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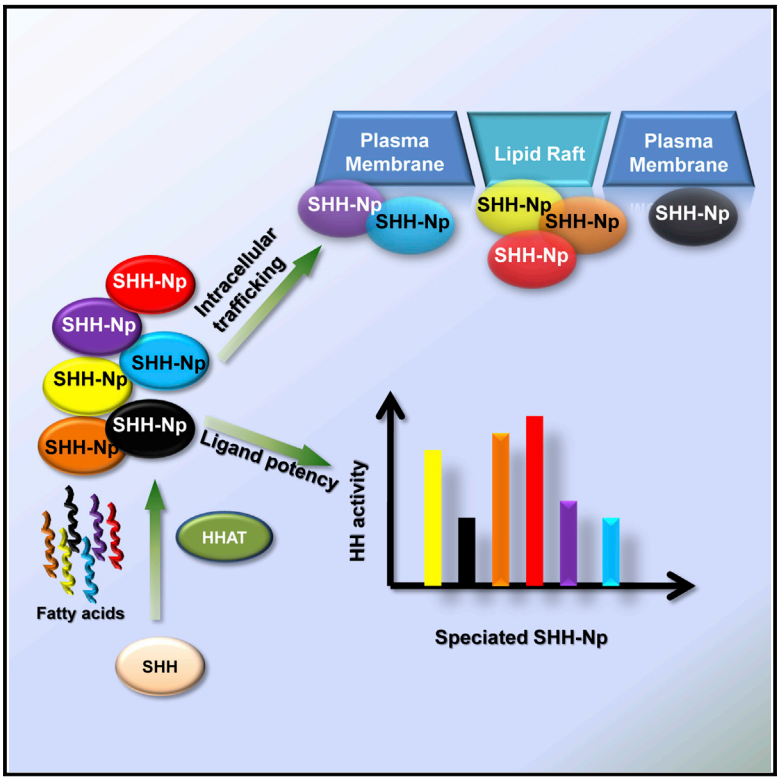
Identification of a Family of Fatty-Acid-Speciated Sonic Hedgehog Proteins, Whose Members Display Differential Biological Properties

Graphical Abstract



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In Brief

Sonic Hedgehog (SHH) is thought to be modified only with palmitate. Long et al. now show that, when SHH is purified from cells expressing endogenous levels, it is modified by a heterogeneous subset of saturated and unsaturated fatty acids. This spectrum of fatty acid modifications on SHH varies depending on the cellular context, and altering the fatty acid composition of SHH in vitro or ex vivo alters its subcellular trafficking and activity.

Highlights

- Sonic Hedgehog was purified from cells expressing endogenous levels
- SHH is covalently modified with a diverse spectrum of fatty acids
- The fatty acid speciation of SHH is cell context dependent
- Fatty-acid-speciated SHH exhibits distinct biological properties

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Identification of a Family of Fatty-Acid-Speciated Sonic Hedgehog Proteins, Whose Members Display Differential Biological Properties

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SUMMARY

Hedgehog (HH) proteins are proteolytically processed into a biologically active form that is covalently modified by cholesterol and palmitate. However, most studies of HH biogenesis have characterized protein from cells in which HH is overexpressed. We purified Sonic Hedgehog (SHH) from cells expressing physiologically relevant levels and showed that it was more potent than SHH isolated from overexpressing cells. Furthermore, the SHH in our preparations was modified with a diverse spectrum of fatty acids on its amino termini, and this spectrum of fatty acids varied dramatically depending on the growth conditions of the cells. The fatty acid composition of SHH affected its trafficking to lipid rafts as well as its potency. Our results suggest that HH proteins exist as a family of diverse lipid-speciated proteins that might be altered in different physiological and pathological contexts in order to regulate distinct properties of HH proteins.

INTRODUCTION

The Hedgehog (HH) family of proteins plays diverse biological roles that are conserved across different classes of animals (Ingham and McMahon, 2001). HH ligands are responsible for embryonic patterning as well as the maintenance, growth, and renewal of various adult structures (Beachy et al., 2004; Ingham and McMahon, 2001). HH proteins harboring missense mutations have also been implicated in human developmental disorders (Bale, 2002), and reactivation of their expression is required for the viability of many cancers (Teglund and Toftgård, 2010). Biochemically, the most extensively studied HH protein is Sonic HH (SHH) (Farzan et al., 2008), which is produced as an ~45 kDa pre-pro-protein that contains an amino-terminal signal sequence targeting SHH to the secretory pathway (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). During SHH's intracellular trafficking, this signal sequence is cleaved off (Bumcrot et al., 1995). A 16 carbon fatty acid, palmitate (C16:0), was reported to modify this newly exposed amino-terminal cysteine via a stable amide bond (Pepinsky et al., 1998) in a reaction catalyzed by the SHH acyltransferase Skinny Hedgehog (Buglino and Resh, 2008; Chamoun et al., 2001). Although this palmitoylated, full-length form of SHH has substantial activity (Tokhunts et al., 2010), the bulk of SHH undergoes additional processing to yield an ~24 kDa amino-terminal form (Chang et al., 1994; López-Martínez et al., 1995; Martí et al., 1995; Roelink et al., 1995). The latter processing step occurs in an intramolecular fashion and results in the addition of cholesterol to the newly exposed carboxyl-terminal glycine via a labile ester bond (SHH-Np) (Lee et al., 1994; Porter et al., 1995).

Very little data regarding the biogenesis of endogenous HH proteins have been published, likely because of the scarcity of endogenous HH proteins and the difficulty of purifying and analyzing such hydrophobic proteins. What is known about the biogenesis of HH proteins is derived from studies in which HH was purified from cells that were engineered to vastly overexpress it (Pepinsky et al., 1998; Porter et al., 1996; Taipale et al., 2000), or from analyses of recombinant HH proteins that lack their hydrophobic modifications (Lee et al., 1994; Pathi et al., 2001; Taylor et al., 2001). Here, we describe the purification of a potent form of SHH-Np from cells that express endogenous-like levels of SHH. Further, we show that this purified SHH-Np actually consists of a family of proteins that are modified at their amino-terminus by a diverse spectrum of saturated and unsaturated fatty acids, and that these novel modifications dictate the biology of HH proteins.

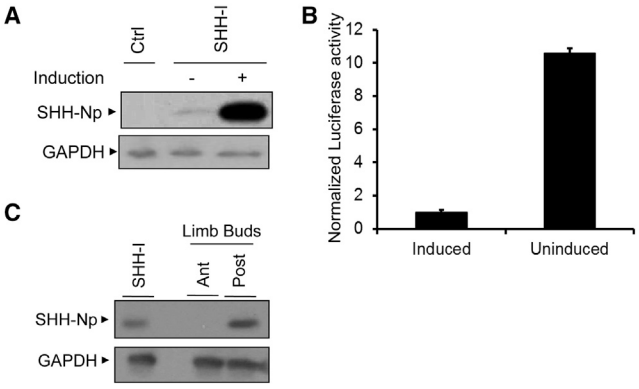

Figure 1. SHH-I Cells Produce Endogenous-like Levels of Potent SHH-Np

(A) An immunoblot showing SHH-Np abundance in SHH-I cells under conditions in which its expression was induced (+) or uninduced (−) with muristerone. SHH-I parental cells, which are not engineered to express *SHH*, were used as a control (Ctrl). GAPDH was used to verify protein normalization.

(B) An aliquot of SHH-I cellular lysate was tested for SHH-Np-associated activity using the Light-II reporter cell line. SHH-Np activity measurements were carried out in the linear range of this assay (see Figure S1B) and these activity results were then normalized to overall SHH-Np levels to determine potency. Error bars represent the SD in one representative experiment.

(C) SHH-Np levels from uninduced SHH-I cells and chick embryo limb buds were compared by immunoblotting. During development, SHH-Np is produced in the posterior portion of limb buds (Post). Here, the anterior (Ant) portion of limb buds serves as a negative control for SHH-Np. As only ~20% of the posterior tissue consists of *SHH*-producing cells (Riddle et al., 1993), we mixed 20% of SHH-I cell lysate with 80% of lysate from anterior limb bud tissue for this comparison. GAPDH was used to verify normalization.

RESULTS

To identify a cell line expressing low levels of *SHH*, we first compared the steady-state levels of SHH-Np for a previously described cell line (SHH-I cells) that expresses *SHH* under the control of a muristerone-inducible promoter (Taipale et al., 2000; Figures 1A and S1A). SHH-I cells produced low levels of SHH-Np in the absence of induction, presumably due to the promiscuity of such inducible promoters, and these levels increased ~20-fold in the presence of muristerone. We next compared the activity of SHH-Np from cell lysates obtained with or without muristerone treatment, measuring the ability of similar amounts of SHH-Np to activate an engineered SHH reporter cell line (Light-II cells) that drives *Firefly* luciferase expression (Taipale et al., 2000; Figures 1B and S1B). The normalized potency of SHH-Np produced under uninduced conditions was significantly higher than that produced when its expression was induced by muristerone. To compare the levels of SHH-Np produced by SHH-I cells with those produced in a physiologically relevant setting (Riddle et al., 1993), we compared the steady-state levels of SHH-Np from uninduced SHH-I cells with those found in dissected posterior and anterior halves of chick limb buds (Figure 1C). SHH-Np levels were similar for uninduced SHH-I cells and posterior chick limb bud tissue.

Using published purification protocols (Pepinsky et al., 1998; Taipale et al., 2000), we were unable to purify SHH-Np from uninduced SHH-I cells expressing such low levels of *SHH*. Thus, we modified these procedures to maximize the yield of active SHH-Np from such cells. Uninduced SHH-I cells were dounce homogenized in an isotonic, detergent-free buffer (Figure 2A) to obtain a total lysate. This cellular lysate was further fractionated by centrifugation at 100,000 × *g*, producing cytoplasmic and membrane-enriched fractions. Similarly prepared lysates of SHH-I parental cells, which do not express detectable amounts of *SHH*, were used as a negative control. The bulk of SHH-Np was in the membrane fraction, consistent with previous reports (Taipale et al., 2000). Aliquots of these lysates, along with the cytoplasmic or membrane-enriched fractions of these cells, were volume normalized and then incubated with Light-II cells to estimate the levels of active SHH in each fraction (data not shown). The bulk of SHH activity was also found in the membrane-enriched pellet.

Detergent extraction of the membrane fraction and purification by centrifugation, ion exchange chromatography, and affinity chromatography resulted in 5 ng of purified SHH-Np per milligram of total cellular lysate (Figure 2B). We estimate the purity of this preparation to be >95%, representing a 200,000-fold purification. The identity of the purified SHH-Np was confirmed by tandem mass spectrometry (MS/MS; data not shown). The vast majority of recovered peptides were derived from the amino-terminal domain of SHH, with coverage against the predicted amino acid sequence of SHH-N approaching 90%. To compare the potency of the SHH-Np isolated here with previously described values (Pathi et al., 2001; Pepinsky et al., 1998; Taipale et al., 2000), we assayed the differentiation of C3H10T1/2 embryonic fibroblasts into osteoblasts (Kinto et al., 1997). The EC_{50} of SHH-Np purified from uninduced SHH-I cells was 0.3 nM, whereas the EC_{50} of recombinant SHH-N was 60 nM (Figure 2C). We also quantified the expression of the SHH target gene *Gli1* as an indicator of activity (Ingram et al., 2002), treating C3H10T1/2 cells with purified SHH-Np (Figure 2D). From this analysis, we estimated the EC_{50} of purified SHH-Np to be 0.2 nM. In both of these assays, the potency of SHH-Np was significantly greater than previously reported (Pathi et al., 2001; Pepinsky et al., 1998; Taipale et al., 2000). Purified SHH-Np was also able to stimulate the proliferation of primary cerebellar granular neuron precursor cells (GPCs) (Dahmane and Ruiz i Altaba, 1999), confirming its activity (Figure S2). Thus, our SHH-Np purification protocol isolates biologically active, potent SHH-Np from cells expressing endogenous-like levels of *SHH*.

We speculated that the increased potency of SHH-Np purified from uninduced SHH-Np cells might result from differential amino-terminal fatty acid modifications, which can alter the activity of recombinant SHH-N in vitro (Pathi et al., 2001; Taylor et al., 2001). To investigate this possibility, we analyzed Lys-C-digested, purified SHH-Np by liquid chromatography (LC)-MS/MS using a high-resolution LTQ Orbitrap mass spectrometer. The mass/charge ratios obtained during these analyses were cross-referenced against expected unmodified masses of individual peptides, and the MS/MS of modified forms was validated manually (Table S1; Figures S3A–S3C). Contrary to previous reports (Pepinsky et al., 1998; Taipale et al., 2000), we identified a diverse assortment of saturated and unsaturated fatty acid

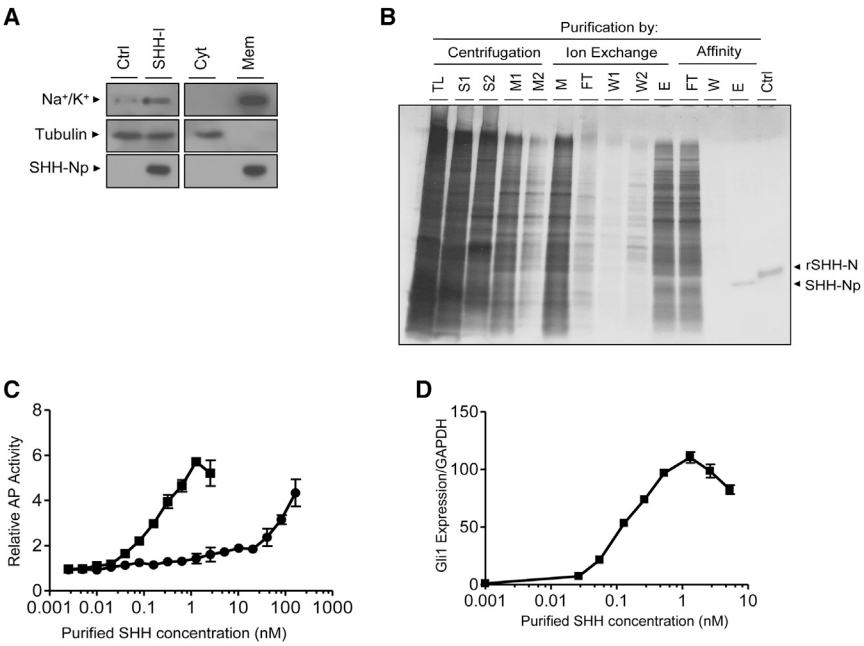

Figure 2. SHH-Np Purified from Low-Level *SHH*-Expressing Cells Is Highly Active

(A) SHH-I cells or the SHH-I parental cell line (Ctrl) were dounce homogenized under isotonic conditions, and total lysate (left panel) was separated by ultracentrifugation at 100,000 × *g* (right panel) to generate a cytosol-enriched fraction (Cyt) and a membrane-enriched fraction (Mem). These fractions were volume normalized to that of the original cellular lysate and immunoblotted as indicated. Tubulin served as a cytosolic protein control and the Na^+/K^+ transporter served as a membrane protein control.

(B) Aliquots of the indicated fractions from various steps of SHH-Np purification were separated by SDS-PAGE, followed by visualization of proteins by silver staining (TL, total lysate; S, 100,000 × *g* supernatant; M, combined 100,000 × *g* pellet detergent extract; FT, non-bound material; W, column wash; E, column eluate). Recombinant, unmodified SHH-N is shown as a control (rSHH-N). Electrophoretic retardation of rSHH-N relative to cholesterol-modified SHH-Np was previously noted (Lee et al., 1994).

(C) The indicated amounts of purified SHH-Np were incubated with C3H10T1/2 fibroblasts, which differentiate into osteoblasts in response to SHH (squares: purified SHH-Np; circles: rSHH-N). Alkaline phosphatase activity, which is an indirect, quantitative measurement of this differentiation, was then measured.

(D) The indicated amounts of purified SHH-Np were incubated with C3H10T1/2 fibroblasts, followed by RNA extraction. The levels of *Gli1* and *GAPDH* were then determined by qRT-PCR. Error bars represent the SD in one representative experiment.

See also Figure S2.

modifications on SHH-Np. Based on extracted ion chromatogram (XIC) peak areas, the most abundant fatty acid-modified forms of SHH-Np were modified with palmitate (C16:0), a palmitoleoyl (C16:1), followed by a stearoleyl (C18:1), a myristoleyl (C14:1), and then to a lesser degree, a stearoyl (C18:0) and myristoyl (C14:0) groups. A number of amino-terminal peptides showed masses encompassing as yet undetermined modifications, consistent with SHH-Np being modified by a diversity of lipid species.

To extend these findings, we also purified SHH-Np from SHH-I cells induced to express higher levels of *SHH* (1) in the presence of 10% fetal bovine serum (FBS), (2) in the presence of serum-free media, and (3) in the presence of serum-free media supplemented with myristate (C14:0). The pattern of lipid speciation on SHH-Np changed significantly under all of these conditions, suggesting that the lipid speciation of SHH is very sensitive to the cellular context (Figure 3). We noted that unsaturated fatty acid-modified SHH-Np was the dominant species regardless of the cellular context, whereas the abundance of SHH-Np forms modified with saturated fatty acids was significantly more variable. Interestingly, the fatty acid modifications on SHH-Np isolated from cells that expressed high levels of *SHH* and were grown in FBS approximately mirrored the abundance of fatty acids found in the membranes of cells grown in FBS, whereas the lipid-speciated forms of SHH-Np purified from cells grown under the other conditions did not. We further noted that SHH-Np isolated from cells grown under serum-free conditions but supplemented with myristate (C14:0) showed an ~500% increase of myristate-modified SHH-Np (data not shown), consistent with our ability to experimentally manipulate the fatty acid speciation of SHH-Np. Significantly, we did not detect an amino-terminal peptide lacking fatty acid modifications in these experiments. Although MS is not generally quantitative, the abundance of the same peptide in different samples may be quantitatively compared (Old et al., 2005). Therefore, the absence of an unmodified amino-terminal peptide in our extracted chromatograms suggests that SHH-Np is quantitatively modified by fatty acids in these cells.

The hydrophobic properties of the various fatty acid modifications we observed on SHH-Np vary over a 500-fold range (Table S1), suggesting that they would alter the biological properties of SHH-Np. Further, unsaturated fatty acids, such as those found on SHH-Np, are known to segregate away from lipid rafts (Levental et al., 2010), where HH proteins are thought to enrich as part of their regulated intracellular movement (Callejo et al., 2011; Creanga et al., 2012; Mao et al., 2009; Rietveld et al., 1999; Taipale et al., 2000). To test this hypothesis, we altered the fatty acid composition of media used with cells or chick limb bud explants expressing *SHH*, and then measured various properties of the resulting SHH-Np. Such lipid-doping experiments have previously been used to alter the covalent lipid modifications of numerous proteins (Hashimoto et al., 2004; Liang et al., 2001; Wolven et al., 1997) before determining changes in their biological function. We therefore incubated serum-deprived SHH-I cells with saturated C14:0, C16:0, C18:0, or unsaturated C16:1 fatty acids, and examined the levels of SHH-Np in both cell lysates and secreted from those cells into conditioned media. Although we did not observe changes in the absolute levels

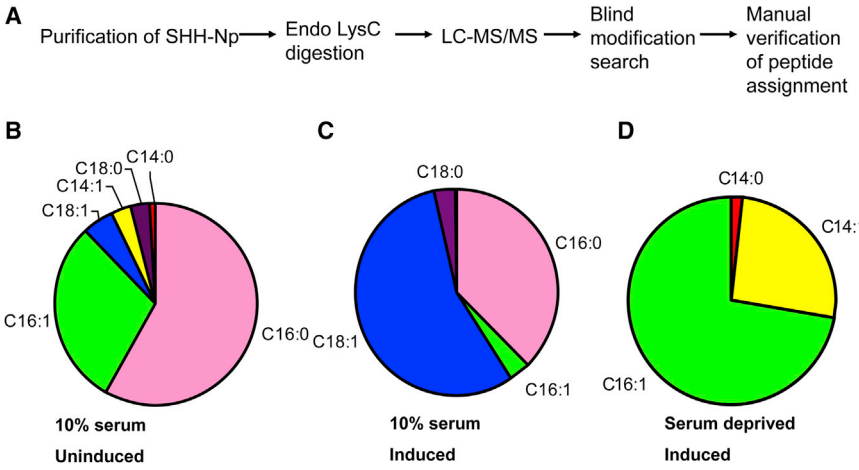

Figure 3. Fatty Acid Speciation of SHH-Np Is Dependent on the Cellular Context

(A) A schematic showing the procedure used to identify the fatty acid modification on SHH-Np.

(B–D) Pie charts showing the relative abundance of lipid species identified on SHH-Np that was purified and isolated under three different cellular contexts: 10% FBS without muristerone induction of SHH expression, 10% FBS and muristerone induction of SHH expression, and serum deprivation and muristerone induction of SHH expression.

See also Figure S3 and Table S1.

of cell-associated SHH-Np when cells were doped with different fatty acids, consistent with previous reports (Bumcrot et al., 1995), we did observe differences in the secretion of SHH-Np forms from cells incubated with different fatty acids (Figure 4A).

Because lipid raft localization is thought to be a prerequisite for the secretion of HH proteins (Callejo et al., 2011; Creanga et al., 2012; Rietveld et al., 1999), we asked whether the various fatty acid-modified SHH-Np forms would differentially localize to lipid rafts. Therefore, we fractionated the cellular lysates of cells incubated with various fatty acids over an OptiPrep gradient to isolate lipid raft-enriched fractions (Figure 4B and data not shown). Incubation of uninduced SHH-I cells with any of the tested saturated fatty acids (C14:0, C16:0, or C18:0) increased the percentage of SHH-Np that was enriched in lipid rafts (Figure 4C). In contrast, incubation of SHH-I cells with palmitoleate (C16:1) reduced the percentage of SHH-Np that was enriched in lipid rafts. Similar experiments were performed on anterior or posterior chick limb bud explants. Consistent with our SHH-I cell-based observations, treatment of the explants with unsaturated C16:1 resulted in decreased SHH-Np localization to lipid rafts in posterior tissue (Figure 5A). Although incubation of tissue explants with saturated fatty acids had no effect on steady-state SHH-Np levels, increased levels of tissue SHH-Np were observed upon palmitoleoyl (C16:1) incubation. The mRNA levels of *SHH* were unchanged by incubation with different fatty acids (data not shown). These results are consistent with decreased secretion of palmitoleoyl (C16:1)-modified SHH-Np, resulting in increased retention in posterior limb bud tissue. We further measured the activity of SHH-Np from these tissues and normalized this activity to their relative abundance (Figures 5B and 5C). This analysis showed that incubation of posterior limb bud fragments with C16:1 reduced SHH-Np activity.

DISCUSSION

We now demonstrate that SHH-Np is actually a family of distinct lipid-speciated forms that exhibit a variety of differential properties. Thus, we favor the idea that modification of SHH-Np by a spectrum of fatty acids provides another biologically relevant layer of SHH-Np regulation. Although the spectrum of fatty acid modifications on SHH-Np was not initially described in previous studies, a similar spectrum of modifications for a small percentage of the SHH mutant SHH-N, which is a non-physiologically relevant form of SHH that is not cholesterol modified, was described (Pepinsky et al., 1998). We speculate that differences in purification protocols or levels of expression resulted in the identification of only the most abundant, palmitoyl-modified form of SHH-Np in these previous reports. For example, one of the previous purification protocols for SHH-Np started with a lipid-raft-enriched fraction (Taipale et al., 2000), which based on our data might exclude SHH-Np modified by unsaturated fatty acids. How such differential forms of SHH-Np might arise is also not yet clear. However, in vitro, Skinny Hedgehog is able to utilize a wide spectrum of fatty acids, many of which have a higher affinity for Skinny Hedgehog than palmitate (Buglino and Resh, 2008). Interestingly, the potency of recombinant forms of SHH-N is altered when it is modified by different fatty acids in vitro (Taylor et al., 2001). This observation is consistent with Skinny Hedgehog being sufficient to modify SHH with the diverse spectrum of fatty acids described here.

Our findings suggest that one consequence of SHH-Np's fatty acid speciation is the regulation of its intracellular trafficking, with decreased localization of SHH-Np modified with unsaturated fatty acids to lipid rafts. This decreased localization is likely the result of unsaturated fatty acids lacking the compactness required to enrich in lipid raft compartments of cellular membranes (Levental et al., 2010), although such fatty acid doping experiments likely result in the production of a number of different fatty acylated species. However, a simple differential localization of SHH-Np proteins to the lipid raft or non-lipid raft compartments of cellular membranes could arise solely by regulating the degree of fatty acid saturation on SHH-Np. One observed functional consequence of this differential localization is the secretion of lipid-raft-localized SHH-Np forms and the retention of raft-excluded forms. Such SHH-Np speciation might then contribute to the gradient of SHH-Np observed in vivo (Gritli-Linde et al., 2001), with SHH-Np modified with the least hydrophobic fatty acids moving farther away from the *SHH*-producing cells than SHH-Np family members modified with more hydrophobic fatty acids. In such a scenario, different fatty acid modifications might modulate SHH-Np's affinity for the various lipoprotein complexes that have been suggested to regulate

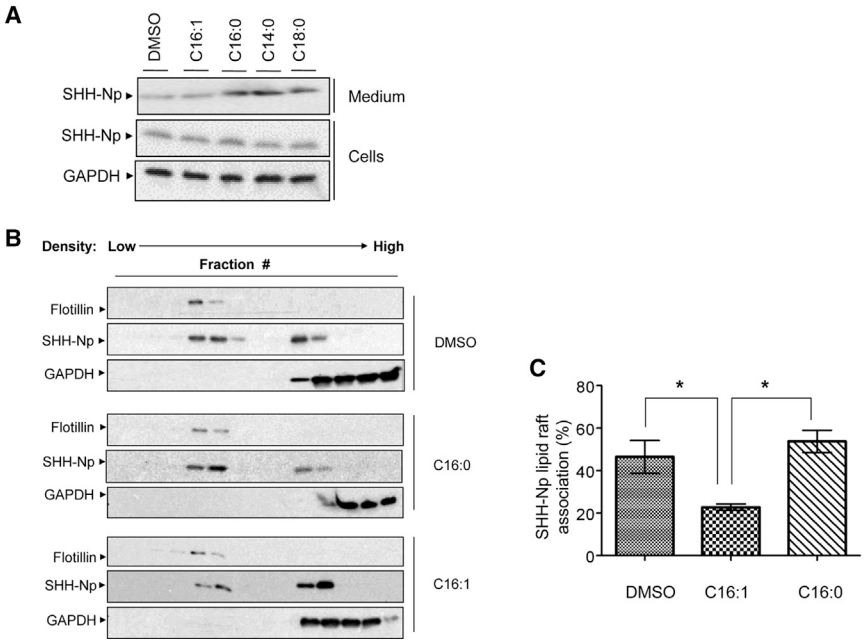

Figure 4. Fatty Acid Speciation of SHH-Np Alters Its Lipid Raft Enrichment

(A) An immunoblot of cell lysates and conditioned media from SHH-I cells incubated in the presence of indicated fatty acids. GAPDH served as a normalization control for cellular lysates. The same volume of conditioned media was subjected to TCA precipitation prior to loading.

(B) Lysates from SHH-I cells incubated with the indicated lipids or DMSO control were separated over an OptiPrep gradient to isolate a lipid-raft-enriched fraction. Fractions from these various OptiPrep density gradients were resolved by SDS-PAGE and then analyzed by immunoblotting for SHH-Np, GAPDH as a cytoplasmic protein marker, or the lipid raft marker flotillin. Note that flotillin localization did not change with various lipid additions.

(C) Quantification of SHH-Np lipid raft enrichment from cells incubated with the indicated fatty acids. Error bars represent the SEM of three independent experiments; p values $\leq$ 0.05 are considered statistically significant and are indicated by an asterisk.

the movement of HH proteins (Callejo et al., 2008; Gradilla et al., 2014; Guerrero and Chiang, 2007; Matusek et al., 2014; Palm et al., 2013; Thérond, 2012; Zeng et al., 2001). Alternatively, fatty acid speciation of SHH-Np might alter its targeting "barcode" (Kornberg, 2011), allowing it to associate with diverse types of lipid microdomains enriched on the cytonemes that are responsible for HH movement (Fifadara et al., 2010; Gupta and DeFranco, 2003; Kornberg, 2013; Sanders et al., 2013). In either model, the fatty acid speciation of SHH described here might be utilized to encode dramatic changes in cellular growth and metabolism, such as those that occur during early development or cancer, directly into the HH proteins that regulate these biological processes.

EXPERIMENTAL PROCEDURES

Comparison of SHH-Np Levels

Fertile, certified research-grade, pathogen-free eggs (Charles River) were incubated at 37.5°C. At Hamburger-Hamilton (H&H) developmental stage 22, embryos were isolated and the limb buds were resected as previously described (Zeng et al., 2001). Resected buds were further divided into SHH-producing posterior and SHH-negative anterior portions, and lysed by suspension in 1% Tx-100, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 7.4, supplemented with protease inhibitors (Roche). Immunoblotting was performed using anti SHH-Np polyclonal H-160 antibodies (Santa Cruz).

Purification of SHH-Np

SHH-I cells (Taipale et al., 2000) were washed with PBS, collected by scraping, dounce homogenized, and centrifuged at 100,000 × *g* for 1 hr. The resultant pellet was resuspended in buffer A and recentrifuged, and the membrane-enriched pellet was extracted twice with buffer B by dounce homogenization and centrifugation at 16,000 × *g* for 30 min. The supernatants were combined, pH adjusted to 5.0 with 1 M 2-[N-morpholino]ethanesulfonic acid (MES), and applied to a bulk SP Sepharose Fast Flow resin. This resin was washed once with buffer C, followed by a second wash with buffer D. SHH-Np was eluted from this resin using buffer E. The eluted fractions were adjusted to pH 7.2 with 1 M HEPES, and then passed through a 5E1 monoclonal antibody (mAb) column (Ericson et al., 1996). After the column was washed with buffer F, the SHH-Np was eluted with buffer G and then immediately neutralized with 1 M HEPES, pH 7.4. Please refer to the Supplemental Experimental Procedures for details regarding buffers A–G.

After SDS-PAGE, the purified SHH-Np was quantified by comparing its concentration against a standard curve of recombinant SHH-II (R&D). This gel was subsequently protein stained using a SilverQuest staining kit (Invitrogen). The optical density of each stained protein was then calculated and compared using ImageJ software (NIH). SHH activity measurements were performed essentially as described previously (Singh et al., 2009), using NIH 3T3 cells expressing an HH reporter gene (Light-II cells). SHH-Np-dependent differentiation and gene expression were assayed using the C3H10T1/2 cell line as previously described (Zeng et al., 2001). All activity measurements were done in triplicate and each experiment was repeated at least three times. The activity data presented are shown as the mean and SD of one representative experiment.

MS Analyses

The identity of the purified SHH-Np was validated by microcapillary liquid chromatography/MS (LC/MS) on a ThermoFinnigan LTQ ion trap mass spectrometer. For identification of fatty acid modifications, SHH-Np was reduced with 4 mM DTT, alkylated with 15 mM iodoacetamide, and EndoLysC digested. Lipid modified peptides were identified by MS/MS analysis on a LTQ-Orbitrap (Thermo) interfaced with an Eksigent nanoLC-2D HPLC. MS/MS spectra were searched against the SHH protein sequence using a Mascot (v 2.1) error-tolerant search with 20 ppm parent mass accuracy, and Inspect/MS-Alignment run in blind modification search mode (Tanner et al., 2006). All MS/MS spectra peptide assignments were manually verified for peptide assignments.

Lipid Treatments

SHH-I cells were serum deprived for 6–7 hr in 0.5% FBS. Prior to lipid treatment, the cells were washed with PBS once and then maintained in the presence of 100 μM fatty acids or DMSO (vehicle) for 16–18 hr (Liang et al., 2001). For embryonic tissue studies, 10–12 pathogen-free H&H developmental stage 22 chick embryos (Charles River) were collected (Zeng et al., 2001) and posterior fragments of the limb buds were dissected as previously described (Zeng et al., 2001). These posterior tissues were incubated with 100 μM fatty acids or DMSO (vehicle) for 16–18 hr in six-well plates. These

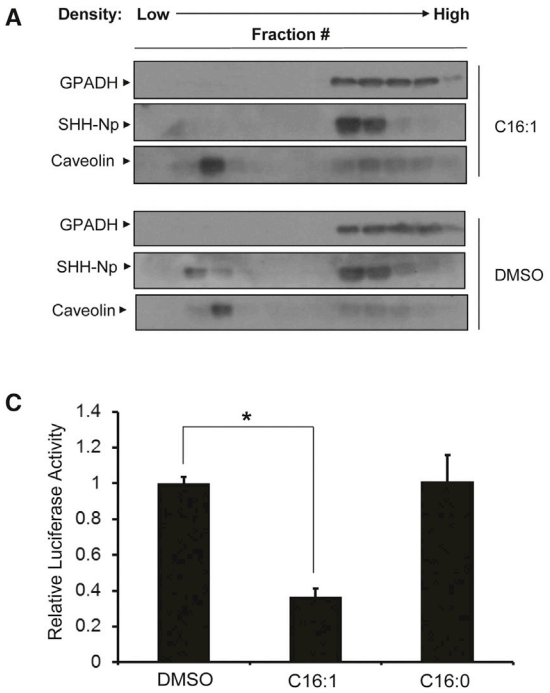

Figure 5. Modification of SHH-Np with Distinct Fatty Acids Alters Its Functionality

(A) Lysates from embryonic limb bud explants exposed to different fatty acids were separated over an OptiPrep density gradient to isolate the lipid raft fraction. Various gradient fractions were resolved by SDS-PAGE and then analyzed by immunoblotting for SHH-Np. Caveolin-1 was used as a lipid raft marker, and GAPDH served to label non-lipid-raft-associated subcellular fractions. Treatment with saturated fatty acids did not change the SHH-Np localization pattern compared with the DMSO control (data not shown).

(B) Upper panel: an immunoblot of limb bud lysates shows the effect of lipid modifications on SHH-Np levels. Lower panel: the lysates contain similar amounts of total protein, as indicated by total protein silver staining.

(C) The potency of SHH-Np was determined by incubating lysates from treated limb buds with Light-II cells and then normalizing this activity to SHH-Np levels. Error bars represent the SEM of three independent experiments; p values ≤ 0.05 are considered statistically significant and are indicated by an asterisk.

tissues were subsequently washed twice in ice-cold PBS and homogenized in a 1% Tx-100, 10 mM sodium phosphate, 150 mM NaCl buffer (pH 6.5).

OptiPrep Density Gradient Ultracentrifugation

Cell or tissue extract was mixed with OptiPrep medium to obtain a 40% fraction. Then two other fractions, composed of 25% and 10% OptiPrep medium or 30% and 0% for tissue extract, were sequentially layered on top followed by centrifugation at 120,000 × *g*, or 160,000 × *g* for tissue extract, for 21 hr (Brusés et al., 2001; Chen et al., 2004; Lisanti et al., 1994). Fractions from the tubes were collected and subjected to SDS-PAGE analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.058.

AUTHOR CONTRIBUTIONS

J.L. performed the purification of SHH from different cellular contexts and performed experiments to determine SHH's potency lipid raft association. R.T. optimized the purification protocol and performed additional experiments to determine SHH potency. W.M.O. performed LC-MS/MS on purified SHH and identified the various fatty acid modifications on SHH described here.

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REFERENCES

Bale, A.E. (2002). Hedgehog signaling and human disease. Annu. Rev. Genomics Hum. Genet. *3*, 47–65.

Beachy, P.A., Karhadkar, S.S., and Berman, D.M. (2004). Tissue repair and stem cell renewal in carcinogenesis. Nature *432*, 324–331.

Brusés, J.L., Chauvet, N., and Rutishauser, U. (2001). Membrane lipid rafts are necessary for the maintenance of the (alpha)7 nicotinic acetylcholine receptor in somatic spines of ciliary neurons. J. Neurosci. *21*, 504–512.

Buglino, J.A., and Resh, M.D. (2008). Hhat is a palmitoylacyltransferase with specificity for N-palmitoylation of Sonic Hedgehog. J. Biol. Chem. *283*, 22076–22088.

Bumcrot, D.A., Takada, R., and McMahon, A.P. (1995). Proteolytic processing yields two secreted forms of sonic hedgehog. Mol. Cell. Biol. *15*, 2294–2303.

Callejo, A., Culi, J., and Guerrero, I. (2008). Patched, the receptor of Hedgehog, is a lipoprotein receptor. Proc. Natl. Acad. Sci. USA *105*, 912–917.

Callejo, A., Bilioni, A., Mollica, E., Gorfinkiel, N., Andrés, G., Ibáñez, C., Torroja, C., Doglio, L., Sierra, J., and Guerrero, I. (2011). Dispatched mediates Hedgehog basolateral release to form the long-range morphogenetic gradient in the Drosophila wing disk epithelium. Proc. Natl. Acad. Sci. USA *108*, 12591–12598.

Chamoun, Z., Mann, R.K., Nellen, D., von Kessler, D.P., Bellotto, M., Beachy, P.A., and Basler, K. (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. Science *293*, 2080–2084.

Chang, D.T., López, A., von Kessler, D.P., Chiang, C., Simandl, B.K., Zhao, R., Seldin, M.F., Fallon, J.F., and Beachy, P.A. (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. Development *120*, 3339–3353.

Chen, M.H., Li, Y.J., Kawakami, T., Xu, S.M., and Chuang, P.T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev. *18*, 641–659.

Creanga, A., Glenn, T.D., Mann, R.K., Saunders, A.M., Talbot, W.S., and Beachy, P.A. (2012). Scube/You activity mediates release of dually lipid-modified Hedgehog signal in soluble form. Genes Dev. *26*, 1312–1325.

Dahmane, N., and Ruiz i Altaba, A. (1999). Sonic hedgehog regulates the growth and patterning of the cerebellum. Development *126*, 3089–3100.

Echelard, Y., Epstein, D.J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J.A., and McMahon, A.P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell *75*, 1417–1430.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T.M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell *87*, 661–673.

Farzan, S.F., Singh, S., Schilling, N.S., and Robbins, D.J. (2008). The adventures of sonic hedgehog in development and repair. III. Hedgehog processing and biological activity. Am. J. Physiol. Gastrointest. Liver Physiol. *294*, G844–G849.

Fifadara, N.H., Beer, F., Ono, S., and Ono, S.J. (2010). Interaction between activated chemokine receptor 1 and FcepsilonRI at membrane rafts promotes communication and F-actin-rich cytoneme extensions between mast cells. Int. Immunol. *22*, 113–128.

Gradilla, A.C., González, E., Seijo, I., Andrés, G., Bischoff, M., González-Mendez, L., Sánchez, V., Callejo, A., Ibáñez, C., Guerra, M., et al. (2014). Exosomes as Hedgehog carriers in cytoneme-mediated transport and secretion. Nat. Commun. *5*, 5649.

Gritli-Linde, A., Lewis, P., McMahon, A.P., and Linde, A. (2001). The whereabouts of a morphogen: direct evidence for short- and graded long-range activity of hedgehog signaling peptides. Dev. Biol. *236*, 364–386.

Guerrero, I., and Chiang, C. (2007). A conserved mechanism of Hedgehog gradient formation by lipid modifications. Trends Cell Biol. *17*, 1–5.

Gupta, N., and DeFranco, A.L. (2003). Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. Mol. Biol. Cell *14*, 432–444.

Hashimoto, Y., Matsuda, T., Matsuura, Y., Haga, T., and Fukada, Y. (2004). Production of N-lauroylated G protein alpha-subunit in Sf9 insect cells: the type of N-acyl group of Galpha influences G protein-mediated signal transduction. J. Biochem. *135*, 319–329.

Ingham, P.W., and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.

Ingram, W.J., Wicking, C.A., Grimmond, S.M., Forrest, A.R., and Wainwright, B.J. (2002). Novel genes regulated by Sonic Hedgehog in pluripotent mesenchymal cells. Oncogene *21*, 8196–8205.

Kinto, N., Iwamoto, M., Enomoto-Iwamoto, M., Noji, S., Ohuchi, H., Yoshioka, H., Kataoka, H., Wada, Y., Yuhao, G., Takahashi, H.E., et al. (1997). Fibroblasts expressing Sonic hedgehog induce osteoblast differentiation and ectopic bone formation. FEBS Lett. *404*, 319–323.

Kornberg, T.B. (2011). Barcoding Hedgehog for intracellular transport. Sci. Signal. *4*, pe44.

Kornberg, T.B. (2013). Cytonemes extend their reach. EMBO J. *32*, 1658–1659.

Krauss, S., Concordet, J.P., and Ingham, P.W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell *75*, 1431–1444.

Lee, J.J., Ekker, S.C., von Kessler, D.P., Porter, J.A., Sun, B.I., and Beachy, P.A. (1994). Autoproteolysis in hedgehog protein biogenesis. Science *266*, 1528–1537.

Levental, I., Grzybek, M., and Simons, K. (2010). Greasing their way: lipid modifications determine protein association with membrane rafts. Biochemistry *49*, 6305–6316.

Liang, X., Nazarian, A., Erdjument-Bromage, H., Bornmann, W., Tempst, P., and Resh, M.D. (2001). Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction. J. Biol. Chem. *276*, 30987–30994.

Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.H., Cook, R.F., and Sargiacomo, M. (1994). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. J. Cell Biol. *126*, 111–126.

López-Martínez, A., Chang, D.T., Chiang, C., Porter, J.A., Ros, M.A., Simandl, B.K., Beachy, P.A., and Fallon, J.F. (1995). Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. Curr. Biol. *5*, 791–796.

Mao, H., Diehl, A.M., and Li, Y.X. (2009). Sonic hedgehog ligand partners with caveolin-1 for intracellular transport. Lab. Invest. *89*, 290–300.

Martí, E., Bumcrot, D.A., Takada, R., and McMahon, A.P. (1995). Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. Nature *375*, 322–325.

Matusek, T., Wendler, F., Polès, S., Pizette, S., D'Angelo, G., Fürthauer, M., and Thérond, P.P. (2014). The ESCRT machinery regulates the secretion and long-range activity of Hedgehog. Nature *516*, 99–103.

Old, W.M., Meyer-Arendt, K., Aveline-Wolf, L., Pierce, K.G., Mendoza, A., Sevinsky, J.R., Resing, K.A., and Ahn, N.G. (2005). Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol. Cell. Proteomics *4*, 1487–1502.

Palm, W., Swierczynska, M.M., Kumari, V., Ehrhart-Bornstein, M., Bornstein, S.R., and Eaton, S. (2013). Secretion and signaling activities of lipoprotein-associated hedgehog and non-sterol-modified hedgehog in flies and mammals. PLoS Biol. *11*, e1001505.

Pathi, S., Pagan-Westphal, S., Baker, D.P., Garber, E.A., Rayhorn, P., Bumcrot, D., Tabin, C.J., Blake Pepinsky, R., and Williams, K.P. (2001). Comparative biological responses to human Sonic, Indian, and Desert hedgehog. Mech. Dev. *106*, 107–117.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. J. Biol. Chem. *273*, 14037–14045.

Porter, J.A., von Kessler, D.P., Ekker, S.C., Young, K.E., Lee, J.J., Moses, K., and Beachy, P.A. (1995). The product of hedgehog autoproteolytic cleavage active in local and long-range signalling. Nature *374*, 363–366.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. Science *274*, 255–259.

Riddle, R.D., Johnson, R.L., Laufer, E., and Tabin, C. (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. Cell *75*, 1401–1416.

Rietveld, A., Neutz, S., Simons, K., and Eaton, S. (1999). Association of sterol- and glycosylphosphatidylinositol-linked proteins with Drosophila raft lipid microdomains. J. Biol. Chem. *274*, 12049–12054.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T.M., et al. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell *76*, 761–775.

Roelink, H., Porter, J.A., Chiang, C., Tanabe, Y., Chang, D.T., Beachy, P.A., and Jessell, T.M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell *81*, 445–455.

Sanders, T.A., Llagostera, E., and Barna, M. (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. Nature *497*, 628–632.

Singh, S., Tokhunts, R., Baubet, V., Goetz, J.A., Huang, Z.J., Schilling, N.S., Black, K.E., MacKenzie, T.A., Dahmane, N., and Robbins, D.J. (2009). Sonic hedgehog mutations identified in holoprosencephaly patients can act in a dominant negative manner. Hum. Genet. *125*, 95–103.

Taipale, J., Chen, J.K., Cooper, M.K., Wang, B., Mann, R.K., Milenkovic, L., Scott, M.P., and Beachy, P.A. (2000). Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature *406*, 1005–1009.

Tanner, S., Pevzner, P.A., and Bafna, V. (2006). Unrestrictive identification of post-translational modifications through peptide mass spectrometry. Nat. Protoc. *1*, 67–72.

Taylor, F.R., Wen, D., Garber, E.A., Carmillo, A.N., Baker, D.P., Arduini, R.M., Williams, K.P., Weinreb, P.H., Rayhorn, P., Hronowski, X., et al. (2001).

Enhanced potency of human Sonic hedgehog by hydrophobic modification. Biochemistry *40*, 4359–4371.

Teglund, S., and Toftgård, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim. Biophys. Acta *1805*, 181–208.

Thérond, P.P. (2012). Release and transportation of Hedgehog molecules. Curr. Opin. Cell Biol. *24*, 173–180.

Tokhunts, R., Singh, S., Chu, T., D'Angelo, G., Baubet, V., Goetz, J.A., Huang, Z., Yuan, Z., Ascano, M., Zavros, Y., et al. (2010). The full-length unprocessed hedgehog protein is an active signaling molecule. J. Biol. Chem. *285*, 2562–2568.

Wolven, A., Okamura, H., Rosenblatt, Y., and Resh, M.D. (1997). Palmitoylation of p59fyn is reversible and sufficient for plasma membrane association. Mol. Biol. Cell *8*, 1159–1173.

Zeng, X., Goetz, J.A., Suber, L.M., Scott, W.J., Jr., Schreiner, C.M., and Robbins, D.J. (2001). A freely diffusible form of Sonic hedgehog mediates long-range signalling. Nature *411*, 716–720.