility is that NRP interacts with other TM

proteins such as VEGF receptors, FGF receptors, PDGF receptors, or Plexins, to regulate HH signaling (Muhl et al., 2017; Prud'homme and Glinka, 2012), although many of these proteins bind to NRP1 through its extracellular domains, which our data indicate are dispensable for NRP1 function in HH promotion. One experiment to address these types of questions might be to replace the NRP1 transmembrane domain with that of an unrelated, single-pass transmembrane protein that is not involved in HH regulation, or perhaps a GPI anchor. This type of experiment would elucidate whether the NRP1 TM is truly required for HH promotion, or whether membrane tethering through another means is sufficient to allow NRP regulation of HH signaling. It would also be interesting to analyze whether NRP1 localization changes in response to addition of HH or SEMA ligand, compared to untreated cells. This could be accomplished, for example, by expressing fluorescently tagged NRP1, perhaps with a C-terminal RFP marker, in NIH-3T3 cells that also contain a GFP or YFP cilia marker and performing confocal super-resolution live cell imaging.

These questions are particularly intriguing given our finding that NRP1 localizes to the primary cilium. Although we find that cilia localization does not correlate with NRP ability to promote HH signaling, other molecules at the ciliary base regulate HH signaling in important ways (Barzi et al., 2010; Tukachinsky et al., 2010; Tuson et al., 2011; Zeng et al., 2010). Interestingly, all of the membrane-tethered NRP constructs we tested were detected broadly throughout the membrane, including at the base of the cilium. Others have shown that reducing NRP expression does not change the amount of GLI accumulation at the tips of primary cilia (Hillman et al., 2011), suggesting that NRPs at the ciliary base might regulate GLI proteins after they have been processed in the cilium. One could imagine a mechanism by which NRPs interfere with GLI activator degradation after ciliary processing. It would be useful to test

whether NRPs affect the accumulation of GLI activator in cells, perhaps using quantitative western blot methods. Alternatively, GSK3β phosphorylates GLI proteins after they have cycled through the cilium (Hui and Angers, 2011). Similar to our approach with PKA phosphorylation in Chapter 2, one could mutate GSK3β phosphorylation sites on GLI proteins and assess whether NRP can promote HH when co-transfected into cells with these modified GLI activators. NRPs also regulate endocytosis (Pang et al., 2014). It would also be interesting to inhibit its ability to do so, to test what role NRP-mediated endocytosis plays in regulating HH signaling. This approach may be tricky, since HH signaling and many other cellular processes require endocytosis. However, many types of endocytosis inhibitors exist (Dutta and Donaldson, 2012), so if one agent specifically blocked NRP-mediated endocytosis, or if the NRP sequences which mediate endocytosis were mutated or deleted, one could begin to address this role of endocytosis in NRP-mediated HH promotion.

If NRP1 localization to primary cilia is not required for HH signaling, why does it traffic there? NRP1 cilia localization may play other important roles in the cell independent of HH regulation. For example, it could be binding to another protein that requires cilia localization for its function, or regulating a different cellular process altogether, such as angiogenesis or endocytosis. In addition, a role for NRP1 cilia localization may be important for Semaphorin signaling. One aspect to consider is that NRP2, unlike NRP1, is not enriched in cilia. As discussed in Chapter 2, NRP1 cilia localization could be important for regulating angiogenesis in arterial endothelial cells, which rely on mechanosensory cilia for homeostasis (Nauli et al., 2011; Prud'homme and Glinka, 2012). Another possibility is that the same mechanisms which allow NRP entry into dendritic spines and axon terminals mediate their cilia localization, which may or may not impact NRP function in HH signaling or other pathways.

4.2.3 Broader Consequences of Neuropilin Regulation of Hedgehog In Vivo

While several studies suggest the importance of NRP-mediated HH signal promotion *in vivo* (Ge et al., 2015; Hayden Gephart et al., 2013; Hillman et al., 2011), this is an area with potential for much more extensive exploration. Given that the NRP1 CD is required for HH promotion (Ge et al., 2015), one intriguing experiment would be to generate mice lacking both the NRP1 CD and the NRP2 CD. Previous groups have deleted the NRP1 CD alone, noting defects in vascular permeability (Roth et al., 2016). However, NRP2 also promotes HH signaling (Ge et al., 2015; Hayden Gephart et al., 2013; Hillman et al., 2011), suggesting the potential for functional redundancy between NRP1 and NRP2 in this model. One approach might be to design CRISPR guides to delete the NRP2 CD in the mice which lack the NRP1 CD, since they are viable and fertile (Roth et al., 2016). One could then analyze any effects on HH-dependent tissues at critical times during mouse embryonic development.

4.3 Plexins and Hedgehog Signaling: Summary of Key Findings

While NRPs have been identified as HH regulators (Ge et al., 2015; Hillman et al., 2011; Pinskey et al., 2017), a role for PLXNs in HH transduction remains unexplored. In Chapter 3, I provide evidence that multiple PLXN family members promote HH signaling in NIH-3T3 cells. My data suggest that, similar to NRPs, the PLXN CD and TM are required for HH promotion. Also, similar to NRPs, my data suggest that PLXNs regulate HH signaling at the level of GLI activator function.

Within its CD, PLXNs contain a split GTPase activating protein, or GAP domain, which regulates the activity of small intracellular GTPases (Puschel, 2007). To investigate the

requirement for GAP activity in PLXN-mediated HH promotion, I mutated key residues within the PLXN GAP domain (Rohm et al., 2000). My data suggest that GAP enzymatic activity is required for PLXN-mediated HH promotion. Furthermore, constitutive activation of the GAP domain (and possibly other signaling pathways) through the deletion of autoinhibitory extracellular domains results in amplified HH pathway activity, suggesting that decreasing or increasing PLXN GAP activity correlates with the level of HH signaling (Takahashi and Strittmatter, 2001). Interestingly, tyrosine phosphorylation by FYN kinases is also required for HH promotion, raising the possibility that multiple regions of the PLXN CD might mediate HH signaling. Many questions remain, however, about how these regions modulate GLI function.

4.4 Plexins and Hedgehog Signaling: Future Directions

4.4.1 Do all Plexins Promote Hedgehog Signaling?

The nine different PLXN family members can be divided into four subfamilies: A, B, C, and D (Neufeld and Kessler, 2008). One common feature of PLXN family members is that they all contain a cytoplasmic GAP domain (Neufeld and Kessler, 2008). I found that GAP activity correlates with HH promotion, raising the question: do all PLXN family members promote HH signaling? Initially, I focused on the A subfamily of PLXN proteins, since they interact with NRP receptors to transduce signals from secreted SEMA ligands (Neufeld and Kessler, 2008). I showed that all members of the A subfamily (PLXNA1, PLXNA2, PLXNA3, and PLXNA4) robustly promote HH signaling. Surprisingly, I found that PLXNB2, which does not interact with NRPs, can also promote HH signaling. To further understand which PLXNs are involved in HH regulation, I will need to compare the ability of the remaining PLXNS (B1, B3, C1, D1) to promote HH signaling in functional assays. Because we show that PLXN GAP activity mediates

HH promotion, and because all of the PLXN family members contain cytoplasmic GAP domains, I would predict that these additional constructs can also promote HH signaling.

Another interesting question to explore is whether GAP domain-containing proteins that do not belong to the PLXN family can promote HH signaling. One way to address this question would be to overexpress other GAP proteins and analyze their effects on HH signaling. By choosing GAPs that are specific for certain small intracellular G proteins, this could also provide insight into which of the many G proteins regulated by PLXN activity might intersect with HH transduction. One caveat with this experiment, however, is that it would be difficult to control for binding specificity and overall activity levels of other GAPs, which could complicate the interpretation of the results. Another caveat is that PLXNs act uniquely at the plasma membrane, whereas most GAPs are cytosolic (Neufeld and Kessler, 2008). To further explore the importance of membrane localization in PLXN regulation of HH signaling, one could: 1) express a cytosolic version of the PLXN CD and assess its ability to promote HH, and/or 2) tether a different GAP protein to the membrane by adding the PLXN TM domain to ask whether any membrane-bound GAP activity is sufficient to promote HH signaling. If GAP activity from other proteins is unable to promote HH signaling, one interpretation might be that HH regulation requires one or more of the various other activities and processes regulated by the PLXN CD. Alternatively, one could utilize Rho and Ras activity assays to better understand G protein regulation downstream of cytosolic and membrane-bound PLXNs and how this correlates with HH pathway activity.

4.4.2 How do Plexins Promote Hedgehog Signaling?

While my data suggest that PLXNs promote HH signaling through their cytoplasmic GAP activity at the level of GLI activator, the precise mechanism of PLXN action in HH signal transduction remains unclear. One question is whether GAP activity is the only part of the PLXN CD that regulates HH signal transduction. In addition to manipulations to the GAP region, I found that mutating key tyrosines phosphorylated by Fyn kinase (St Clair et al., 2018) also blocks PLXN promotion of HH signaling. One of these tyrosines lies within the PLXN GAP domain, while the other is outside of the GAP region (St Clair et al., 2018). An unanswered question is whether these tyrosine mutations simply disrupt GAP activity, or whether they may function independently of GAP regulation. Most functional assays for PLXN activity test for cytoskeletal collapse, but teasing apart the cytoplasmic activities downstream of arginines within the GAP domains vs tyrosines phosphorylated by Fyn kinases will require a more sensitive and specific approach, perhaps measuring GTP-bound vs GDP-bound RAS and RAP. This will establish a framework to decipher whether GAP activity can be distinguished from Fyn kinase phosphorylation and provide further insight into how PLXN cytoplasmic activity mediates the HH response.

Downstream of PLXNs, another obvious question is where does GAP regulation or other cytoplasmic activity intersect with HH signal transduction? My data suggest that PLXNs may regulate GLI activator, but how this regulation is achieved remains completely unexplored. One possibility is that PLXN GAP activity indirectly impacts GLI function through one of its many downstream effectors. However, exactly which intracellular PLXN effectors mediate HH promotion will be complicated to dissect.

Considering this question from the standpoint of GLI regulation, several possible candidates can be identified that may intersect with HH signal transduction downstream of PLXN cytoplasmic activity. As previously discussed, GLI proteins are regulated by PKA, GSK3β, and CK1 phosphorylation (Pan et al., 2006; Pan and Wang, 2007; Pan et al., 2009; Tempe et al., 2006; Wang et al., 2000; Wang and Li, 2006). Therefore, PLXNs could regulate GLI proteins by mediating the activity of one or more of these kinases. Interestingly, SEMA signaling through PLXNs requires activation of many of these same proteins. For example, SEMA3A activates GSK3β at the leading edge of growth cones, which in turn phosphorylates key microtubule regulatory proteins and is essential for radial cell migration and dendritic orientation in the developing cortex (Eickholt et al., 2002; Morgan-Smith et al., 2014; Uchida et al., 2005). In addition, SEMA3A signaling induces both anterograde and retrograde axonal transport in dorsal root ganglia neurons via kinesins and dyneins, whose motor function in axons is controlled by GSK3, protein phosphatase1 (PP1), And Cdk5 (Goshima et al., 1999; Goshima et al., 1997; Morfini et al., 2004; Niethammer et al., 2000). Therefore, PLXNs may regulate GLI phosphorylation or their intracellular transport via kinesins and dyneins – a possibility that could be potentially be explored through existing collaborations here at the University of Michigan.

Another important negative regulator of GLI function is SUFU, which binds to and inhibits GLI proteins and regulates their processing within the primary cilium (Ding et al., 1999; Svard et al., 2006). One could therefore imagine PLXN proteins interrupting GLI/SUFU interactions, or perhaps influencing their trafficking relative to the primary cilium to modulate HH signaling. Future experiments could address these possibilities by testing whether PLXN overexpression, constitutive activation, or deletion impacts GLI-SUFU interactions using immunoprecipitation, FRET, or some other approach. Another interesting experiment would be

to visualize fluorescently tagged SUFU and GLI proteins within cells and observe how their distribution changes with HH activation, PLXN overexpression, or a combination of the two.

While connections to GLI regulation are just beginning to be explored, another possibility is that small intracellular GTPases downstream of PLXN GAP activity influence HH signal transduction. As discussed in Chapter 1, G-proteins downstream of PLXN GAP domains play a number of important roles within the cell. While it remains unclear how G-proteins may influence GLI regulation, several studies have focused on how HH signaling may influence G-protein activity. Interestingly, SHH can stimulate small Rho GTPases, including Rac1 and RhoA, through SMO interactions with G_i family G-proteins to regulate fibroblast migration (Polizio et al., 2011; Riobo et al., 2006). Strikingly, this process is not dependent upon GLI transcriptional activity (Polizio et al., 2011). These studies raise the question: could SMO act together with PLXNs to signal in this non-canonical way? One could imagine that PLXN binding to SMO could mitigate PTCH inhibition, or somehow change SMO regulation of GLI activity to favor HH transcription. This could be an exciting area to explore with future experiments.

It is important to consider that small intracellular GTPases downstream of PLXN GAP regulation induce cytoskeletal collapse via integrin signaling and disassembly of focal adhesions (Bos and Pannekoek, 2012; Kinbara et al., 2003; Oinuma et al., 2004; Serini et al., 2003; Wang et al., 2012). Therefore, global cytoskeletal instability may mediate PLXN-dependent HH promotion. Interestingly, however, transfecting a constitutively active form of PLXN that robustly causes cytoskeletal collapse ($PLXNA1^{\Delta ECD}$) fails to induce HH signaling on its own without another means of HH pathway induction. One experiment to examine the role of cytoskeletal instability in HH regulation might be to add different types of cytoskeletal disruptors, such as nocodazole or cytochalasin, for varying amounts of time and assess HH

signaling using our NIH-3T3 luciferase reporter assay. One might predict that cytoskeletal disruption might impact multiple signaling pathways simultaneously. Therefore, it will be important to include other pathway reporters as controls for these experiments. My very preliminary results using a WNT luciferase reporter (courtesy of Dr. Ken Cadigan) suggest that PLXNA1$^{\Delta ECD}$ does not strongly promote WNT signaling as it does HH (Figure 4-1). In one experiment, PLXNA1$^{\Delta ECD}$ promotes WNT signaling when stimulated with 12.5 ng of β–catenin (Figure 4-1). However, another experiment using 20 ng of β–catenin shows that PLXNA1$^{\Delta ECD}$ significantly inhibits WNT signaling (Figure 4-1). Notably, across experiments I only see about a five-fold induction of WNT signaling, and quite a bit of variation exists between even pCIG-transfected cells treated with 20 ng of β–catenin (Figure 4-1). These experiments should be further optimized, and these results will need to be repeated and confirmed before making any conclusions.

If PLXN-mediated cytoskeletal collapse indeed affects HH signaling specifically, an important aspect to consider is the specific requirement for primary cilia in vertebrate HH signaling. Similar to neuronal projections in some ways, cilia are microtubule-based structures, and may therefore be very sensitive to PLXN-mediated cytoskeletal disruptions. Thus, PLXN GAP activation may mediate GLI processing in the primary cilium. While one might predict that disruption of primary cilia may result in a decrease in HH signaling, the opposite has been found to be true, due to the inability of GLI3 to be processed into its repressor form (Wong et al., 2009). Therefore, PLXN-mediated cytoskeletal disruption could be impacting primary cilia in a way that boosts HH target gene expression. Interestingly, my preliminary data suggest that PLXNA1 is not enriched in the primary cilium. However, the effect of PLXNs on cilia length and morphology have not been explored, and how PLXNs regulate GLI processing is completely

unknown. Fixed or live cell imaging with stained or fluorescently tagged GLI constructs and cilia markers could be used to investigate these questions.

Yet another aspect to consider is the possibility for interactions between PLXNs and additional proteins at the cell surface to play some role in HH regulation. Similar to NRPs, PLXNs interact with a variety of cell surface proteins, including tyrosine kinases Met and ERBB2, and Off-track receptors (Giordano et al., 2002; Winberg et al., 2001). Currently, it is unknown whether PLXNs bind directly to any of the HH pathway components or cell-surface regulators. While I cannot formally exclude the possibility that each PLXN or PLXN subfamily regulates HH signaling via a unique mechanism, it is more likely that PLXNs from the A and B subfamilies converge upon a similar regulatory process. Therefore, focusing on interacting proteins and intracellular mechanisms that are shared between PLXN subfamilies will be useful moving forward.

4.4.3 Do Plexins Regulate Hedgehog Signaling In Vivo?

PLXNs strongly regulate HH signaling in NIH-3T3 fibroblasts, raising interesting possibilities about their potential roles in other cell types and tissues *in vivo*. Unfortunately, functional redundancy between the nine members of the PLXN family vastly complicates loss of function analyses, particularly given our finding that PLXNs from multiple subfamilies can regulate HH signaling. A first step toward an *in vivo* loss of function approach might be to carefully characterize PLXN expression at different stages in mouse development using in whole mount and section in situ hybridization and/or antibody staining. While this will be a significant amount of work, a more comprehensive analysis of PLXN expression will help identify areas that may express fewer PLXNs, making genetic deletion experiments much more feasible.

Particular tissues that may be interesting to consider in light of their reliance on HH signaling during development include the neural tube, limb buds, intestines, and forebrain/craniofacial tissues, although many additional tissues rely on HH signaling for patterning.

Another approach to consider might be designing CRISPR guides targeting the GAP domains of multiple (or potentially all) of the PLXN family members. Generating and validating mice with so many deletions would be challenging to say the least, but with rapid advances in CRISPR technology, this approach may be feasible in embryonic stem cells (ES cells) or immortalized cell lines. ES cells can be cultured and differentiated into multiple lineages, which may provide insight into PLXN regulation of HH signaling in different tissues should appropriate mouse models be inaccessible. My initial attempts using CRISPR to delete PLXNA1 and PLXNB2 in NIH-3T3 cells have highlighted some of the challenges of this approach, including screening for effective guides, screening for cells in which the intended deletion has taken place, and designing experimental methodologies that will allow us to control for off-target effects. However, successfully deleting PLXNs using CRISPR will dramatically improve our understanding of PLXN function in HH signal transduction, and could also be applied to HH-dependent cancer cell models. Alternate approaches may be to design a small-molecule GAP domain inhibitor that would simultaneously constrain all PLXN activity within a given cell, or perhaps generate antibodies against a conserved region within the extracellular SEMA domain shared between PLXN family members.

4.5 Neuropilins and Plexins Within the Broader Signaling Landscape

Considering NRP and PLXN cooperation in SEMA signaling raises many important questions about their function in HH signal transduction. Do NRPs and PLXNs work together to

regulate HH signaling? Do they converge on the same downstream mechanism to achieve GLI regulation, or do they function through completely separate cascades? As discussed in Chapter 3, these possibilities are not mutually exclusive. To further define the contributions of different SEMA receptors in more detail, it will be important to generate HH-responsive cell lines lacking either PLXNs or NRPs. While CRISPR experiments with PLXN proteins are challenging based on the sheer number of PLXN family members, generating *Nrp*$^{-/-}$ cell lines may be much more feasible, considering that only two deletions would be required (NRP1 and NRP2). Several studies suggest that loss of NRP1 and NRP2 result in a severe reduction in HH signaling (Ge et al., 2015; Hillman et al., 2011). A HH-responsive *Nrp*$^{-/-}$ cell line would therefore allow for important rescue experiments with NRP deletion constructs to confirm and extend my findings in Chapter 2 and also allow me to determine whether PLXNs require endogenous NRPs to promote HH signaling. Experiments like these will provide an improved understanding of NRP and PLXN roles and contributions to HH regulation.

Another question that arises from the discovery that both NRPs and PLXNs promote HH signaling is whether SEMA ligand is involved in this process. As discussed in Chapter 3, SEMA ligand activation of PLXN GAP activity may contribute to increased HH signaling, although several lines of evidence suggest that SEMAs are not required for NRP-mediated HH promotion. Again, here a *Nrp*$^{-/-}$ or *Plxn*$^{-/-}$ HH-responsive cell line would be a useful tool to investigate SEMA ligand requirements, as the presence of endogenous NRPs and PLXNs complicates existing studies. Even so, this is a tricky question to address, as many variables, including the level of NRP or PLXN overexpression, the amount and duration of SEMA ligand added to the system, as well as the sensitivity of the assay could all impact the results. In this case, modulating levels of SEMA ligand *in vivo* and analyzing the impact on HH target gene

expression using qRT-PCR or other methods may be useful, in that NRPs and PLXNs will be present at endogenous levels.

Many additional questions remain when considering the broader implications of this work. Do NRPs and PLXNs promote HH signaling in other cell types beside NIH-3T3s? Do NRPs and PLXNs influence other developmental signaling pathways, or is their regulation specific to HH? What about other axon guidance pathways like Ephrins, Netrins, and Slits – do they also influence HH signaling? Are there differences between repulsive and attractive axon guidance cues that determine their effects on HH or other developmental signaling pathways? What factors determine whether a NRP or PLXN receptor acts within the HH signaling cascade or the SEMA signaling cascade, or are they able to do both simultaneously? In the words of Ramón y Cajal, "It is fair to say that, in general, no problems have been exhausted; instead, men [and women] have been exhausted by the problems" (Ramón y Cajal, 1999). It is my hope that fresh talent approaching these questions without prejudice will see many new possibilities.

4.6 Figures

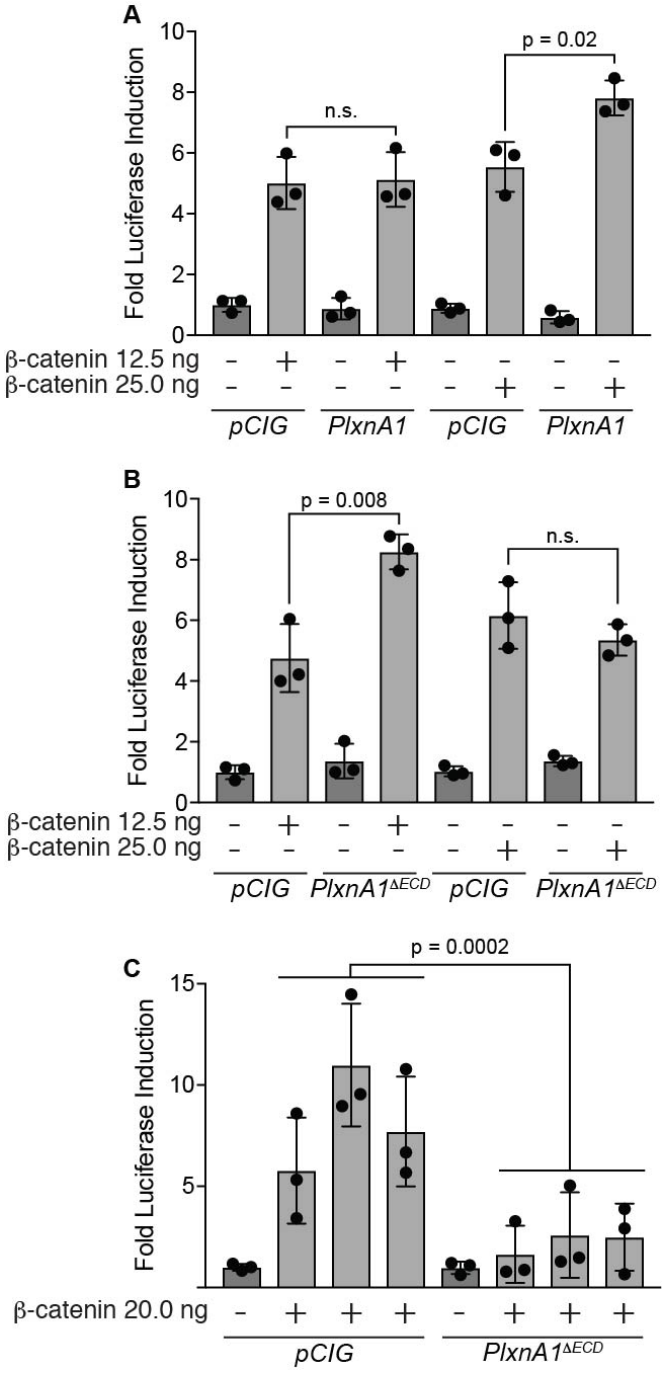

Figure 4-1. Plexin-A1 and Plexin-A1 lacking its extracellular domain do not consistently promote WNT signaling.
WNT-dependent luciferase reporter activity measured in NIH-3T3 cells transfected with the indicated constructs and stimulated with β-catenin. Data are reported as mean -fold induction ± S.D., with *p* values calculated using two-tailed Student's *t* test. *n.s.*, not significant.

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Appendix 1:
Developing Future Biologists: Creating and Assessing a Portable Short Course to Engage Underrepresented Undergraduate Students in Developmental Biology

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Abstract:

Many barriers discourage underrepresented students from pursuing science careers. To access graduate education, undergraduate students must first gain exposure to a particular subject and subsequently accumulate related coursework and research experience. Many underrepresented students lack exposure to developmental biology due to limited undergraduate course offerings and finite resources at smaller institutions. To address this disparity, a group of University of Michigan graduate students and postdoctoral fellows created a portable short course focusing on developmental biology, titled "Developing Future Biologists" (DFB). This weeklong educational initiative provides hands-on laboratory sessions, interactive lectures, and professional development workshops to teach students about developmental biology and increase awareness of scientific career options. To evaluate course effectiveness, we developed a pre-post assessment, incorporating main ideas from the BioCore Guide. Student understanding of basic concepts and perceived experience in developmental biology increased in DFB participants, despite the abbreviated nature of the course. Here, we provide all course materials and an in-depth analysis of the assessment we created. The DFB portable short course model is an easily adaptable tool that connects undergraduate students with opportunities for advanced study and lowers barriers for underrepresented students in science, technology, engineering, and mathematics.

Introduction:

Similar to other science, technology, engineering, and mathematics (STEM) fields, developmental biology trainees do not proportionally represent the diversity of our nation (NSF (National Science Foundation), 2015). Many barriers contribute to this lack of diversity, including limited opportunities to partake in relevant science coursework and gain research experience (Hurtado et al., 2010). It is widely accepted that increased diversity enhances graduate student training and development through the integration of a variety of cultural perspectives (Aguilera, 2012). For outstanding students from all backgrounds to join the developmental biology community, however, they need to be made aware of opportunities in science and develop a passion for this exciting field.

To address this issue, a team of University of Michigan graduate students and postdoctoral fellows created Developing Future Biologists (DFB), an educational initiative designed to lower the cultural barriers to graduate education, increase awareness of science careers, and teach students core concepts of developmental biology. The program centers on a weeklong short course that includes developmental biology instruction, hands-on laboratory exercises, professional development activities, and networking sessions. A main focus of the course is to incorporate active learning strategies, which enhance student learning in STEM fields (Freeman et al., 2014; Haak et al., 2011). Additionally, the course aimed to assist students from a variety of backgrounds build long-term mentoring relationships with University of Michigan graduate students, postdoctoral fellows, and faculty members. Previous research suggests that similar mentoring efforts have helped students succeed in scientific endeavors (Tsui, 2007). Importantly, DFB was designed to be portable and scalable so that future iterations could be adaptable to a wide variety of subjects and locations.

In May of 2015, our team implemented the first DFB course in Ponce, Puerto Rico, where several of our instructors had completed undergraduate studies in biology and noticed a need for developmental biology instruction. Only three of the ten University of Puerto Rico (UPR) undergraduate campuses offer a developmental biology course on a regular basis, with only two of the three offering laboratory-based instruction. Therefore, after a successful pilot program focused on UPR Ponce students, we returned to Puerto Rico in 2016, opening the application to undergraduate students from all UPR campuses and providing room and board for students from other cities. To accomplish this, we compiled a variety of external and internal funding from sources such as the Society for Developmental Biology Non-SDB Educational Activities Grant, American Society for Cell Biology Committee for Postdocs and Students Outreach Grant, the Department of Cell and Developmental Biology at the University of Michigan, and the Rackham Graduate School Dean's Strategic Initiative at the University of Michigan, among others (see Acknowledgements). In addition, we offered a local iteration of the course to underrepresented undergraduate students from the state of Michigan in 2017, allowing us to validate our assessment on a second student demographic.

Since 2010, the Vision and Change Call to Action has been instrumental in guiding innovations in biology education (AAAS, 2010). The DFB course model incorporates many aspects of Vision and Change, including integrating core concepts into the curriculum, focusing on student-centered learning through active participation, and encouraging students and professors from the University of Michigan to embrace high quality, innovative teaching methods. To gauge the effectiveness of the course and measure students' grasp of core concepts in developmental biology, we developed and incorporated formal pre-post assessments using the BioCore guide's interpretation of the Vision and Change core concepts in Biology (Brownell et

al., 2014). To assess student attitudes, we also included a pre-post survey asking students to self-rate their experience in developmental biology, their interest in graduate school, and their awareness of science career options.

The purpose of this report is two-fold: 1) To present data addressing the effectiveness of the 2016 and 2017 DFB course iterations, and 2) To provide resources for the development of similar initiatives. Throughout the course, our goals were to build relationships with students, improve attitudes about graduate education and scientific careers, and effectively teach students the core concepts of developmental biology. Here, we present our course design, teaching materials and assessment data, demonstrating that this short course model can enhance student understanding and perceived experience in developmental biology.

Course Development and Methods:

Instructor Selection and Preparation

Graduate student, postdoctoral fellow, and faculty instructors for the DFB course were selected eight months before the course start dates. Faculty instructors were selected based on involvement with undergraduate and graduate education and were invited to participate via email. All other instructors submitted a cover letter and curriculum vitae, and selected applicants were interviewed. Instructors were selected based on teaching experience, interest in social justice/inclusion, and ability to commit two years to the program. A two-year commitment was required to help with turnover and ensure the continuation of the initiative. Over the course of the academic year, instructors met weekly to develop the course curriculum, design lab activities, create the course applications and advertisements, design assessments, review applications,

practice lab instruction, and perform other tasks related to creating this course. Meeting notes, as well as all other materials created for the course, were organized and stored on a shared drive.

Student Applications & Selection

Approximately three months before the course start date, advertisements were sent out via email, flyers (Figure A1-S1), the course Facebook page (https://www.facebook.com/developingfuturebiologists), and the course website (http://developingfuturebiologists.com). Applications, which were designed using Google Forms and linked through the course website, were open for one month. The course was restricted to 24 students due to equipment limitations and to maximize the personal interactions among enrolled students and DFB instructors. Students were selected for participation based on grade point average (minimum of 2.8 on a 4.0 scale) year in college, major, career goals, and reason for course interest. While senior students participated in the course, we sought to accept a larger portion of first and second year students based on previous studies demonstrating that early research experiences increase continued participation in the sciences, particularly for students from underrepresented minority groups (Nagda B.A., 1998; Rodenbusch et al., 2016). Preference was given to students who communicated enthusiasm and a clear personal benefit from participation in DFB.

Lectures and Labs

One of our main goals in developing this course was to teach students the core concepts of developmental biology. To achieve this objective, we developed interactive, discussion-based lectures and hands-on laboratories surrounding main ideas and experimental techniques in

developmental biology. Each day focused on a single theme, including early embryonic development, cell signaling, gene expression, organogenesis, and development and disease. Instructional sessions were held daily from 9:00 AM to 5:00 PM, with discussion-based lectures in the morning, interactive labs in the afternoon, and occasional evening networking activities (Figure A1-S2).

Prior research indicates that courses with hands-on activities, such as labs, increase enthusiasm and learning in students (Basey et al., 2014). Therefore, our course was designed to focus on lab activities, with discussion-based lectures serving to introduce core content that was later incorporated into laboratory material. Experienced faculty, postdocs, and graduate students led discussions, and slide presentations were combined with active-based learning to encourage student involvement. For example, one discussion session included reenactment of the Wnt cellular signaling pathway, where students acted out the functions of specific pathway components. In addition, the use of iClicker remotes allowed instructors to pose questions throughout the lecture to further engage students in the material (Caldwell, 2007; Crossgrove and Curran, 2008).

Afternoon labs served as the main hands-on component of the course, with the objective of exposing students to basic research methods and tools used in the field of developmental biology. Instruction in basic laboratory safety and record keeping was provided prior to lab participation. Due to space limitations in 2016, the course was designed to have two lab sessions covering the same material each afternoon; half of the students attended lab, the other students attended professional development sessions, and then the groups switched sessions after 90 minutes. This format was kept in 2017, as positive feedback from 2016 professional development sessions demonstrated a strong need for such instruction. Each lab session utilized

materials from common model organisms, including worms, frogs, flies, chickens, and mice. To guide participation, students were provided a lab workbook containing background information, experimental protocols, questions about each specific exercise, and space for students to record their observations (workbook available upon request. Please email jpinskey@umich.edu). Workbooks were not graded, and students were allowed to keep their workbooks following the conclusion of the course.

Professional Development for Participants

The importance of mentoring in underrepresented student success is well established (Nagda B.A., 1998; Villarejo et al., 2008; Whittaker and Montgomery, 2012). To facilitate mentoring relationships between DFB instructors and participants, students were split into groups and assigned two team leaders from the University of Michigan. The team leaders mentored their assigned students both during the course and after its conclusion. To encourage bonding within these teams, friendly competitions including questions about course content and lab-based challenges were held throughout the week for small prizes. In addition to the team assignments, networking activities at which students could informally interact with their assigned mentors as well as other instructors were held. These included an ice cream social, a bowling night, dinner with additional faculty from outside of the course, and an end-of-course networking dinner. These events created a welcoming environment for undergraduates to speak with graduate students, postdocs, and faculty, encouraging the formation of meaningful mentoring relationships.

We further aimed to help students envision themselves as researchers and learn about career opportunities by incorporating a series of career development and informational sessions.

Topics included *curriculum vitae* review, effective networking skills, interview skills, presentation skills, and program-based opportunities offered at institutions like the University of Michigan (Figure A1-S2). Additionally, career panels were incorporated into the course: one with current graduate students, and one with and faculty/postdoctoral instructors. The panels began with introductory statements from each member describing their personal scientific career paths, and proceeded with questions focused on careers in research. To allow students to gain further perspective on the types of projects and scope of work done by graduate students, the graduate student instructors gave short research talks about their specific projects daily during lunch.

Assessment tools and statistical analyses

Previous reports have outlined the major concepts in biology, including an adaptation for developmental biology in particular (Brownell et al., 2014; Rossi et al., 2013). Using these as a guide, we created pre-post assessments to evaluate student understanding of core concepts in developmental biology as well as lab techniques used throughout the course (Figure A1-1). Questions corresponded to each of the major topics of the course, each with five possible answers (Assessments available upon request, please email jpinskey@umich.edu). Question order was randomized from pre- to post-test to avoid memorization of the questions. A score of 0 and 1 was assigned for each incorrect and correct answer, respectively. Additionally, five items on the pre-post examination were included to ascertain students' general perspectives about scientific careers and developmental biology. Each answer was given a value of 0-4 with 0 corresponding to least familiar/positive about the question topic and 4 being most familiar/positive about the question topic. All values are reported as mean ± standard deviation.

Normality of the data were tested with D'Agostino & Pearson normality tests, and statistical comparisons between pre-test and post-test results were made using paired two-tailed *t*-tests, Wilcoxon matched-pairs tests, or one-way ANOVA with Sidak's *post hoc* test as dictated by design and data distribution. Importantly, all DFB instructors completed a training certification for research in human subjects through the Program for Education and Evaluation in Responsible Research and Scholarship (PEERRS) at the University of Michigan, and this study was formally exempt from ongoing Institutional Review Board (IRB) review. Furthermore, participants were provided the option of signing a release form for photography and videography taken during the course.

Feedback for the course was collected on a combination of iClicker data, pre-post tests, and written feedback surveys. Student feedback was collected for each lecture and lab session at its conclusion, via a standard series of iClicker questions and a written feedback survey (Survey available upon request, please email jpinskey@umich.edu). To gain qualitative insight into the students' experiences, we also encouraged open-ended reflections on the back of the course evaluations.

Results:

Student Understanding of Core Concepts Improved over the Course of the Week

To measure understanding of core concepts in the field of developmental biology, we analyzed scores from pre- and post-test questions addressing our five content areas (Figure A1-2). In 2016, students' background knowledge varied widely, with pre-assessment scores ranging from 17-50% (4 to 12 correct out of 24) and an average score of 33% (8 correct out of 24). Student background knowledge prior to the course in 2017 was similar, with pre-assessment

scores ranging from 13-60% (4 to 18 correct out of 30) and average score of 36% (11 correct out of 30). Excitingly, for both course iterations, cumulative student understanding of the core concepts in developmental biology improved significantly following instruction (Figure A1-2A). Post-test scores rose from an average of 33% to 57% (raw score of 7.9±2.7 to 13.8±3.7, $p<0.0001$) in 2016 and from an average of 36% to 66% (raw score of 10.9±3.7 to 19.9±4.6, $p<0.0001$) in 2017. Overall, 22 out of 24 students in 2016 and all 15 students in 2017 improved their scores on the post-assessment, with increases ranging from an additional 1 to 10 points out of 24 in 2016, and 3 to 22 out of 30 in 2017 (Figure A1-2B). In 2016, a single student's test score decreased (from 11 to 8 of 24 points), and one did not change (7 of 24 points on both assessments). The average percent improvement per individual was 24% in 2016 and 30% in 2017. Accordingly, the distribution of test scores shifted to more positive values on the post-test (Figure A1-2 C-D). Together, these results suggest an improved overall understanding of core developmental biology concepts after participation in DFB.

In addition to the overall increase in test score, it was important to investigate whether a net improvement existed for each of the individual concept areas. Each test was divided into an equal number of questions assigned to each of the five concepts and a section on lab based techniques, with question order randomized. To analyze content-specific changes, questions were sorted and changes in pre-post scores were examined for each concept section (Figure A1-3). Understanding of all concepts improved during both iterations of the course (two-tailed t-test, $p<0.05$), with some concepts improving more than others. In 2016, organogenesis improved the most (40% average increase in score for that concept), followed by early embryo (32%), techniques (27%), development & disease and cell signaling (18% each), and finally gene expression (10%). In 2017, questions covering techniques improved the most (41%), followed by

early embryo (40%), organogenesis (31%), development & disease (25%), cell signaling (24%), and finally gene expression (19%). These results suggest that intervention was successful for student learning each day of the course.

Although scores improved for the test as a whole as well as for each individual concept area, post-assessment score in 2016 was still only 57%. While conducting our initial analysis of the 2016 data, we observed that some items on the 2016 assessment were simply not covered during the course. In some cases, instructors changed their lecture content after the assessments had been designed, whereas in other cases, instructors simply ran out of time to discuss all of their material. To address this consideration, we asked instructors to self-rate coverage of each assessment item relevant to their topic on a numerical scale. After receiving all instructor analyses, questions were divided into two equally-sized groups, labeled "less instructor coverage" and "more instructor coverage". As predicted, the topics that were covered more in depth or by multiple instructors had better learning outcomes than those topics that were not as well-discussed (Figure A1-4). We found that, while the "more covered" portion and the "whole" test improved from pre to post, there was no improvement in the "less covered" group of questions (percent correct mean±SD, less covered pre 37.9±14.7, post 46.5±15.5, more covered pre 27.8±16.2, post 68.4±20.0, whole test pre 32.8±11.1, post 56.9±15.2, $p=0.162$ less covered, $p<0.0001$ more covered and whole test, one-way repeated measures ANOVA/Sidak). These results demonstrate that in 2016, students improved to a greater extent on the material that the course rigorously covered than on material that was less-discussed. These findings were taken into consideration during planning of the 2017 course, leading to improvement of post-assessment scores from an average of 57% in 2016 to an average of 66% in 2017.

Item Analysis Provided Recommendations for Assessment Improvement

To measure the validity and effectiveness of our assessments, we performed item analyses for difficulty for both the 2016 and 2017 pre-post exams (Tables 1 and 2). Item difficulty measures the proportion of students who answer an item correctly (Allen and Yen, 2002). Therefore, a higher difficulty score signifies that an item was "easier", because a higher percentage of students answered that item correctly. Importantly, overall difficulty scores increased from pre to post for both years, indicating that more students answered post-test items correctly following instruction (Tables 1 and 2). In 2016, 5 out of 24 items on the pre-assessment (21%) had difficulty scores above 0.5, with an overall average difficulty of 0.33, compared to 15 out of 24 items on the post-assessment (63%), which had an overall average difficulty of 0.57 (Table 1). Similar trends were observed in 2017, with 6 out of 30 items above 0.5 on the pre-test (20%) with an average score of 0.36, compared to 25 out of 30 items on the post-test (83%), which had an overall average difficulty of 0.66 (Table 2). Several questions had difficulty scores that decreased, including items 4, 19, and 24 in 2016 and items 4 and 10 in 2017 (Tables 1 and 2). These items should be revised in future assessments.

In addition, item discrimination was used to measure how well each assessment question distinguished between high- and low-performing students (Allen and Yen, 2002). Discrimination scores below 0.2 reflected the need for item revision. Interestingly, in 2016, the pre-test had 10 out of 24 questions (42%) adequately discriminating between low and high performing students, while the post-test displayed an increase in discrimination scores, with 18 out of 24 items (75%) above 0.2 (Table 1). Mean discrimination improved from 0.16 on the pre-assessment to 0.37 on the post assessment (Table 1). In 2017, however, both the pre- and post-assessments contained more questions with discrimination scores above 0.2: 22 out of 30 items on the pre-assessment

(73%) and 25 out of 30 questions on the post-assessment (83%) (Table 2). In addition, mean discrimination improved for the 2017 pre-assessment (from 0.16 to 0.29), whereas the post-test discrimination mean remained unchanged at 0.37 (Table 2, c.f. Table 1). Six items in 2016 showed decreased discrimination from pre to post (1, 9, 14, 20, 21, and 22), whereas eight items decreased in 2017 (3, 5, 8, 13, 16, 26, 27, and 29). Any item with a discrimination score below 0.2 on the post-test should be considered for revision. Together, these item statistics will guide improvements to the pre-post assessment for future iterations of the course.

Lastly, we used the Blooming Biology Tool to categorize assessment items depending on required cognitive domains (Crowe et al., 2008). Because the course was only a week in length, most items measured lower order cognitive skills at the knowledge level (Tables 1, 2). However, we did slightly improve the taxonomy of the test in 2017, with the amount of higher order items increasing from 33% to 40% of the assessment (c.f. Tables 1,2).

Student Experience, but not Interest in Developmental Biology Improved after DFB

Because our ultimate goal is to lower barriers to graduate education, we wanted to assess whether students felt they gained experience as a result of the course, and whether the course influenced their intended career pathway in some way. Student perspectives in these areas were assessed via five items in the pre-post analysis. Over the duration of both the 2016 and 2017 course, students felt they became more familiar with career options in the sciences ($p<0.02$). In 2016, the average pre to post test score in this area notably increased 75% in raw point value (2.88 to 3.36 out of 4). Students also felt that they had gained developmental biology experience in the classroom and the lab in both the 2016 Puerto Rico and 2017 Michigan courses (Figure A1-5, $p< 0.01$). Surprisingly, overall interest in developmental biology did not increase either

year (Figure A1-5, $p \geq 0.4$). These data may reveal a selection bias for students with a strong interest in developmental biology prior to their participation in DFB. Also of interest, the likelihood of students attending graduate school did not change (Figure A1-5, $p \geq 0.2$). Together, these results indicate that the DFB course improved students' perceived experience in developmental biology, but not their immediate likelihood of pursuing graduate education.

Discussion:

In this report, we describe the development, implementation, and assessment of Developing Future Biologists, a portable short course in developmental biology. Our mission centers around engaging underrepresented undergraduate students in an active learning environment, while also providing professional development and continued mentorship. Two of our major goals during the course were to teach students the core concepts of developmental biology and to increase awareness of career options in the sciences. Using a pre-post method of assessment, we found that student understanding of core concepts in developmental biology improved, particularly in content areas of organogenesis and early embryonic development. Item analyses indicated that students were able to perform better on post-tests in both 2016 and 2017, and that discrimination and taxonomy improved with the revised test given for the second iteration of the course. Furthermore, students indicated an increased awareness of career options in the sciences, although their interest in developmental biology and likelihood of attending graduate school did not change. Overall, this course represents a novel educational model that could be widely adopted by other universities and departments to help reduce barriers to graduate education.

Portable Course Design and the Alumni Connection

Many universities and research institutions offer paid summer research opportunities or internship programs that allow students to gain experience in developmental biology laboratories. For students to pursue these opportunities, however, they must first be motivated to seek out and apply for these programs, and they also must be willing to travel long distances and commit considerable time to these programs (typically around 10 weeks). Arguably, students with little background in developmental biology and with limited access to laboratory resources might be unaware of these summer research programs. Additionally, students may be hesitant to commit an extensive period of time to such endeavors without knowing if they are truly interested in the field. Our unique course model allows us to engage students who might be curious about developmental biology but are unable to commit to a full summer research opportunity in the field. By bringing this course directly to the students, we lower the activation energy required for participation. For students who are interested in pursuing additional research opportunities after the course, our professional development sessions and long-term mentoring model allows us to help them identify subsequent summer research programs and prepare successful applications.

The initial geographical location for the course arose naturally from our instructor alumni connections to UPR Ponce. Not only did our instructors from Ponce recognize the need for this type of initiative based on their own experiences, but they also were able to help tremendously with the course logistics. Given the portable nature of the course, great care was taken to ensure that laboratory activities were feasible with the equipment and resources available at UPR Ponce. Thus, it was incredibly helpful to have alumni involved who were familiar with the institution and facilities. Our alumni instructors also facilitated networking connections between the DFB

team and Ponce faculty members, who helped with advertising, coordinating space, and receiving material shipments (all tissues samples used in the labs were shipped to Ponce, with the exception of the locally obtained chicken eggs). Their experience and institutional knowledge was invaluable to the success of the course.

Many groups have demonstrated that shared social identities, including visual identity, have a profound impact on students' perceptions of themselves in a certain career path (Whittaker and Montgomery, 2012). Our Ponce alumni instructors as well as team members from other UPR campuses enabled course participants to instantly build connections on the basis of shared identity and common experience. DFB participants are therefore able to receive mentoring from positive role models from similar backgrounds to their own, who have already successfully navigated the path to graduate school and a career in science. Overall, we found that developing a partnership with the UPR Ponce community through alumni connections was crucial to the success of the program, and strongly advise that others trying to develop similar programs take this into consideration.

Course Impact on Participants and Instructors

Although a weeklong course seems like a very short period of time, the outcomes we present here strongly suggest the potential for a lasting impact, both on the UPR students participating in the course and the instructors from the University of Michigan. Within a single week, participants improved their understanding of the core concepts of developmental biology and became more aware of career options in the sciences. While the course was focused on developmental biology, the laboratories and lecture materials exposed UPR and Michigan students to a wide variety of model organisms and research techniques used broadly throughout

many fields in biology and biomedical science. Overall, we provided students with a basic introduction to developmental biology, highlighted some of the exciting ongoing research in the field, and helped students more easily envision career paths for themselves in STEM.

Our pre-post assessment indicated that the application process selected for students who were highly interested in developmental biology prior to formal instruction, and that student interest remained high throughout the course of the week. While the course did not increase students' interest in developmental biology, it did allow interested students to access this topic in a meaningful way, as evidenced by significant increases in participants' level of developmental biology experience in both the laboratory and the classroom. While these initial assessments show promising results, future courses could largely expand and improve assessment beyond the pre-post test mechanism. Several published indices exist to measure scientific integration (Estrada, 2009) as well as scientific self-efficacy (Chemers, 2006). These and other tools will allow us to improve our understanding of student attitudes and performance in the future.

We observed that DFB did not improve the item score pertaining to likelihood of DFB student to attend graduate school. One complication of this metric was that our students had varying ideas of the definition of graduate school, some which included medical and veterinarian school, and others which did not. Additionally, a short term educational initiative that is only a week in length may not be long enough to change undergraduate students' career plans. Perhaps a more appropriate question for future surveys might be whether students would be interested in attending a longer course or summer research program in developmental biology. Students were able to have honest conversations with graduate students, postdoctoral fellows, and faculty members to help them make more informed decisions about their career aspirations in the future. For students who do decide to pursue additional career development in the sciences, the extended

mentorship aspect of the initiative facilitates access to additional resources and continued support, including letters of recommendation, personal advice, and assistance with applying to research programs. The mentorship model also provides a mechanism to follow up with DFB participants and observe which careers they choose to pursue in the long-term. Continuing assessment will be crucial for past and future DFB courses to analyze impact on recruitment and retention of underrepresented students in scientific fields.

In addition to the impact of the course on enrolled students, DFB also had a profound impact on the team that created, planned, and implemented the course. Few opportunities exist for graduate students to create original learning modules and laboratory activities. This experience has also been invaluable for the graduate students and postdoctoral fellows involved, allowing instructors to develop exceptional teaching, communication, and organizational skills. University of Michigan instructors involved with DFB have a unique opportunity to learn more about students from diverse backgrounds and to better serve in mentoring capacities for these students. Presumably, this will make instructors more comfortable and more likely to engage in cross-cultural mentoring in the future, which will be critical for expanding diversity in the sciences overall. Future iterations of DFB should focus on improving instructors' mentorship skills through resources like the National Research Mentoring Network (NRMN) and other current initiatives.

Course Design and Scalability

The DFB initiative provides a framework for the development of similar courses with flexible content and length. At the heart of this program are hands-on, active learning approaches and relationship-building between undergraduate students and their near-peer counterparts

(Ramani et al., 2006). Information gathered from instructor evaluations suggests that these approaches were highly valued by our students. Our assessment data suggest that a week of interactive learning is a sufficient amount of time to teach students core concepts of developmental biology. Our hope is that graduate students, postdocs, and faculty members from other departments and institutions will adapt this model to create similar courses in areas of need. To meet these ends, we formatted the course to be modular in nature so that it could easily be applied in any field of science education.

Conclusion

To improve diversity outcomes in the sciences, underrepresented students need to be engaged in a meaningful way and shown pathways to success. The weeklong DFB initiative addresses these issues through active learning strategies that have been previously shown to reduce the gap between students of different educational backgrounds (Haak et al., 2011) and by providing mentorship from individuals who have faced similar disadvantages and succeeded, a mentoring strategy demonstrated to increase retention of minority groups in other STEM fields (Dennehy and Dasgupta, 2017; Keller et al., 2017). Through these methods, DFB was able to build on personal connections, leverage existing diversity, and provide high quality long-term mentoring. We believe these aspects are crucial to the success of DFB and, more importantly, the improvement of the cultural climate in science as a whole.

Acknowledgements:

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planning and implementing this course, providing laboratory space, equipment, and materials as needed. In particular, we thank the administrative personnel of the UPR Ponce: Maritza Torres and Delia Martínez; the professors Heidi Reyes, Dr. Wilfredo Ayala, Dr. Cynthia Rivera, David Forestier, and Dr. Abigail Ruiz; and our invited speakers Dr. Pedro Santiago and Dr. Jose García Arrarás. At the University of Michigan, we thank members of the Allen, Barolo, Spence, Denver, Miller, Innis, and Wellik labs for providing helpful reagents and materials. We also thank Dr. Leah Bricker, Dr. Ken Cadigan, and Dr. Larry Gruppen for helping with course and assessment design, and Dr. Sue Moenter for helping edit the manuscript.

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Lastly, we would like to thank all of the amazing students who took time out of their summers to enroll in this course and showed up every day full of excitement and curiosity.

Figures

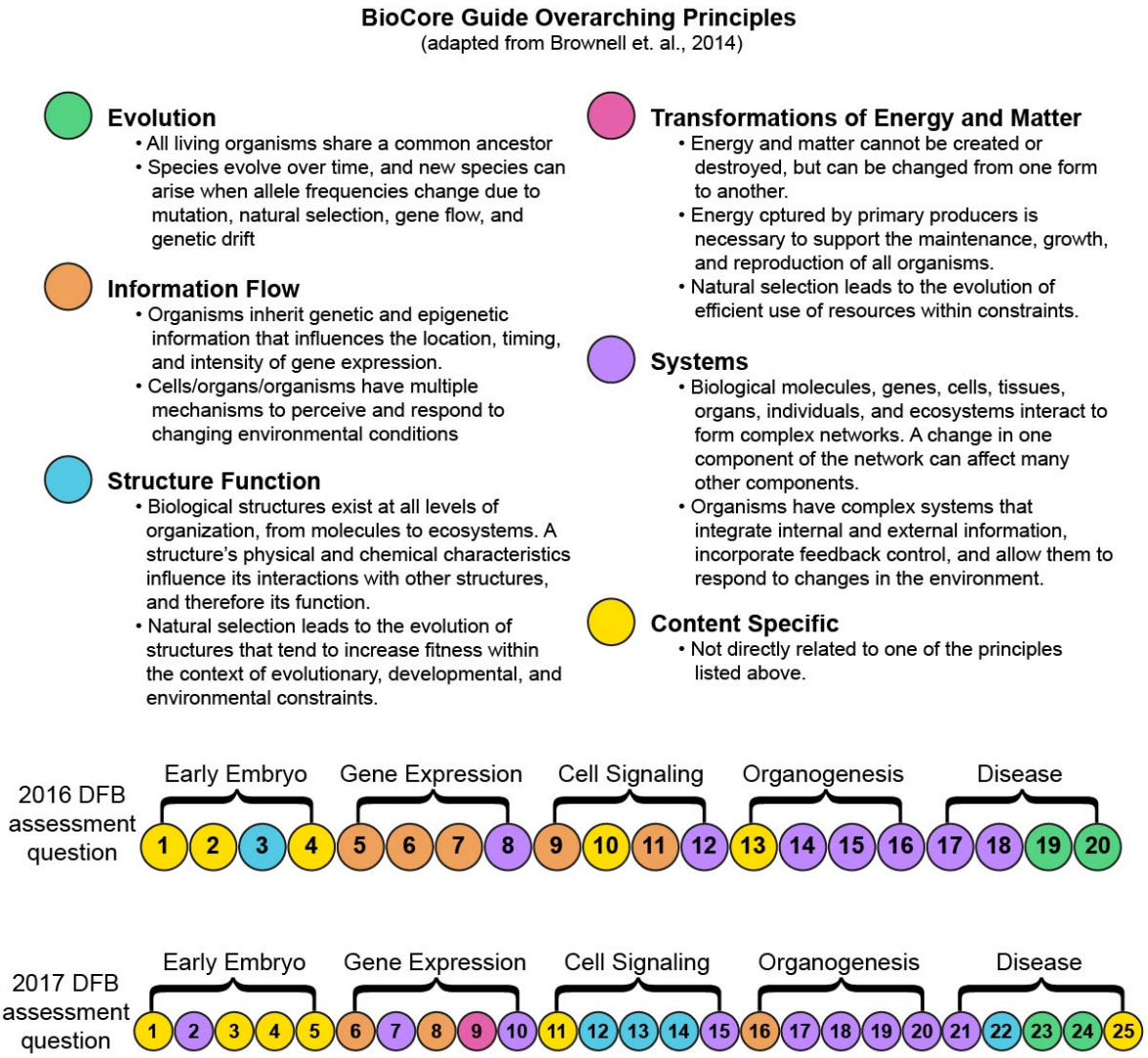


Figure A1-1: The Developing Future Biologists pre-post assessment covers the core concepts of developmental biology.
The core concepts in Biology were defined by Vision and Change (AAAS, 2010) and further adapted into overarching principles by the BioCore Guide (Brownell et. al. 2014). Each question within the DFB pre-post assessment from 2016 (top row) or 2017 (bottom row) either relates directly to an overarching principle or relates to specific content covered in lecture or lab, as indicated by color coordination. Please contact jpinskey@umich.edu for full assessments.

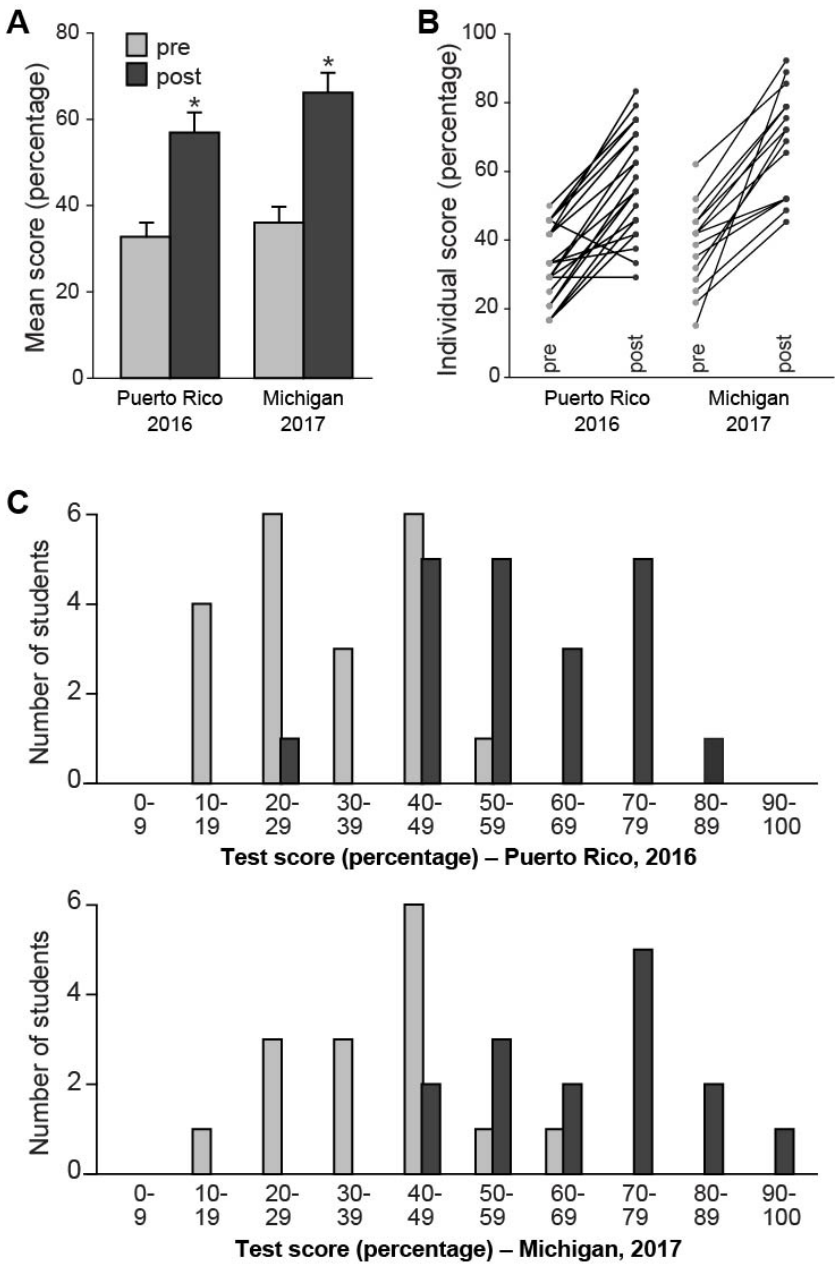


Figure A1-2. Student understanding of developmental biology core concepts improved following course participation.

(A) Aggregate test scores on pre- and post-assessment. Data reported as mean ± SD. *$p<0.0001$ for both years, using a paired two-tailed Student's t test. Score ranges: 2016, pre-assessment: 4 to 12 points out of 24 (17% to 50%; median 33%), post assessment: 7 to 20 points (29 to 83%; median 54%). 2017, pre-assessment: 4 to 18 points out of 30 (13% to 60%; median 40%), post assessment: 13 to 27 points (43% to 90%; median 70%). (B) Individual student pre- and post-assessment scores. Black lines connect each individual's pre and post score. (C-D) Range of scores on the pre (light gray) and post (dark gray) assessments for each year.

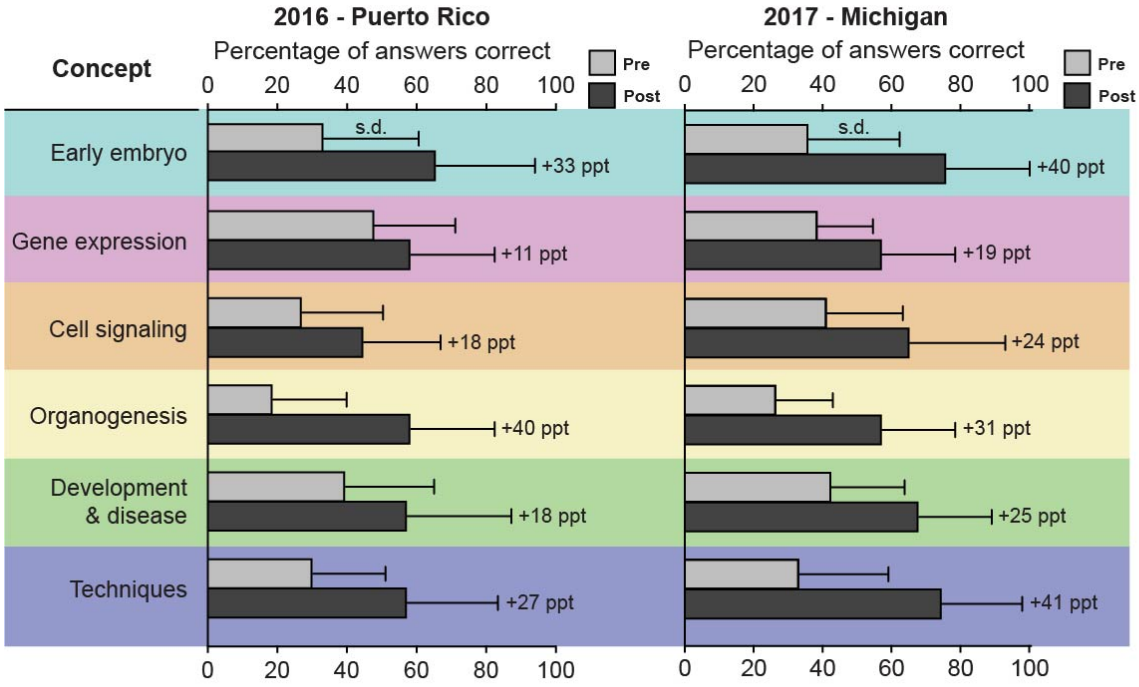


Figure A1-3. Student understanding of developmental biology improved within each conceptual area of instruction.
The percentage of correct answers on the pre- and post-tests increased for all core concepts in both 2016 (left) and 2017 (right). The percentage of students who answered correctly on the pre (light grey) and post (dark grey) tests are displayed for each concept (4 questions per concept). Each concept showed significant improvement (two-tailed t-test, $p<0.05$). Average percent increase for each concept listed to the right of post-test bars (ppt = percentage point).

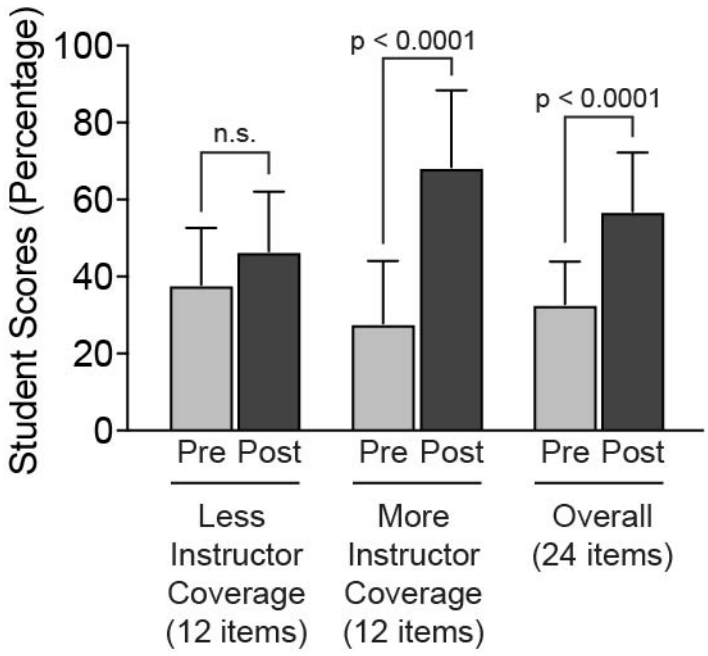


Figure A1-4. 2016 Student performance correlates with increased instructor coverage. Item coverage scores were collected from instructors, and test items were divided into two equal groups reflecting less instructor coverage (less than 60% instructor coverage score), and more instructor coverage (greater than 70% instructor coverage score). Student scores by item coverage changed based on instructor coverage scores (one-way repeated-measures ANOVA, $p<0.0001$). Post hoc analysis results suggest students scored similarly on pre and post items for which less instruction was given (Sidak $p=0.1620$), while scores improved on items for which more instruction was given (Sidak $p<0.0001$) and for the test overall (Sidak $p<0.0001$).

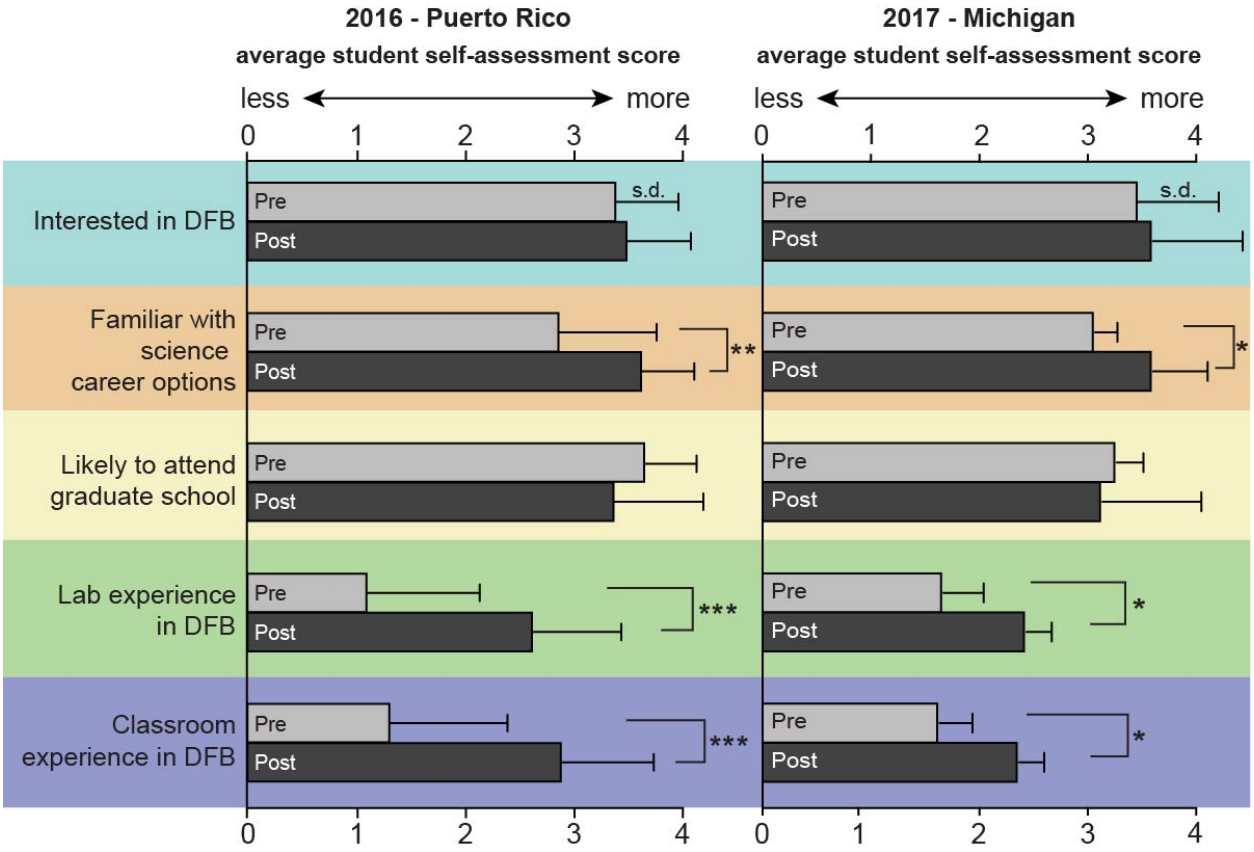


Figure A1-5. Student attitudes about developmental biology and careers in science.
Pre (top, lighter bars) and post (bottom, darker bars) test analysis sorted by item from 2016 (left) and 2017 (right). Student reflection on familiarity with career options in the sciences and experience gained in the classroom and lab significantly improved (career options: $p<0.02$, and lab and classroom: $p< 0.01$). Overall interest in developmental biology and likelihood to attend graduate school did not significantly change ($p\geq0.2$) *$p<0.02$, **$p<001$, ***$p<0.0001$.

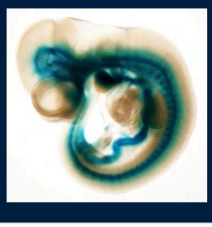
Figure A1-S1 (A). Advertisement for 2016 Developing Future Biologists course.

Developing Future Biologists

is pleased to announce our third annual

Introductory Short Course in Developmental Biology

UNIVERSITY OF MICHIGAN



- Hands on Laboratories
- Interactive Lectures
- Networking Opportunities

Who should apply?

Any undergraduate currently attending college in the state of Michigan and interested in developmental biology. Students identifying with under-represented minority groups are especially encouraged to apply *

When is the course?

May 15-20, 2017

Where is it?

University of Michigan at Ann Arbor

* Refer to www.developingfuturebiologists.com for more details on student eligibility

Applications open March 1, 2017

Meals and accommodations are provided for all admitted students

Find out more information at www.developingfuturebiologists.com

Any Questions? Connect with us!

contactdfb@umich.edu

dfb

Figure A1-S1 (B). Advertisement for 2017 Developing Future Biologists course.

Figure A1-S2 (A). Schedule for 2016 Developing Future Biologists course.

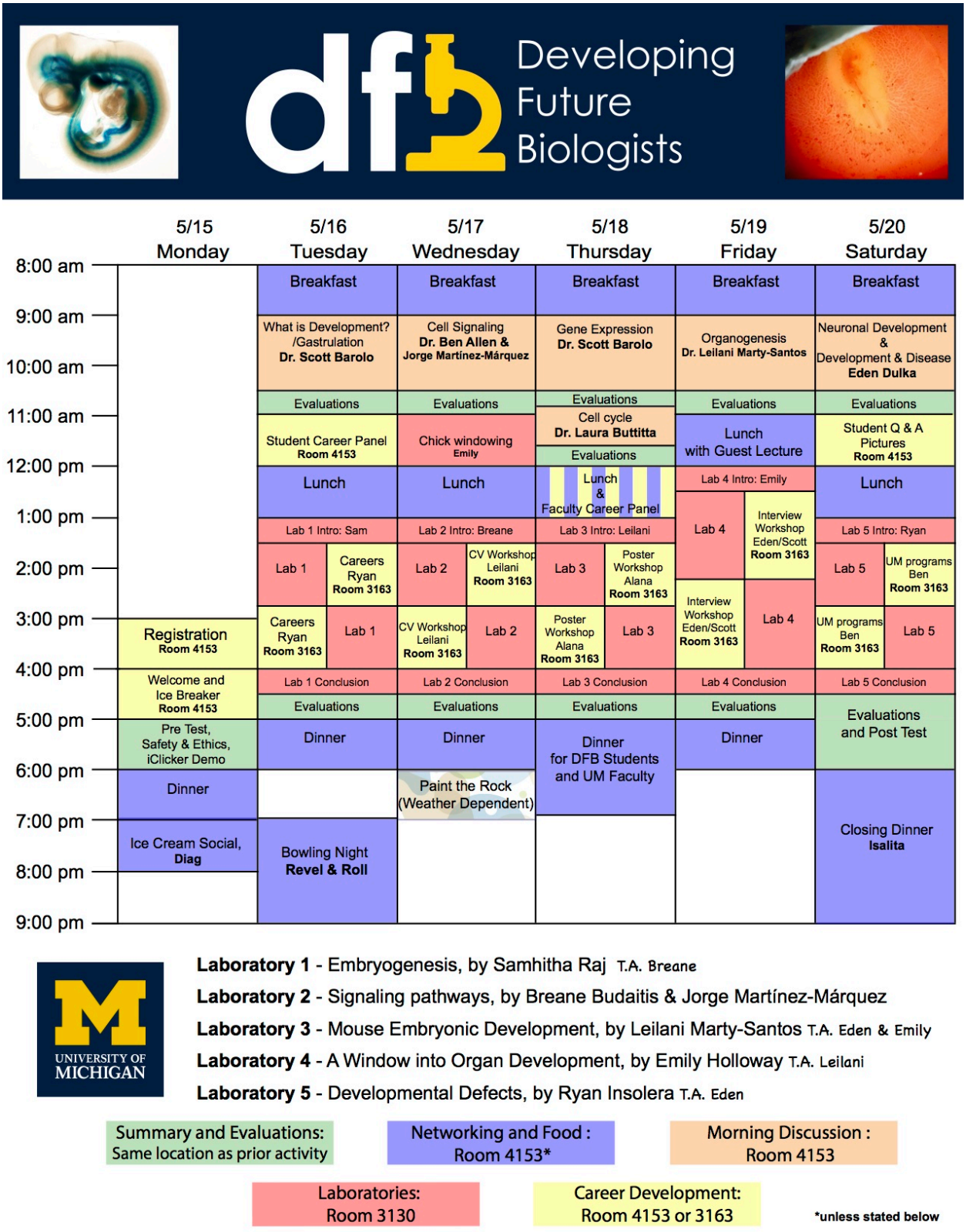


Figure A1-S2 (B). Schedule for 2017 Developing Future Biologists course.

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