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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, which is covalently modified by cholesterol and palmitate. However, most studies of HH biogenesis have characterized protein from cells in which HH is over-expressed. We purified Sonic Hedgehog (SHH) from cells expressing physiologically relevant levels, and showed that it was more potent than SHH isolated from over-expressing cells. Furthermore, the SHH in our preparations were

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AUTHOR CONTRIBUTIONS

Jun Long performed the purification of SHH from different cellular contexts, and performed experiments to determine SHH's potency lipid raft association. Robert Tokhunts optimized the purification protocol, and performed additional experiments to determine SHH potency. Will Old performed LC-MS/MS on purified SHH and identified the various fatty acid modifications on SHH described here.

modified with a diverse spectrum of fatty acids on their 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, which might be altered in different physiological and pathological contexts to regulate distinct properties of HH proteins.

Keywords

Sonic Hedgehog; purification; fatty acids; lipids; covalent modifications

INTRODUCTION

The Hedgehog (HH) family of proteins play 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 Toftgard, 2010). Biochemically, the most extensively studied HH protein is Sonic HH (SHH) (Farzan et al., 2008), which is produced as a ~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 sixteen 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 a ~24 kDa amino-terminal form (Chang et al., 1994; Lopez-Martinez et al., 1995; Marti et al., 1995; Roelink et al., 1995). This 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, 1995; Lee et al., 1994; Porter et al., 1995).

Very little data regarding the biogenesis of endogenous HH proteins has 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 where HH has been purified from cells engineered to vastly overexpress it (Pepinsky et al., 1998; Porter et al., 1996; Taipale et al., 2000), or from the 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 modified at its amino-

terminus by a diverse spectrum of saturated and unsaturated fatty acids, and that these novel modifications dictate the biology of HH proteins.

EXPEIRMENTAL 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 limb buds 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 suspending 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, recentrifuged, and the membrane enriched pellet 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 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 washing the column 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 Supplementary Materials for details of buffers A-G.

Purified SHH-Np was quantified after SDS-PAGE, 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 Image J software (NIH). SHH activity measurements were performed essentially as described, using NIH-3T3 cells expressing a HH reporter gene (Light-II cells) (Singh et al., 2009). SHH-Np dependent differentiation and gene expression were assayed using 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 is shown as the mean and the standard deviation (SD) of one representative experiment.

Mass Spectrometry Analyses

The identity of the purified SHH-Np was validated using microcapillary 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 tandem mass spectrometry analysis on a LTQ-Orbitrap (Thermo) interfaced with an Eksigent nanoLC-2D HPLC. Tandem mass spectra (MS/MS) 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 hrs 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 hrs (Liang et al., 2001). For embryonic tissue studies, 10-12 pathogen free Hamburger-Hamilton (H&H) developmental stage 22 chick embryos (Charles River) were collected (Zeng et al., 2001), and posterior fragments of the limb buds dissected as previously described (Zeng et al., 2001). These posterior tissues were incubated with 100 μM fatty acids or DMSO (vehicle) for 16-18 hrs in 6 well plates. These 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, sequentially layered on top followed by centrifugation at 120,000 × g, or 160,000 × g for tissue extract, for 21 hrs (Bruses et al., 2001; Chen et al., 2004; Lisanti et al., 1994). Fractions from the tubes were collected and subjected to SDS-PAGE analysis.

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 control of a muristerone inducible promoter (Taipale et al., 2000) (**Fig. 1A** and **Fig 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 approximately twenty-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) driving *Firefly* luciferase expression (Taipale et al., 2000) (**Fig. 1B** and **Fig 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 to that produced in a physiologically relevant setting (Riddle et al., 1993), we compared the steady-state levels of SHH-Np from uninduced SHH-I cells to dissected posterior and anterior halves of chick limb buds (**Fig. 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

(**Fig. 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 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 (see Supplementary Experimental Procedures) resulted in 5 ng of purified SHH-Np per mg of total cellular lysate (**Fig. 2B**). We estimate the purity of this preparation to be greater than 95%, representing a 200,000-fold purification. The identity of the purified SHH-Np was confirmed by tandem mass spectrometry (data not shown). The vast majority of recovered peptides were derived from the amino-terminal domain of SHH, with a coverage against the predicted amino-acid sequence of SHH-N approaching 90%. To compare the potency of the SHH-Np isolated here to those previously described (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, while the EC_{50} of recombinant SHH-N was 60 nM (**Fig. 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 (**Fig. 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 (GPC) (Dahmane and Ruiz i Altaba, 1999), confirming its activity (**Fig. 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 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 validated manually (**Table S1 and Fig. S3A-C**). Contrary to previous reports (Pepinsky et al., 1998; Taipale et al., 2000), we identified a diverse assortment of saturated and unsaturated fatty acid 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% FBS, 2) in the presence 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 cellular context (**Fig. 3)**. We noted that unsaturated fatty acid modified SHH-Np is the dominant species regardless of the cellular context, whereas the abundance of SHH-Np forms modified with saturated fatty acids were significantly more variable. Interestingly, the fatty acid modifications on SHH-Np isolated from cells expressing high levels of *SHH*, 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 us being able 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 its 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), prior to determining their changes in 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. While we did not observe changes in the absolute levels 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 (**Fig. 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. The cellular lysates of cells incubated with various fatty acids were therefore fractionated over an OptiPrep gradient to isolate lipid raft enriched fractions (**Fig. 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 enriched in lipid rafts (**Fig. 4C**). In contrast, incubation of SHH-I cells with palmitoleate (C16:1) reduced the percentage of SHH-Np 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 (**Fig. 5A**). While 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 different fatty acid incubation (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 (**Fig. 5B** and **Fig. 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, which 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, 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 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 further 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 suggested to regulate 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; Therond, 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 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 occurring during early development or cancer, directly into the HH proteins regulating these biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Changes

- Editing **Title** into 3 lines of text at no more than 50 characters per line
- Reduction of **Abstract** size to comply with the restriction (less than 150 words)
- Moving **Table 1** to supplemental materials as **Table S1** to comply with the restriction of the number of Figures/tables for a Report (no more than 5)
- Editing Experimental Procedures of SHH-Np purification and Mass Spectrometry
- Reduction of manuscript length to comply with the general length restriction for a Report (less than 42,000 characters)

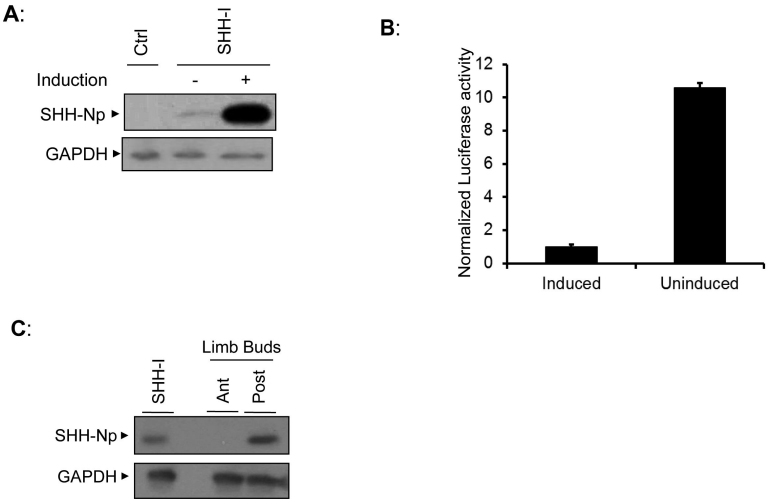

Figure 1. SHH-I cells produce endogenous-like levels of potent SHH-Np

(**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 standard deviation (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 approximately 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.

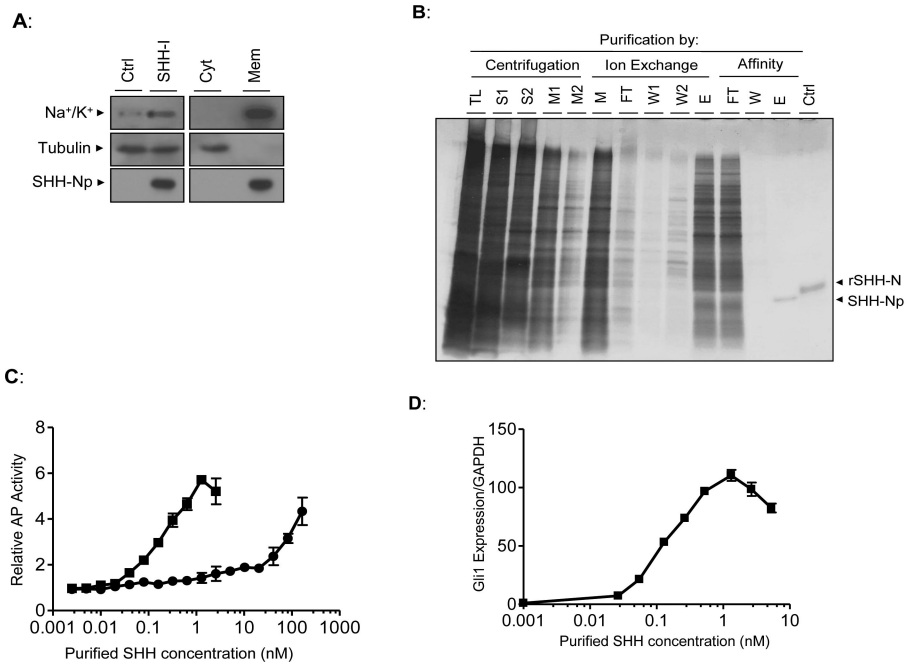

Figure 2. SHH-Np purified from low-level *SHH* expressing cells is highly active

(**A**) SHH-I cells, or the SHH-I parental cell line (Ctrl), were dounce homogenized under isotonic conditions and total lysate (left panel) 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 serves as a cytosolic protein control while the Na^+/K^+ transporter serves 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, has been 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 q-RT-PCR. Error bars represent the SD in one representative experiment.

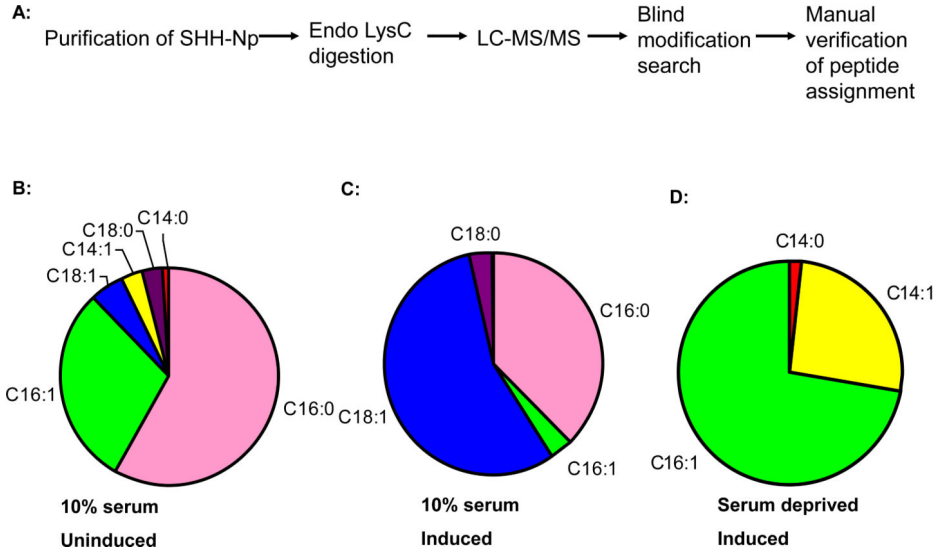

Figure 3. Fatty acid speciation of SHH-Np is cell context dependent

(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 purified isolated under three different cell 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.

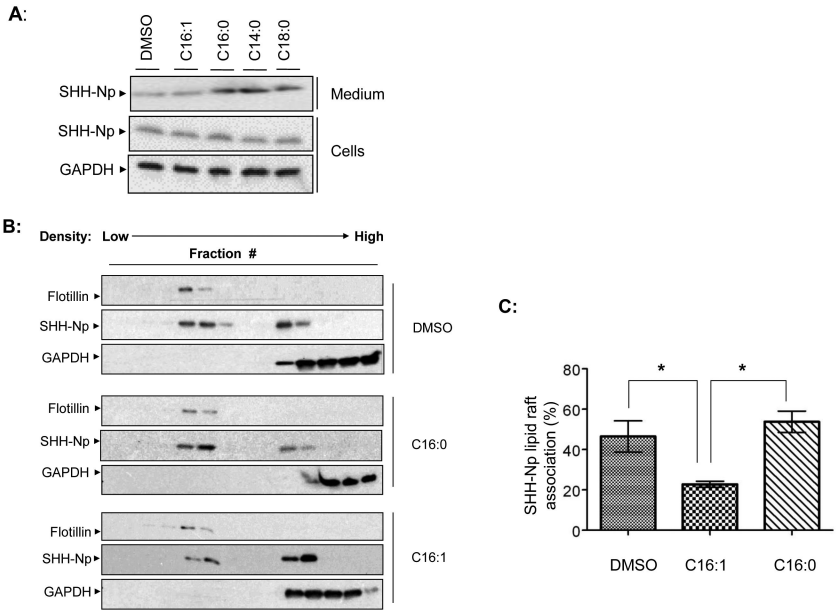

Figure 4. Fatty acid speciation of SHH-Np alters its lipid raft enrichment

(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 medium 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 then analyzed by immunoblotting for SHH-Np, GAPDH as a cytoplasmic protein marker, or the lipid raft marker flotillin. Note that flotillin localization does not change with various lipid additions. Quantification of SHH-Np lipid raft enrichment from cells incubated with the indicated fatty acids is shown in **(C)**. Error bars represent the standard error of the mean (SEM) of three independent experiments. p values ≤ 0.05 are considered statistically significant, and indicated by a asterisk.

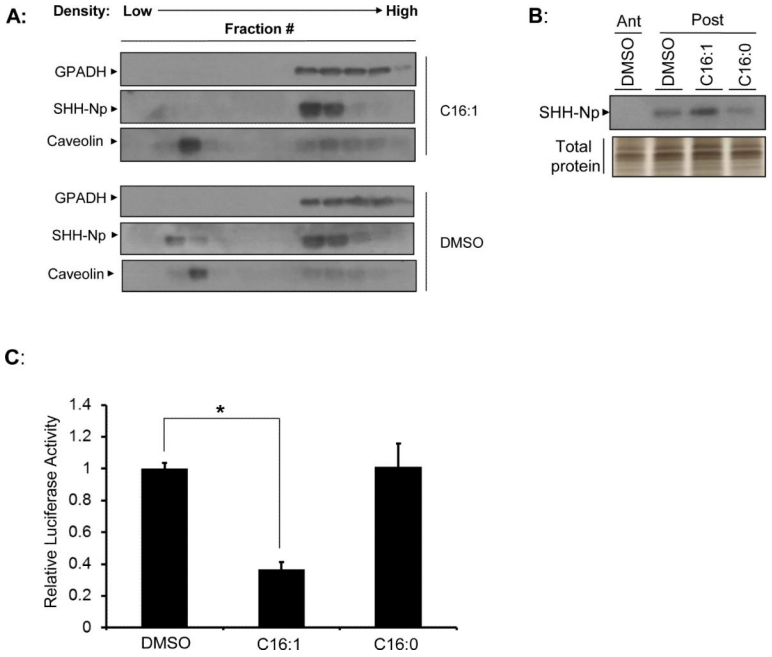

Figure 5. Modification of SHH-Np with distinct fatty acids alters its functionality

(**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 than analyzed by immunoblotting for SHH-Np. Caveolin-1 was used as a lipid raft marker while GAPDH served to label non-lipid raft associated subcellular fractions. Treatment with saturated fatty acids did not change SHH-Np localization pattern over the DMSO control (data not shown). (**B**) An immunoblot (upper panel) of limb bud lysates showing effect of lipid modifications on SHH-Np levels. Lysates contain similar amount of total protein as indicated by total protein silver staining (lower panel). **(C)** The potency of SHH-Np was determined by incubating lysate 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 indicated by an asterisk.