Hedgehog (HH) signaling is critical for regulating 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 characterized, 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.

Hedgehog (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. 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. In this “off” state, GLI2 and GLI3 are phosphorylated by PKA, GSK3β, and CK1. As a consequence of this phosphorylation, GLI2 is largely degraded, whereas GLI3 is processed into a transcriptional repressor (13, 14). HH ligand binding to PTCH1 results in derepression of SMO, which initiates a signal transduction cascade that culminates in GLI protein processing in a context-specific manner (8, 15–17).

Multiple cohorts of cell surface proteins regulate HH pathway activity by binding to HH ligand. DISP1 and SCUBE2 tightly control ligand secretion (18–20), whereas trafficking and turnover are regulated by LRP2 and GPCs (21–23). The cell surface components GAS1, CDON, and BOC function as essential co-receptors at the level of signal reception (24, 25). Additionally, ligand interactions with PTCH1, PTCH2, and HHIP1 result in pathway antagonism (26–28). 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 (29–34) and act to positively regulate HH signaling at the cell surface (35, 36). NRPs are expressed in a variety of HH-responsive tissues during critical periods of HH-related developmental patterning (37, 38). Importantly, loss-of-function experiments demonstrated that Nrp1a knockdown in zebrafish disrupts HH-dependent somite development (35), whereas genetic deletion of Nrp1 and Nrp2 in the mouse suppresses HH-driven cerebellar granule neuron progenitor proliferation (36). Additionally, NRPs exacerbate HH-related cancers, suggesting that they impact both HH-dependent development and HH-driven disease (36, 39–42). Notably, NRPs are thought to act downstream of HH ligands (36), distinguishing their mode of action from most other cell surface regulators of the HH pathway. Previous reports suggest that...
NRPs regulate Hedgehog signaling by modulating GLI activator function

A previous study showed that NRPs increase ligand-stimulated HH pathway activity in HH-responsive fibroblasts (35). To confirm and extend these findings, we first tested whether NRPs promote HH signaling using a luciferase-based reporter assay system in NIH-3T3 fibroblasts (43). Although the addition of HH ligand is sufficient to induce a transcriptional response, we found that NRPs significantly increase ligand-activated HH pathway activity, as detected by GLI-dependent luciferase output (Fig. 1A and supplemental Fig. S1), consistent with a previous report (35). Notably, co-expression of *Nrp1* and *Nrp2* does not significantly change the level of NRPs (35). Western blot analysis confirmed that HA-tagged NRPs are expressed at similar levels in NIH-3T3 cells (Fig. 1B). Although NRP1 significantly promotes HH signaling in ~90% of assays (n = 8, average-fold change = 2.04), NRP2 significantly promotes HH signaling in only 40% of assays (n = 8, average-fold change = 1.36, supplemental Fig. S1). Because of this variability, we decided to focus on NRPs for further analysis.

To determine whether HH ligand is required for NRPs to promote HH signaling, we activated HH signaling by adding exogenous smoothened agonist (SAG), co-transfecting a constitutively active form of Smo (Smo2; Fig. 1C–F) (44–46). Strikingly, NRPs significantly increase the HH-dependent luciferase output, regardless of the means of pathway activation (Fig. 1D–F). In contrast, NRPs do not alter GLI3-mediated repression of Hedgehog signaling (Fig. 1G and H). Together, these data support a model in which NRPs act to selectively regulate GLI activator function downstream of HH ligand. The membrane-anchored NRPs are necessary and sufficient to promote HH signaling.

**Results**

**NRP1 and NRP2 promote HH signaling by modulating GLI activator function**

Neuropilin TM dimerization is mediated by a double GXXG motif in the TM domain that stabilizes signaling complexes for both Semaphorin and VEGF ligands (47). Mutating the three glycine residues within the double GXXG motif to valines completely disrupts dimerization and blocks NRP1 function in Semaphorin signaling (47). To determine whether NRP1 TM dimerization is required for HH signal promotion, we recreated these three glycine mutations in both NRP1 (Fig. 3A) and NRP1ACD (Fig. 3B). Strikingly, these mutations do not impair the ability of either construct to promote HH signaling (Fig. 3), suggesting that NRP1 membrane attachment, but not TM dimerization, is required for its function in HH signaling.

**NRP1 promotes HH signaling independently of GLI2 phosphorylation by PKA**

Previous work has suggested that NRPs regulate HH signaling by recruiting PDE4D to the cell membrane (36). PDE4D negatively regulates PKA activity by locally reducing levels of cAMP (48). 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 (13, 14, 49). To test whether the NRPs mediate HH signaling activity through PKA-dependent GLI phosphorylation, we generated serine-to-alanine mutations at the six consensus PKA phosphorylation sites critical for GLI2 repression (13, 14, 49) (GLI2SA–6, Fig. 4A). As expected, GLI2SA–6 expression results in a significant increase in HH signaling compared with WT GLI2.
Although GLI2 stimulates HH signaling less effectively than its constitutively active counterpart GLI2\textsuperscript{N}, we still observed a reliable increase in activity with Nrp1 co-expression (Fig. 4B). This increase was not observed when we co-expressed Nrp1\textsuperscript{CD}, consistent with previous results (Fig. 4B, cf. Fig. 2B). Surprisingly, Nrp1 still promotes HH signaling when co-ex-
Figure 2. NRP1 cytoplasmic and transmembrane domains are necessary and sufficient to promote HH signaling. A, schematic of full-length NRP1, NRP1ΔCD, and NRP1ΔECD. 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 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 (α-β-Tub) was used as loading control. Right, quantitation of NRP1 and NRP1ΔCD 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, NRP1ΔCD, and NRP1ΔECD. 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.
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Figure 3. NRP1 TM dimerization is not required to promote HH signaling. A, left, schematic of full-length NRP1 and NRP1ΔCD, 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. B, left, schematic of NRP1 and NRP1ΔCD, G-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.

pressed with GLI2P1–6, suggesting that NRP1 regulates GLI activity independently of PKA phosphorylation. Importantly, Nrp1ΔCD does not promote signaling when co-expressed with GLI2P1–6, indicating that this PKA-independent promotion of HH signaling still requires the NRP1 CD (Fig. 4B).

Identification of a novel NRP1 cytoplasmic motif that mediates HH 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 (50). 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 (Fig. 2; data not shown). Furthermore, deleting the NRP1 SEA (NRP1Δ890–922, Fig. 5A) did not impair NRP1-mediated promotion of HH signaling in NIH-3T3 cells (Fig. 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 (NRP1Δ883–902, Fig. 5A) and assessed function in NIH-3T3 HH signaling assays. Strikingly, NRP1Δ883–902 failed to promote HH signaling (Fig. 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 NRP1Δ883–902 (Fig. 5F), and immunofluorescent staining under non-permeabilizing conditions confirmed that NRP1Δ883–902 properly localizes to the cell surface (Fig. 5G). We then asked whether restoring part of this region would rescue NRP1 function in HH signaling; however, NRP1Δ890–922 still failed to promote signaling (Fig. 5D). Ultimately, adding back 12 additional residues from amino acid 890–902 (NRP1Δ890–922) rescued NRP1-mediated promotion of HH signaling equivalently to full-length NRP1 (Fig. 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 (supplemental Fig. S4). 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.

NRP1, but not NRP2, localizes to primary cilia in HH-responsive fibroblasts

The primary cilium is a highly regulated subcellular compartment into which molecules over 40 kDa cannot freely diffuse (51), and an intact cilium is important for HH signaling to proceed normally (52). Given that NRP1 regulates HH signaling...
Figure 5. Identification of a 12-amino acid motif in the NRP1 CD required for HH 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, NRP1ΔECD, NRP1Δ883–902, and NRP1Δ890–922. Nuclear GFP (green) indicates transfected cells, whereas DAPI (blue) marks all nuclei. Note that NRP1ΔECD lacks the NRP1 antibody epitope and served as a negative control. Scale bar = 10 μm.
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![Image](image_url)

**Figure 6.** NRP1, but not NRP2, localizes to primary cilia in HH-responsive fibroblasts. A–H, antibody detection of HA/YFP (green) and primary cilia (red, AcTub) in WT (top) and Dynaev-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.

Through the modulation of GLI activity, and that GLI proteins localize to cilia and require intact cilia for their processing and function (53), we asked whether NRPs localize to the primary cilium.

To assess primary cilia localization, we expressed Nrp1 and Nrp2 in WT and Dynaev-mutant (Dync2h1^{lln/lln}) mouse embryonic fibroblasts (MEFs) (Fig. 6). Dynaev motors mediate retrograde transport of ciliary components; thus, cilia-localized proteins accumulate within the primary cilium of Dync2h1^{lln/lln} MEFs, allowing for more robust detection (54). We found that NRP1, but not NRP2, localizes to primary cilia (identified with anti-acetylated tubulin, AcTub) in WT and Dync2h1^{lln/lln} MEFs (Fig. 6). NRP1 was detected in 51% of cilia in WT MEFs and further enriched in Dync2h1^{lln/lln} MEFs, with 68% of cilia positive for NRP1 (Fig. 6, A, E, I, and J). NRP2, on the other hand, was only detected in primary cilia in 9% of Dync2h1^{lln/lln} MEFs, with no ciliary localization observed in WT MEFs (Fig. 6, B, F, I, and J). As a positive control, SMOM2 robustly localizes to the primary cilium in both WT and Dync2h1^{lln/lln} MEFs (98% of cilia in each group; Fig. 6, C, G, I, and J), consistent with previous findings (55). In contrast, BOC, a cell surface–localized HH co-receptor (56, 57), was detected broadly throughout the cell surface but was not observed in primary cilia (Fig. 6, D and H–J). Importantly, no HA staining was observed in the cilia of vector-transfected cells (supplemental Fig. S5). To further confirm these data, we stained WT and Dynaev-mutant MEFs for endogenous NRP1 and detected NRP1 localization to primary cilia (Fig. 6K). These results suggest that NRP1, but not NRP2, localizes to the primary cilium of HH-responsive fibroblasts.

**NRP1 cilia localization does not correlate with HH 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 (supplemental Fig. S1). 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^{ΔECDS} and Nrp1^{Δ902–922}. Notably, both of these constructs robustly promoted HH signaling (Figs. 2B, 5E, and 7E). We performed immunofluorescent staining to
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Figure 7. Ciliary localization of NRP1 does not correlate with HH signal promotion. A–C, antibody detection of NRP1 (A), NRP1ΔECD (B), and NRP1Δ902–922 (C) in NIH-3T3 cells (HA, green; AcTub, red; DAPI, blue). D, chart summarizing the structure (top) and cillum localization (bottom) of each construct imaged on the left. n = 165, 166, and 162 cells for Nrp1, NRP1ΔECD, and NRP1Δ902–922, 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.

Examine the ciliary localization of NRP1, NRP1ΔECD, or NRP1Δ902–922 in NIH-3T3 cells (Fig. 6). Although NRP1 localizes to primary cilia in roughly 40% of transfected cells (Fig. 7, A and D), both NRP1ΔECD and NRP1Δ902–922 displayed significantly reduced localization to primary cilia (Fig. 7, B–D). Taken together, these results suggest that cilia localization does not correlate with NRP1-mediated promotion of HH signal transduction.

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 (4, 24, 25, 58–60). Conversely, deregulation of HH cell surface components contributes to HH-driven birth defects and cancers (61–66). NRPs are also implicated in numerous human cancers (40, 67). Notably, Nrp2 knockdown increases survival in a HH-dependent mouse model of medulloblastoma (39). 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.

NRPs as a novel class of ligand-independent HH cell surface regulators

Numerous cell surface proteins promote HH pathway activity through interactions with HH ligands (23–28, 65, 68, 69). 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 (70). Importantly, we find that Nrp1 still promotes HH pathway activity when co-transfected with GLI2P1–6, a version of GLI2 that cannot be phosphorylated by PKA at six critical repressive sites. Therefore, in contrast to previous work (36), 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 (49) or affect GSK3β activity, which is also regulated by cAMP (71); 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 (35), 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 Semaphorins ligands can contribute to HH signal promotion. Class 3 Semaphorins interact with the extracellular domains of NRP1 and NRP2 (72, 73). Although two previous studies present contrasting results regarding Semaphorin ligand involvement in HH signal regulation (35, 36), 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 (67). It is possible that NRP interactions
Identification of a cytoplasmic motif in NRP1 that mediates HH signal transduction

Our data indicate that the membrane-attached NRP1 CD is necessary and sufficient to promote HH signaling. Notably, 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.

Identification of a cytoplasmic motif in NRP1 that mediates HH signal transduction

Our data indicate that the membrane-attached NRP1 CD is necessary and sufficient to promote HH signaling. Notably, 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.

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 (79). 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 (47, 80). 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 (47). 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 (67, 81), 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.
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NRP1 ciliary localization does not correlate with increased HH 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, NRP1 may play an important role elsewhere in the cell, perhaps even at the ciliary base. Accordingly, NRP1, NRP2, may be sufficient to impact HH signal transduction. Alternatively, the possibility that the low levels of cilia localization we observe may be sufficient to impact HH signal transduction. NRP1 ciliary localization does not correlate with increased HH signal promotion. 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, NRP1 may play an important role elsewhere in the cell, perhaps even at the ciliary base. Accordingly, NRP1, NRP2, may be sufficient to impact HH signal transduction. Alternatively, the possibility that the low levels of cilia localization we observe may be sufficient to impact HH signal transduction. NRP1 ciliary localization does not correlate with increased HH signal promotion.

Cell culture

Cell lines were maintained in DMEM (Life Technologies, 1965) supplemented with 10% bovine calf serum (ATCC, 30-2030) and 1000 penicillin—streptomycin—l-glutamine (Life Technologies, 10378016). Cultures were kept at 37 °C with 5% CO₂ 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 pGL3 reporter construct (43). Briefly, cells were seeded at 2.5 × 10⁵ 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 (NSSH)-conditioned medium. Alternatively, SAG (Sigma-Aldrich, SML1314) was added at a concentration of 300 ng/μl to activate HH signaling. Luciferase reporter activity and β-galactosidase activity were measured 48 h later on a Spectra-max 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 (Dyn2h11αβ/αβ) and wild-type littermate control MEFs (generously provided by Dr. Kathryn V. Anderson, Memorial Sloan Kettering (54)), were plated at 1.5 × 10⁵ cells/well in a 6-well dish with a coverslip at the bottom of each
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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 anti-mouse IgG1 (γ1), 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 Immuno-Mount mounting medium (Fisher, 9990412). Immunofluorescence image analysis and imaging were performed on a Leica SP5X upright two-photon confocal microscope using LAS AF software (Leica) and a Leica ×63 (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 radioimmunoprecipitation 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 × 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 reagents, AF566), and mouse IgG1 anti-Neuropilin1 (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 (Den- ville, E3018) and developed using a Konica Minolta SRX-101A medical film processor.

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.

Acknowledgments—We thank Dr. A. L. Kolodkin (Johns Hopkins University) for providing Neuropilin constructs and Dr. K. V. Anderson (Memorial Sloan Kettering Cancer Center) for providing wild-type and Dyn2h, MEFs. Members of the B. L. A. and R. J. G. laboratories contributed technical assistance, insightful comments, and helpful suggestions. We also thank Drs. K. Sue O’Shea, K. J. Verhey, J. D. Engel, K. F. Barald, S. Barolo, and J. R. Spence for sharing equipment and reagents. Confocal imaging was performed in the Microscopy and Image Analysis Laboratory at the University of Michigan.

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