Supplemental Fig. S1. Validation of RNAi efficacy and specificity.

A

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B

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C

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Supplemental Fig. S2. Sequence target and specificity of Nrp RNAi reagents.

A

Nrp1 isoform 1 (NM_008737)

B

Nrp2 isoform 1 (NM_001077403)
Nrp2 isoform 2 (NM_001077404)
Nrp2 isoform 3 (NM_010939)
Nrp2 isoform 4 (NM_001077405)
Nrp2 isoform 5 (NM_001077406)
Nrp2 isoform 6 (NM_001077407)

C

Normalized Hh Pathway Luciferase Reporter Activity

+Shh

RNAi: Non-Targeting | Nrp2#2 | Nrp2#3 | Nrp2#4 | Nrp2#5 | Nrp2#6
Supplemental Fig. S3. *Nrp* RNAi inhibits the transcriptional induction of Hh pathway target genes.
Supplemental Fig. S4. *Nrp1* RNAi does not inhibit Wnt3A-stimulated Wnt pathway luciferase reporter activity.

A

B

RNAi: GFP, Luciferase, Nrp1

+Shh

+Wnt3A

Normalized Hh Pathway Luciferase Reporter Activity

Normalized Wnt Pathway Luciferase Reporter Activity
Supplemental Fig. S5. Nrp1 expression co-localizes with dermal papilla and endothelial cell markers in developing mouse skin at E17.5.
Supplemental Fig. S6. *Nrp1* transcript abundance is increased by Shh treatment in NIH3T3 fibroblasts and primary dermal cells.
Supplemental Fig. S7. Diagram of protein products used in over-expression experiments.

A

B

C

+Shh

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Normalized Hh Pathway Luciferase Reporter Activity

Normalized Hh Pathway Luciferase Reporter Activity
Supplemental Fig. S8. Nrp1 over-expression potentiates Hh reporter activity induced by co-transfection of SmoM2.

Normalized Hh Pathway Luciferase Reporter Activity

Co-Transfected Agonist: None SmoM2 None
Shh: - - +
Supplemental Figure S9. Hh signaling and known Nrp ligands.

A

Normalized Hh Pathway Luciferase Reporter Activity

ShhN (ng/mL)

B

Normalized Hh Pathway Luciferase Reporter Activity

ShhN (ng/mL)

C

Normalized Hh Pathway Luciferase Reporter Activity

Untreated +ShhN

D

Normalized Hh Pathway Luciferase Reporter Activity

Mw (kDa)

D’

VEGFR-R2/Kdr
Supplemental Fig. S10. Nrps are not required for primary cilium formation or Shh-stimulated Smo translocation to the primary cilium.
Supplemental Fig. S11. Nrp1 over-expression does not potentiate Hh pathway activity in SuFu<sup>−/−</sup> MEFs.
Supplemental Fig. S12. Phylogenetic tree of selected chordate Nrp protein sequences.
Supplementary Figure Legends

**Supplemental Fig. S1. Validation of RNAi efficacy and specificity.** (A) *Smo* siRNAs efficiently reduced Smo protein in NIH3T3 cells, as measured by immunoblotting of whole cell protein extracts with an anti-Smo antibody capable of detecting endogenous protein. *Smo* siRNA #4 (red) demonstrated the greatest potency and was selected for use in all subsequent investigations. (B) Individual siRNAs targeting *Nrps*. A series of *Nrp1* and *Nrp2* DSPs and synthetic siRNAs were tested for efficacy in NIH3T3 fibroblasts. Note that the *Nrp1* and *Nrp2* antibodies are specific, as siRNAs targeting each gene do not perturb the abundance of the other. Unless otherwise stated, *Nrp1* siRNA #1 and *Nrp2* siRNA #5 (both in red) were used in all subsequent experiments. (C) The *Nrp1* antibody detected additional bands that were not affected by *Nrp1* RNAi. To test the provenance of these bands, we derived MEFs from embryonic day E12.5 mouse embryos homozygous for a conditional Nrp1 allele (*Nrp1* c/c) (Gu et al. 2003). After recombining these MEFs *in vitro* using Cre recombinase delivered by recombinant adenovirus, the additional bands detected by our *Nrp1* antibody persisted. This result indicates these bands are non-specific and unrelated to *Nrp1* gene products.

**Supplemental Fig. S2. Sequence target and specificity of Nrp RNAi reagents.** (A) The mouse *Nrp1* (NM_008737) gene structure based on the mm9 genome assembly is illustrated. The sequence target of the primary *Nrp1*
synthetic siRNA used in this manuscript (#1), a second Nrp1 synthetic siRNA to the 3' UTR used in rescue experiments (#2), and a Nrp1 shRNA hairpin used in lentivirus experiments (H) are indicated. Exons are shown as blocks and introns as shown as lines. Scale bar represents 50 kb. (B) The gene structures of mouse Nrp2 isoform 1 (NM_001077403), isoform 2 (NM_001077404), isoform 3 (NM_010939), isoform 4 (NM_001077405), isoform 5 (NM_01077406), and isoform 6 (NM_001077407) are illustrated. Nrp2 isoforms 1-4 share a 3' UTR that is homologous to that of Nrp1. The sequence target of the primary Nrp2 synthetic siRNA used in this manuscript (#1), a series of Nrp2 synthetic siRNAs to the 3' UTR (#2-6), and a Nrp2 shRNA hairpin used in lentivirus experiments (H) are indicated. (C) Shh-stimulated Hh pathway reporter activity in Shh-LIGHT2 cells treated with non-targeting or Nrp2 RNAi. Error bars represent ± 1 SD.

Supplemental Fig. S3. Nrp RNAi inhibits the transcriptional induction of Hh pathway target genes. Following Nrp1+2 RNAi, Shh-stimulated induction of the Hh pathway target genes Gli1 and Ptc1 was reduced in NIH3T3 fibroblasts, as measured by qPCR (see Materials and Methods). Data in each case were analyzed using the relative Ct value method and normalized on a per-well basis to the housekeeping gene Gapdh. Error bars represent ± 1 SD.

Supplemental Fig. S4. Nrp1 RNAi does not inhibit Wnt3A-stimulated Wnt pathway luciferase reporter activity. (A) Normalized Hh pathway luciferase
reporter activity following diced pool RNAi against GFP (negative control), firefly luciferase (positive control) and Nrp1. Error bars represent ± 1 SD. (B) Normalized Wnt pathway luciferase reporter activity following diced pool RNAi against GFP (negative control), firefly luciferase (positive control) and Nrp1. Error bars represent ± 1 SD.

**Supplemental Fig. S5.** Nrp1 expression co-localizes with dermal papilla and endothelial cell markers in developing mouse skin at E17.5. (A) Co-localization of Nrp1 in early-stage (stage 2) hair follicles with a dermal papilla marker P75NTR and endothelial cell marker CD31. (B) Nrp1 expression in mid-stage (stage 4) hair follicles is primarily in dermal sheath cells. Scale bars represent 20 μm.

**Supplemental Fig. S6.** Nrp1 transcript abundance is increased by Shh treatment in NIH3T3 fibroblasts and primary dermal cells. (A) Baseline and Shh-stimulated Nrp1 and Nrp2 transcript abundance in NIH 3T3 fibroblasts, as measured by quantitative PCR (qPCR) normalized to Gapdh expression. Error bars represent ± 1 SD. (B) Baseline and Shh-stimulated Nrp1 and Nrp2 transcript abundance in mouse primary dermal cells (see Materials and Methods), as measured by quantitative PCR (qPCR) normalized to Gapdh expression. Error bars represent ± 1 SD.
Supplemental Fig. S7. Diagram of protein products used in over-expression experiments. (A) The coding region of yellow fluorescent protein (YFP) was fused to the carboxyl termini of mouse CD4 and Nrp1 cDNAs. Nrp1 is comprised of two CUB domains (A1, A2), two coagulation factor-like domains (B1, B2), and a MAM domain. CD4 was chosen as a negative control because it is a single-pass transmembrane protein of similar molecular weight to Nrp1, yet composed of multiple Ig domains. (B) Endogenous Nrp1 and over-expressed Nrp1-YFP in transfected NIH 3T3 fibroblasts, as detected by immunoblot for Nrp1. Control cells were transfected with CD4-YFP expression vector. (C) Normalized Hh pathway luciferase reporter activity in NIH3T3 fibroblasts treated with non-targeting or Nrp1 3’ UTR RNAi, then transfected with either CD4-YFP, Nrp1, or Nrp1-YFP expression vectors. Error bars indicate mean ± 1 SD.

Supplemental Fig. S8. Nrp1 over-expression potentiates Hh reporter activity induced by co-transfection of SmoM2. SmoM2 (also referred to in the literature as SmoA1) is a W539L Smo mutant capable of strong, agonist-independent Hh pathway target gene induction (Xie et al. 1998). Nrp1-YFP or YFP expression vectors were co-transfected with a SmoM2-YFP expression vector into NIH 3T3s, along with Gli-dependent firefly luciferase and constitutive Renilla reporter plasmids. Co-expression of Nrp1 with SmoM2 resulted in increased maximal reporter activity, as compared to controls. Error bars indicate mean ± 1 SD.
Supplemental Fig. S9. **Hh signaling and known Nrp ligands.** (A) No functional interaction was observed when recombinant ShhN was titrated against a fixed, high concentration of recombinant Sema3A-Fc in a Hh pathway luciferase-based reporter assay in Shh-LIGHT2 cells. Error bars indicate mean ± 1 SD. (B) Similarly, no functional interaction was observed between recombinant ShhN and high levels of recombinant VEGF_{164}. The “No Ligand” curves in (A) and (B) are identical but shown on two separate axes for clarity. Error bars indicate mean ± 1 SD. (C) Recombinant Sema3F did not functionally interact with recombinant ShhN when added to Shh-LIGHT2 cells. Error bars indicate mean ± 1 SD. (D) VEGF-R2/Kdr protein was not detected in whole cell extracts prepared from the NIH3T3 fibroblasts, even after extreme immunoblot over-exposure (D'). VEGF-R2/Kdr was readily detectable when an identical amount of protein from human umbilical vein endothelial cells (Huvecs) was loaded on the same gel. This antibody detects both the mouse and human VEGF-R2/Kdr proteins.

Supplemental Fig. S10. **Nrps are not required for primary cilium formation or Shh-stimulated Smo translocation to the primary cilium.** (A) No difference in ciliation frequency was observed between Nrp1+2 RNAi and control RNAi conditions. Cilia were identified by Immunofluorescent staining of NIH 3T3s treated with RNAi. Ciliation frequency was quantified by counting the number of cilia visible in the acetylated tubulin channel and dividing this number by the number of whole nuclei in the same field. At least 30 nuclei were counted
for each condition. Error bars indicate mean ± 1 SD (B) Quantification of Smo translocation to the primary cilium in response to Shh, demonstrating no change following Nrp1+2 RNAi (p>0.2, Two-tailed Student’s T-test). Error bars indicate mean ± SEM for 10-20 cilia. (C) No defect in ciliary morphology or Shh-stimulated Smo (green) translocation was observed by cell immunofluorescence following Nrp1+2 RNAi. Nrp1 (red) was stained to confirm RNAi efficacy. Cilia were detected by staining acetylated tubulin (cyan) and nuclear DNA was highlighted with Hoescht dye (blue). Scale bars represent 10 µm. (D) HA-Gli2 cells (see Materials and Methods) treated with Shh following control or Nrp1+2 RNAi (Kim et al. 2009). HA-Gli2 (red), Smo (green), acetylated tubulin (cyan), and Hoescht dye to highlight nuclei (blue). Images are shown in shifted overlay to highlight simultaneous ciliary staining for multiple proteins (Milenkovic et al. 2009). (E) Immunofluorescent staining of NIH3T3 fibroblasts treated with Shh following Nrp1+2 RNAi. Gli2 (red), Smo (green), acetylated tubulin (cyan), and Hoescht dye to highlight nuclei (blue). Images are shown in shifted overlay. (F) Immunofluorescent staining of NIH3T3 fibroblasts without permeabilization. Nrp1 (red) and Hoescht dye to highlight nuclei (blue).

Supplemental Fig. S11. Nrp over-expression does not potentiate Hh pathway activity in SuFu−/− MEFs. Over-expression of Nrp1-YFP in SuFu−/− MEFs does not significantly increase activation of a co-transfected Gli-dependent firefly luciferase reporter construct. Tripling the dose of Nrp1-YFP used in NIH 3T3s still produced no significant effect on pathway activity (300 ng/well on a 24
well plate). To control for transfection efficiency, a hemagglutinin epitope-tagged SuFu expression construct was transfected and restoration of Shh sensitivity was demonstrated. Error bars indicate mean ± 1 SD.

**Supplemental Fig. S12. Phylogenetic tree of selected chordate Nrp protein sequences.**

Phylogenetic tree of Nrp protein sequences from mouse (m), human (h), and zebrafish (z). NCBI reference sequences are: mNrp1= NP_032763.2; mNrp2= NP_001070871.1; hNrp1= NP_001019799.1; hNrp2= NP_003863.2; zNrp1a= NP_001035416.1; zNrp1b= NP_991237.1; zNrp2a= NP_998130.1; zNrp2b= NP_998131.1. Scale bar represents 0.05 amino acid substitutions per site.


