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Robert Tokhunts, Samer Singh, Tehyen Chu, Gisela d'Angelo, Valerie Baubet, et al.. The Full-length Unprocessed Hedgehog Protein Is an Active Signaling Molecule. *Journal of Biological Chemistry*, 2010, 285 (4), pp.2562-2568. 10.1074/jbc.m109.078626 . hal-03033990

HAL Id: hal-03033990

<https://hal.science/hal-03033990>

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The Full-length Unprocessed Hedgehog Protein Is an Active Signaling Molecule^{*S}

Received for publication, October 22, 2009; Published, JBC Papers in Press, November 17, 2009; DOI 10.1074/jbc.M109.078626

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The hedgehog (HH) family of ligands plays an important instructional role in metazoan development. HH proteins are initially produced as ~45-kDa full-length proteins, which undergo an intramolecular cleavage to generate an amino-terminal product that subsequently becomes cholesterol-modified (HH-Np). It is well accepted that this cholesterol-modified amino-terminal cleavage product is responsible for all HH-dependent signaling events. Contrary to this model we show here that full-length forms of HH proteins are able to traffic to the plasma membrane and participate directly in cell-cell signaling, both *in vitro* and *in vivo*. We were also able to rescue a *Drosophila* eye-specific *hh* loss of function phenotype by expressing a full-length form of *hh* that cannot be processed into HH-Np. These results suggest that in some physiological contexts full-length HH proteins may participate directly in HH signaling and that this novel activity of full-length HH may be evolutionarily conserved.

Underscoring the importance of sonic hedgehog (SHH)³ in developmental patterning, inherited and sporadic mutations in *SHH* are frequently found in patients diagnosed with the human developmental disorder holoprosencephaly (1–5). A number of *SHH* point mutations have been identified from holoprosencephaly patients, providing a unique opportunity to test the hypothesis that these mutations will reveal novel mechanistic insights into SHH biogenesis.

EXPERIMENTAL PROCEDURES

SHH Activity Assays—The full-length cDNA of human *SHH* (a gift from Dr. Cliff Tabin) was cloned into pcDNA3.1. All of the

SHH mutants were made by site-directed mutagenesis using the QuikChange II kit (Stratagene) and expressed as described (6). Cell-to-cell signaling was determined by co-culturing SHH mutant-expressing cells with cells expressing SHH-dependent *luciferase* reporter gene (7, 8). Total cellular SHH activity was determined by incubating whole cell lysate from *SHH*-expressing cells with cells expressing SHH-dependent *luciferase* reporter gene (9) (lysate assay) as described (10). All of the activity assays were done in triplicate, and each experiment was repeated at least three times. The activity data are presented as mean ± standard deviation (*error bars*) in the various figures.

SHH Carboxyl-terminal Immunoprecipitation—Bosc cells expressing *SHH-U* or wt *SHH* were lysed in 1% Triton X-100 lysis buffer (150 mM NaCl, 10 mM Na₂HPO₄, 1% Triton X-100, pH 6.5) supplemented with a protease inhibitor mixture for 10 min on ice. Following nuclei removal by centrifugation, the samples were immunoprecipitated for 3 h at 4 °C using either antibodies raised against a SHH carboxyl-terminal domain peptide (R & D Systems) or control IgG and protein G beads (Sigma). The various immunoprecipitates were washed twice with PBS and incubated with confluent cells expressing SHH-dependent *luciferase* reporter genes overnight to assay SHH activity as described above or analyzed by immunoblotting.

Immunofluorescence—To examine PTC-dependent internalization of SHH-U, Bosc cells transiently expressing *GFP*, *SHH-U*, the SHH receptor *PATCHED* (*PTC*) (FLAG-tagged), or a control plasmid were co-cultured on microscope slides for 2 days in various combinations. These slides were washed with cold PBS, fixed in 4% paraformaldehyde for 10 min at room temperature, and permeabilized/blocked with PBS containing 5% donkey serum and 0.1% Tween 20. The various samples were incubated with goat anti-SHH carboxyl-terminal domain peptide (R & D Systems) or mouse anti-FLAG peptide (Sigma) antibodies followed by incubation with donkey anti-goat IgG-Alexa fluor 594 or donkey anti-mouse IgG-Alexa fluor 488 (Molecular Probes), respectively. All of the images were acquired with a Leica DMIRE2 confocal laser-scanning microscope, using 1 airy unit intensity of collected light.

Immunoblotting Tissues—Stage 22 chicken limb buds or rabbit gastric glands were isolated (11) and then lysed in 3 ml of radioimmune precipitation assay buffer (150 mM NaCl, 10 mM

* This work was supported, in whole or in part, by National Institutes of Health Grant GM64011 (to D. J. R.). This work was also supported by an award from the American Lung Association/LUNGevity Foundation (to D. J. R.) and a Graduate Assistance in Areas of National Need (GAANN) predoctoral fellowship in nanomedicine (to R. T.).

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental "Experimental Procedures" and Figs. S1–S6.

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³ The abbreviations used are: SHH, sonic hedgehog; wt, wild type; PBS, phosphate-buffered saline.

Na_2HPO_4 , 1% sodium deoxycolate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1% Triton X-100, pH 7.2, and freshly added 200 μM Na_3VO_4) supplemented with protease inhibitor mixture. The samples were immunoprecipitated using anti-SHH 5E1 monoclonal antibody or control IgG (Sigma) and True-blot beads. The immunoprecipitates were analyzed by immunoblotting with anti-SHH antibodies H-160 (Santa Cruz Biotechnology). Oregon-R (wt), *hh^{ac}* (12), and *UAS-hh-M4* lines of *Drosophila* were also used to examine the expression of various forms of HH in different tissues. *hh^{ac}* is a null allele. The *UAS-hh-M4* line (13) specifically expressed HH in larval tissues. Wild type embryos, homozygous *hh^{ac}* embryos, and heads of wt third instar *hh-Gal4>UAS-hh-M4* larvae enriched in imaginal discs were dissected in ice-cold PBS and homogenized in lysis buffer (150 mM NaCl, 50 mM Tris, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, pH 7.5) supplemented with a protease inhibitor mixture for 30 min on ice. The proteins were separated by SDS-PAGE and analyzed by immunoblotting with rabbit "Calvados" polyclonal anti-HH antibody (14) or mouse anti- α -tubulin monoclonal antibody (Sigma).

Drosophila Analyses—For eye analysis three transgenic constructs *UAS-hh-U* (15), *UAS-hh* (16), and *UAS-hh-N* (16) were specifically expressed in the developing retina of *hh¹* animals with the eye-specific promoter *GMR-GAL4*, and rescue of the phenotype was evaluated. For leg disc analysis, animals with the following genotypes were generated: *y, hsflp/+; tub<<Gal4, UAS-CD8-GFP/+; ptc/lacZ*. They were then heat-shocked for 22 min at 37 °C between the L1 and L3 stages. The discs were fixed and analyzed as previously described (15). The images were collected on a Zeiss LSM510 confocal microscope.

RESULTS

We have examined a set of *SHH* holoprosencephaly point mutations that encode a mutated carboxyl-terminal domain of SHH (2–4). During the processing of SHH into SHH-Np, this carboxyl-terminal domain functions to regulate the covalent addition of cholesterol to its amino-terminal domain and subsequent cleavage of full-length SHH (Fig. 1A for a schematic of the various forms of HH described here). We engineered these *SHH* holoprosencephaly mutations, *SHH-D222N*, *SHH-T267I*, *SHH-G290D*, *SHH-A373T*, and *SHH-P424A*, into a human *SHH* cDNA construct. Plasmids expressing these *SHH* holoprosencephaly mutations, wt *SHH*, or a control plasmid, were transfected into Bosc cells, a human embryonic kidney cell derivative (17), and the abundance and processing of the mutant proteins were examined from cell lysates. The processing pattern of three *SHH* holoprosencephaly mutants, *SHH-D222N*, *SHH-T267I*, and *SHH-A373T*, was significantly different from that of wt *SHH*, showing an increased abundance of their full-length form (SHH-F) and a corresponding decrease in their processed SHH-Np form (Fig. 1B). Thus, three of the five *SHH* holoprosencephaly mutations examined showed accumulation of full-length SHH and decreased SHH-Np. Next we estimated the activity of these *SHH* mutants by co-culturing cells expressing these mutant proteins with NIH-3T3 cells stably expressing a SHH-dependent *luciferase* reporter gene (8) (Fig. 1C). Interestingly, two of the processing-deficient mutants, *SHH-D222N* and *SHH-A373T*, exhibited an activation of the

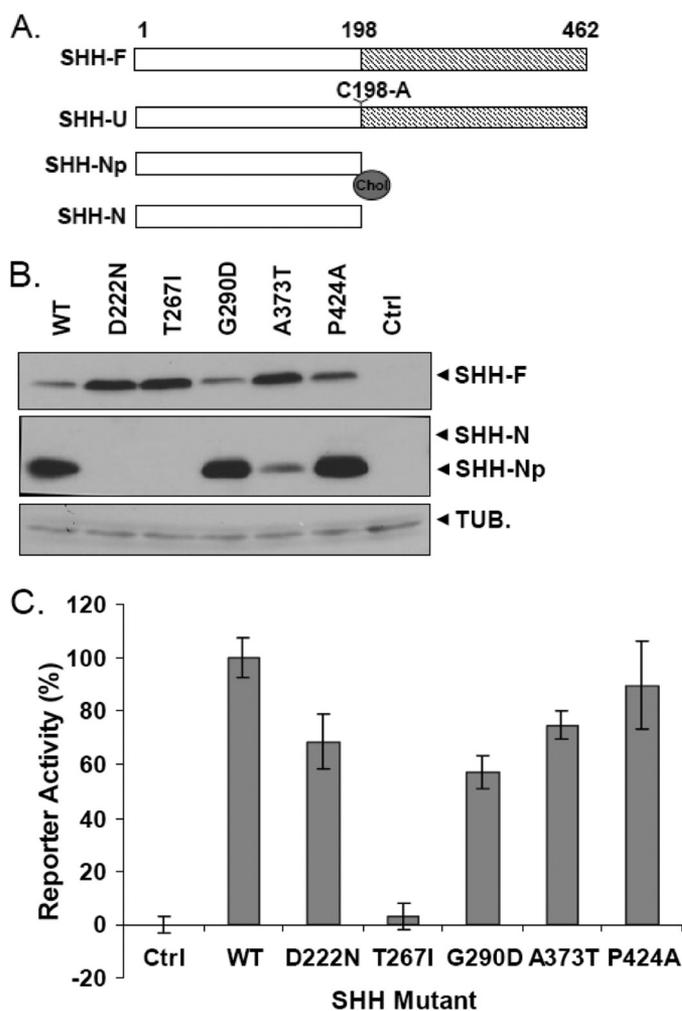


FIGURE 1. SHH holoprosencephaly mutations that encode carboxyl-terminal domain point mutants affect the processing and activity of the SHH protein. A, schematic diagram showing protein encoded by different *SHH* constructs. SHH is produced as a full-length protein (*SHH-F*) comprised of an amino-terminal domain (open box) and a carboxyl-terminal domain (hatched box) that regulates auto-processing of the two domains. During the processing of full-length SHH, cholesterol (Chol) is covalently added to the newly generated carboxyl terminus of SHH (*SHH-Np*). *SHH-U* refers to a mutant form of full-length SHH in which the critical processing site amino acid Cys¹⁹⁸ has been changed to Ala (*C198-A*) to prevent autoprocessing. *SHH-N* refers to a form of amino-terminal SHH peptide that is not cholesterol-modified but used in various assays as a substitute for *SHH-Np*. B, lysate from cells transfected with plasmids expressing wt *SHH* or the various *SHH* holoprosencephaly mutations, *SHH-D222N*, *SHH-T267I*, *SHH-G290D*, *SHH-A373T*, *SHH-P424A*, or a control (*Ctrl*) plasmid was analyzed for the expression of the unprocessed full-length SHH (*SHH-F*), the processed *SHH-Np*, or tubulin (*TUB.*), by immunoblotting. The *SHH-N* arrowhead shown indicates where the non-cholesterol-modified protein migrates on this immunoblot. On average, we see a small amount of *SHH-Np* in the cell lysate of *SHH-D222N*-expressing cells, unlike the individual experiment shown here. C, cells expressing the indicated *SHH* mutants were assayed for their SHH activity by co-culturing them with cells expressing SHH dependent *luciferase* reporter gene. The activities of the various SHH mutants are shown as a percentages of wt SHH activity.

SHH reporter gene ~65% of wt SHH. Together these results confirm the importance of the SHH carboxyl-terminal domain to the processing and activity of SHH.

The observed levels of *SHH-D222N* and *SHH-A373T* activity were unexpected. The simplest explanation for this result was that the full-length form of these proteins, which appears to be the predominant form, is active. However, this surprising

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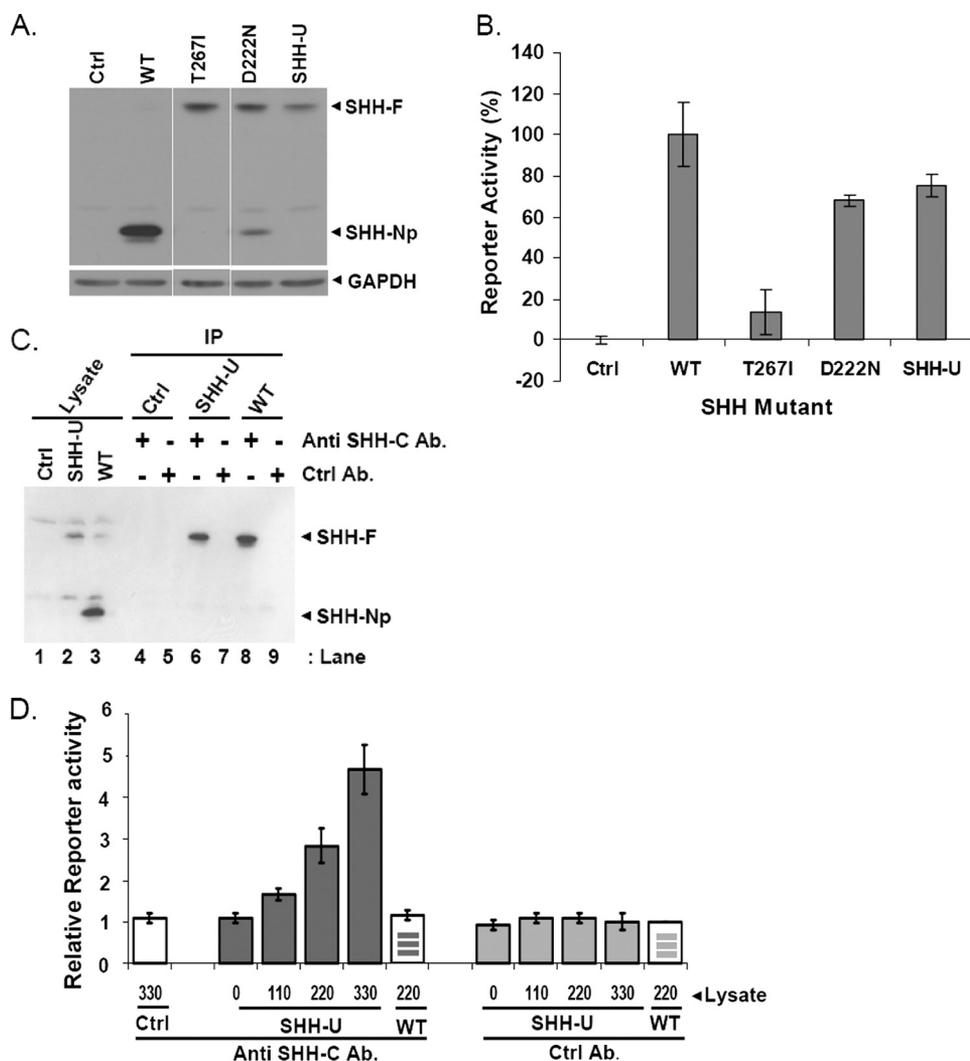


FIGURE 2. Full-length unprocessed forms of SHH are active. *A*, the processing efficiency of wt SHH, SHH-D222N, SHH-T2671, and SHH-U was analyzed by immunoblotting the lysate of cells expressing the indicated constructs. The expression of unprocessed full-length SHH (SHH-F), the processed form of SHH (SHH-Np), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are indicated. *B*, cell lysate, normalized to total cell protein (as described in Fig. S3), containing the indicated SHH mutants was added to cells expressing SHH-dependent *luciferase* reporter gene to determine their relative SHH activity. This comparison was done under conditions where the correlation between wt SHH activity and amount of cellular lysate assayed was linear. The activities of the various SHH mutants are shown as percentages of wt SHH activity. *C*, SHH was isolated from the cell lysate of SHH-U or wt SHH-expressing cells using antibodies specific to the SHH carboxyl-terminal domain and then immunoblotted with antibodies specific for the SHH amino-terminal domain. Lanes 1–3 show aliquots of the indicated cellular lysate, whereas lanes 4–9 show various specific or control immunoprecipitates (IP). For the wt SHH (WT) immunoprecipitation (lanes 8 and 9), the amount of lysate used is four times that used for SHH-U, because full-length SHH levels are lower in wt SHH-expressing cells (compare lane 2 with lane 3). *D*, full-length SHH was immunoprecipitated from indicated amounts of SHH-U or wt SHH-expressing cell lysate (depicted as μ g of protein) using antibodies specific to the SHH carboxyl-terminal domain (dark gray bars, unstriped or striped) or control antibodies (light gray bars, unstriped or striped). Antibodies to the SHH carboxyl-terminal domain were also used to immunoprecipitate from 330 μ g of cell lysate from cells transfected with a control plasmid (white bar). The various immunoprecipitates were then incubated with cells expressing SHH-dependent *luciferase* reporter gene to assay SHH activity. Ctrl, control.

outcome might also result from some low abundance processed form of SHH-Np inefficiently produced from the mutant full-length SHH, as is observed for SHH-A373T. To distinguish between these possibilities, we engineered a SHH mutant that is unable to process into SHH-Np, SHH-U, by mutating the highly conserved Cys¹⁹⁸ that is absolutely essential for full-length SHH processing (18, 19) (Fig. 1A). We expressed SHH-U, SHH-D222N, SHH-T2671, and wt SHH in Bosc cells and examined the ability of these mutant proteins to process into their

SHH-Np forms (Fig. 2A). Consistent with what has been previously described for SHH-U (18), the ability of this mutant to process into its SHH-Np form is completely attenuated, whereas the SHH-D222N mutant yields some small amount of its processed form. We next examined the activity of SHH-U by co-culturing cells expressing various SHH mutants with cells expressing a SHH-dependent *luciferase* reporter gene (supplemental Fig. S1). Cells expressing SHH-U were able to activate the SHH pathway ~44% as well as wt SHH. Thus, although the SHH-U mutant is unable to convert into its processed SHH-Np form, it still exhibits significant levels of SHH activity when measured in co-culture assay. Consistent with this activity in a cell-cell contact assay, SHH-U localized to the plasma membrane of these cells (supplemental Fig. S2).

To further validate the ability of SHH-U to activate the SHH pathway, we compared its activity to wt SHH using a cellular lysate assay (9), which allows us to compare wt SHH and SHH-U under conditions where we could easily determine the linear range of our assay. The ability of different amounts of cell lysate from wt SHH transfected Bosc cells to activate cells expressing a SHH-dependent *luciferase* reporter gene was first used to determine the linearity of this assay (supplemental Fig. S3A). Activation of the SHH reporter gene by wt SHH-containing cell lysate was inhibited by the SHH antagonist cyclopamine (20, 21) (supplemental Fig. S3B), consistent with this assay being specific for SHH. Lysates from Bosc cells transfected with plasmids expressing SHH-U, SHH-D222N, SHH-T2671, wt SHH, or a plasmid control

were normalized to total protein content (Fig. 2A) and assayed for SHH activity (Fig. 2B) under conditions where this assay was linear (supplemental Fig. S3A). SHH-U and SHH-D222N activated the SHH pathway to ~70% of wt SHH, whereas SHH-T2671 exhibited only minimal levels of activity.

Our results suggest that the full-length form of SHH is capable of signaling. However, one could still argue that a small amount of full-length SHH is being proteolytically clipped in a nonspecific manner to reveal an active SHH fragment. To fur-

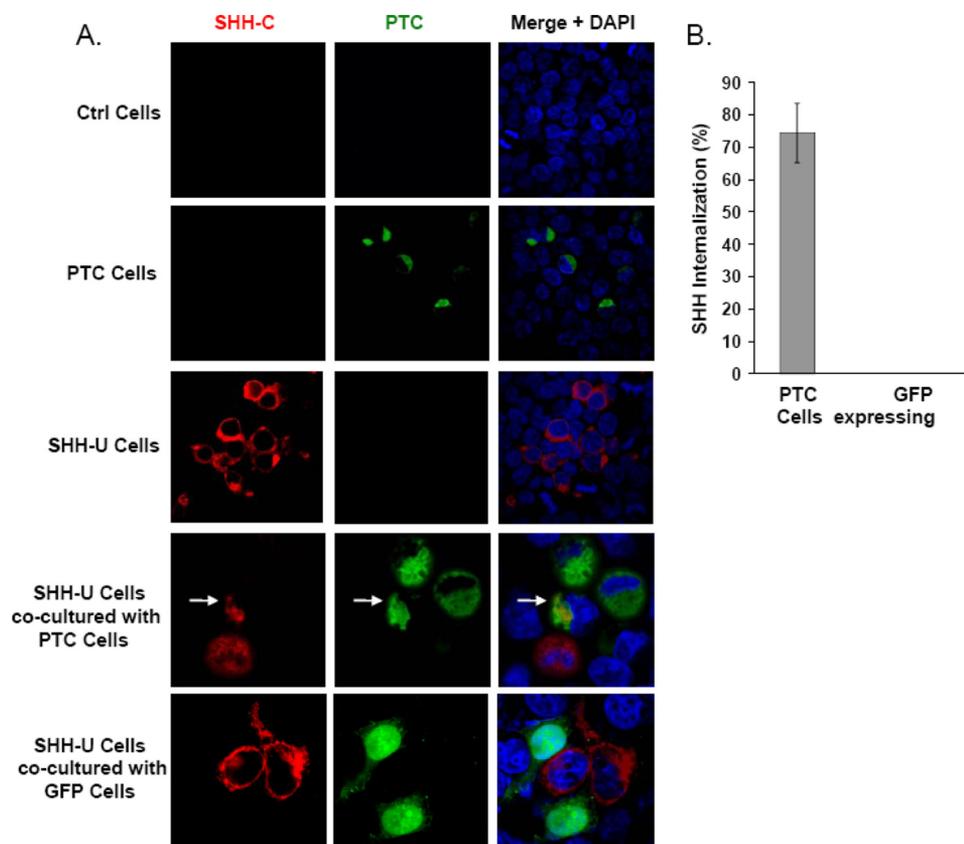


FIGURE 3. SHH-U interacts with the hedgehog receptor Patched. *A*, cells expressing either control plasmid (*Ctrl* cells), *PTC* (*PTC* cells), *SHH-U* (*SHH-U* cells), or *GFP* (*GFP* cells) were generated. Each population of cells was stained with 4',6'-diamino-2-phenylindole (*DAPI*, blue) to mark the cells and antibodies specific to the SHH carboxyl-terminal domain (red) and *PTC* (green). Representative images are shown. *First row*, control cells; *second row*, *PTC* cells; *third row*, *SHH-U* cells; *fourth row*, *PTC* cells co-cultured with *SHH-U* cells; *fifth row*, *GFP* cells co-cultured with *SHH-U* cells. *SHH-U* is endocytosed by *PTC* when these two cell types are physically associated (*fourth row*, arrow). The co-culture figure (*fourth row* and *fifth rows*) is displayed at 4 \times enhanced magnification, using Photoshop, relative to the various control panels. *B*, the ability of *PTC* cells to endocytose *SHH-U* from adjacent *SHH-U* cells was quantified in three separate experiments. On average, we observed *SHH-U* being endocytosed by 134 *PTC*-expressing cells out of 180 *PTC*-expressing cells in physical association with *SHH-U*-expressing cells counted. Conversely, 0 of 180 *GFP*-expressing cells in physical association with *SHH-U*-expressing cells internalized *SHH-U*.

ther confirm that full-length SHH can itself activate the SHH pathway, we immunoprecipitated SHH-U using two unrelated antibodies targeted to its carboxyl-terminal domain and determined its activity. The specificity of the antibodies used in this analysis was validated by evaluating their ability to immunoprecipitate full-length SHH, but not SHH-Np, from the lysate of Bosc cells expressing *wt SHH* or *SHH-U* (Fig. 2C and supplemental Fig. S4 show results for different antibodies). The various immunoprecipitates were resolved by SDS-PAGE and immunoblotted using antibodies to the amino-terminal domain of SHH. This result verified that the antibodies used here can specifically immunoprecipitate full-length SHH but not SHH-Np. We next determined the activity of these various immunoprecipitates by assaying their ability to activate the SHH-dependent *luciferase* reporter gene. The SHH-U immunoprecipitate activated SHH signaling in a dose-dependent manner (Fig. 2D and supplemental Fig. S4B), giving up to 3–5-fold more activity than a similar immunoprecipitate from control cell lysate or from immunoprecipitates using nonspecific antibodies. Moreover, the immunoprecipitate from lysates of *wt SHH*-expressing cells, in which the full-length SHH species is less

abundant, resulted in less activity. The decreased activity observed in this latter control is consistent with the activity we describe here being derived from full-length SHH and not from some SHH fragment associating nonspecifically with the SHH carboxyl-terminal antibodies. Taken together these results confirm that the full-length form of SHH has the ability to activate SHH signaling.

If the activity of SHH-U we observe is derived from the full-length protein, we would expect to observe SHH-U interact with the SHH receptor *PATCHED* (*PTC*). We could then monitor this direct interaction as *PTC*-dependent endocytosis using antibodies to the SHH carboxyl-terminal domain and *PTC* (22). We first expressed a control plasmid, *GFP*, *SHH-U*, or the SHH receptor *PTC* in individual populations of Bosc cells and then verified that these distinct populations of cells only showed reactivity to antibodies directed against the proteins they were engineered to express (Fig. 3A, top three rows). We then co-cultured these different cell populations expressing control plasmid, *GFP*, *SHH-U*, or *PTC* (Fig. 3A, bottom two rows). Our results show that SHH-U is specifically taken up by *PTC*-expressing cells when *SHH-U*-expressing cells

are physically associated with *PTC*-expressing cells ~75% of the time (Fig. 3A, fourth row from top, and 3B) but not by *GFP*-expressing cells (Fig. 3A, bottom row, and 3B). This result suggests that SHH-U can interact with *PTC* in a functional manner. Consistent with this suggestion, the SHH inhibitory 5E1 monoclonal antibody was also able to attenuate the *PTC*-dependent endocytosis of SHH-U (supplemental Fig. S5).

SHH plays an important role in the dorsal-ventral patterning of the neural tube, regulating cell fate through the induction or repression of homeodomain transcription factors such as *Nkx2.2* and *Pax6* (23). Thus, the extent to which these transcription factors are regulated by ectopically expressed SHH can serve as an indicator of SHH activity *in vivo*. Therefore, we electroporated *SHH-U*, *wt SHH*, or a control plasmid into one side of the developing chick neural tube to determine whether SHH-U exhibited activity in this *in vivo* system (supplemental Fig. S6). Consistent with our *in vitro* data, *SHH-U* expression resulted in ectopic repression of the normal expression of *Pax6* when compared with the nonelectroporated contralateral side of the neural tube (supplemental Fig. S6, O and T). SHH-U was also able to induce the ectopic expression of *Nkx2.2* in more

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TABLE 1

SHH-U induces different cell fates in the developing neural tube

SHH-U induces different cell fates in the developing neural tube. Plasmids expressing *SHH-U* or wt *SHH* were electroporated into one side of a Hamburger and Hamilton stage 10–12 chick neural tube. The ectopic induction or repression of the SHH-regulated homeodomain transcription factors *Nkx2.2* and *Pax6* was then determined by *in situ* analyses, relative to the nonelectroporated contralateral side of the neural tube in three separate experiments.

SHH constructs	<i>Nkx2.2</i> induction	<i>Pax6</i> repression	Total embryos
	%	%	
wt SHH	79	68	28
SHH-U	48	32	25

dorsal regions of the neural tube than observed in the contralateral control half of the neural tube (supplemental Fig. S6, N and S). The ability of SHH-U to regulate the expression of *Pax6* and *Nkx2.2* was ~50% of that of wt SHH, when approximately equal amounts of expression plasmid were electroporated into a chick neural tube (Table 1). However, when the amount of wt *SHH* or *SHH-U* expressed was compared with the level of endogenous chicken *SHH* expression in the floor plate and neural tube, using the anti-SHH 5E1 monoclonal antibody, we noted that wt SHH appears to be more highly expressed than SHH-U (supplemental Fig. S6, compare C and H with M and R). This observation was similar to our *in vitro* observations (Fig. 2C), which also shows that wt *SHH* is more highly expressed than *SHH-U*. Based on this differential expression of wt *SHH* and *SHH-U*, we assume that the regulation of *Nkx2.2* and *Pax6* expression by SHH-U represents an underestimate of its inherent activity relative to wt SHH. These results demonstrate that SHH-U also has substantial activity in an *in vivo* setting, ectopically regulating the expression of two homeodomain genes normally regulated by SHH in the neural tube.

If the activity of full-length HH protein is physiologically relevant, one might expect to observe its differential accumulation in various tissues. To test this prediction we examined distinct tissues for their relative amounts of full-length HH proteins. First we decided to probe adult rabbit gastric glands for the presence of full-length SHH, in which full-length SHH has been suggested to play a role in the maintenance of gastric function (11, 24), as well as stage 22 chick limb buds, in which SHH-Np has been shown to be involved in limb patterning (25). Cell lysates were generated from these primary tissues, followed by immunoblot analyses. Our results show that under conditions in which the levels of SHH-Np are normalized, increased levels of full-length SHH were found in gastric gland tissue relative to limb bud tissue (Fig. 4A). These results suggest that the ratio of different SHH species may be differentially regulated. To explore the evolutionary conservation of such differential processing, we performed a similar analysis on *Drosophila* tissues. Thus, we collected *Drosophila* embryos and imaginal disc tissue from third instar larvae and compared the ratio of full-length HH protein to HH-Np by immunoblot analyses (Fig. 4B). Our results show that full-length HH proteins are the dominant species of HH during fly embryonic development, in which multiple species of HH co-migrate at the molecular size of full-length HH. Consistent with these immunoreactive proteins being full-length HH, these bands were absent in lysate from embryos deficient in *hh* (*hh^{ac}*). In contrast, HH-Np was the dominant species of HH in imaginal disc tissue, although full-length HH

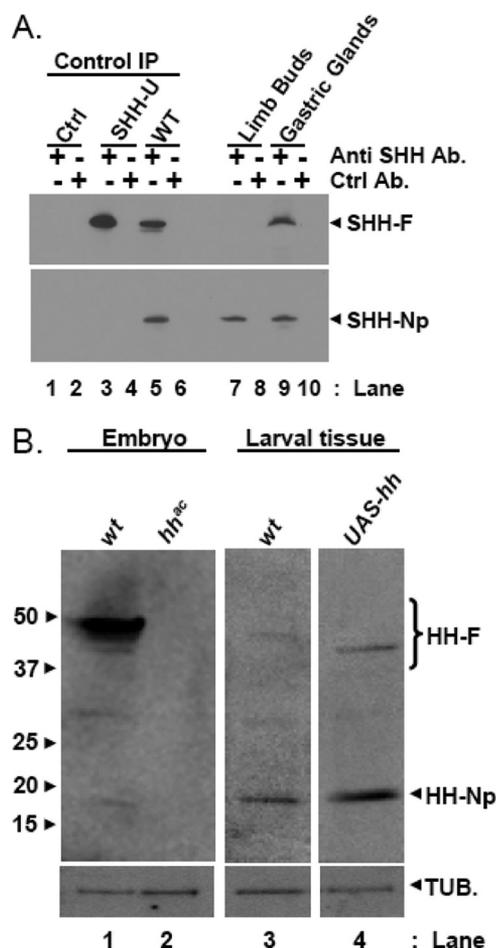


FIGURE 4. Full-length hedgehog proteins enrich differentially in tissues. A, SHH was isolated from the lysate of cells expressing *SHH-U* or wt *SHH*, limb buds of stage 22 chicken embryos, or rabbit gastric glands using antibodies specific to the SHH amino-terminal domain and then immunoblotted with another antibody specific for the SHH amino-terminal domain. Lanes 1–6 show various specific or control immunoprecipitates for SHH from control (Ctrl), *SHH-U*, and wt *SHH* overexpressing cells, whereas lanes 7–10 show endogenous SHH from specific tissues. The lower panel is a shorter exposure of the same blot to visualize SHH-Np normalization. B, immunoblot analysis of lysates from *Drosophila* expressing *UAS-hh*, *hh^{ac}*, or wt *Drosophila* reveals the differential expression of full-length HH (HH-F) to HH-Np in wt embryos relative to wt imaginal discs or *UAS-hh*-expressing heads (compare lane 1 with lanes 3 and 4). *hh^{ac}* is a null allele, so it acts as a control for the specificity of the anti-HH antibody used. All of the panels are part of the same blot, shown at different exposure levels (lanes 3 and 4 showing full-length HH and HH-Np are longer exposure than lanes 1 and 2, whereas all lanes showing tubulin are same exposure). The size standards are in kDa. A portion of the membrane was probed for tubulin (TUB.) as a loading control.

was still observed in this tissue. Collectively, these results show that the accumulation of full-length HH protein is consistent with it playing a physiologically relevant role, albeit in a spatially and temporally defined manner.

To determine whether the inherent activity of full-length HH proteins was evolutionarily conserved, we examined the activity of analogous *Drosophila* HH mutants during eye development, where HH is thought to act predominantly in a localized manner (26). Using an eye-specific *GAL4* driver, we expressed *UAS* constructs *hh-U* (15), wt *hh* (16), and *hh-N* (16) (analogous to SHH-U, wt SHH, and SHH-N (Fig. 1A)) in the eye imaginal disc and examined their ability to rescue the ommatidia development defect found in *hh¹* mutant *Drosophila* (27, 28). Because the summation of individual ommatidia development

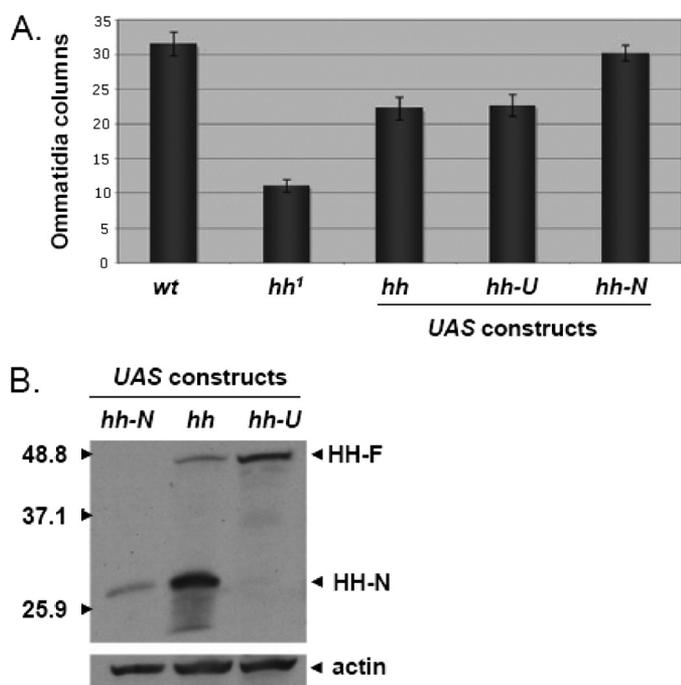


FIGURE 5. The activity of full-length, unprocessed hedgehog proteins is evolutionarily conserved. *A*, the extent of rescue of the eye *hh*¹ phenotype was established by quantification of ommatidial development in the wild type, *hh*¹, and *hh*¹ animals bearing one of the transgenes: wt *hh*, *hh-U* or *hh-N* driven by eye-specific *GAL4* driver *GMR-GAL4*. Between 4 and 10 retinas were counted for each genotype. *B*, immunoblot analysis reveals the predicted protein products from the three transgenes wt *hh*, *hh-U*, and *hh-N* expressed in eye imaginal discs. Notably, the product from *hh-U* appears to migrate at a similar molecular weight to the full-length unprocessed wt HH polypeptide (*HH-F*) product prior to its auto-cleavage. The size standards are in kDa. A portion of the membrane was probed for actin expression, as a loading control.

within each adult eye gives a quantitative determinant of HH activity, we were able to quantify the relative activity of these various HH proteins by determining the number of developed ommatidia per retina in transgenic flies (Fig. 5A). From these analyses we observed that the ommatidia and lamina developmental defects of these mutant flies (Fig. 5A, second column) were substantially rescued by the expression of *hh-U* (Fig. 5A, fourth column), wt *hh* (Fig. 5A, third column), and *hh-N* (Fig. 5A, last column). Notably, the ability of HH-U and wt HH to rescue ommatidia development was quite similar. Moreover, *hh-N* expression resulted in more correctly developed ommatidia (Fig. 5A, last column), consistent with this *in vivo* activity assay not being saturated for HH. The inability of the HH-U protein to be processed into HH-Np *in vivo* was verified by immunoblotting imaginal discs removed from the various transgenic flies (Fig. 5B). These results also clearly demonstrate the ability of full-length HH proteins to activate some aspects of HH signaling *in vivo*, without the requirement to process full-length HH to its cholesterol-modified HH-Np form. In addition, these results show that the inherent activity of full-length HH proteins is evolutionarily conserved, implicating the importance of this novel HH activity to its function.

DISCUSSION

The well accepted model of HH biogenesis suggests that the cholesterol-modified, processed form of HH, HH-Np, is the

only known active HH species. Contrary to this model, our results suggest that full-length unprocessed HH proteins also exhibit substantial levels of activity. We speculated that the two most likely reasons for our results are that the full-length unprocessed HH proteins retain some substantial level of activity or that we were observing the activity of some small undetectable amount of inefficiently processed HH-Np. We favor the former model because of our analyses of HH-U proteins, which are unable to process into their cholesterol-modified forms. However, it remains possible that these full-length HH-U proteins are subject to some small amount of nonspecific proteolytic cleavage that liberates an active amino-terminal product, which would then be responsible for the bulk of activity we observe here. Our results are not consistent with this latter hypothesis for three main reasons: 1) the quantitative nature of the assays used here, both *in vitro* and *in vivo*, to determine the activity of the full-length HH proteins are inconsistent with the majority of the activity observed being contributed by a small undetectable fraction of the protein, 2) we were able to specifically immunoprecipitate active SHH-U using two distinct antibodies to the SHH carboxyl-terminal domain, and 3) the holoprosencephaly mutant SHH-T267I, which should be subject to the same putative nonspecific proteolytic clipping as SHH-U and SHH-D222N, exhibited negligible activity.

Contrary to the results presented here, it has been previously suggested that full-length HH proteins are not active (18, 19, 25). We do not know the reasons for the apparent discrepancy between the results of the various groups involved but speculate that the level of expression and type of assay used may be important to visualize the activity of full-length HH proteins.

Our ability to assay the activity of HH-U proteins was limited to assays that involved cell-cell contact, with the activity contributed by any SHH-U in the conditioned media being negligible (data not shown). Consistent with this observation, SHH-U was only internalized by PTC when cells expressing each construct were in direct contact. Our ability to rescue the ommatidia defect of *hh*¹ flies might also be due to more localized signaling, because HH is only thought to act over small distances in the fly eye (26). This suggests that the biological functions of HH-U proteins might be limited to those that do not require HH to act far from its site of synthesis. Although it is generally accepted that long and short range HH signaling is differentially regulated, this research has focused on how other distinct proteins direct the processed, cholesterol-modified form of HH proteins through different mechanisms (7, 14, 16, 29–33). Our results broaden this discussion, because we suggest that unprocessed full-length HH proteins might be directly responsible for a subset of localized HH signaling. In this model, long and short range HH signaling would be controlled by distinct forms of HH proteins, with long range signaling regulated solely by the cholesterol-modified processed protein and short range signaling controlled, at least in part, by full-length unprocessed HH proteins. Consistent with this model, we did not observe significant accumulations of full-length HH proteins in tissues thought to depend primarily on ability of HH to signal over extended distances, such as chicken limb buds (34, 35). In contrast we detected large amounts of full-length HH protein in *Drosophila* embryos, in which HH is thought to act in a more

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localized manner (34, 36). In conclusion, our results suggest that the relationship between HH processing and activity is more complex than previously thought and that in some biological contexts the full-length forms of HH family members may play a significant physiological role.

Acknowledgments—We are grateful to the Dartmouth microscopy core for expert assistance. We are also grateful to Drs. Yashi Ahmed, Stacey Ogden, and members of the Robbins laboratory for thoughtful discussion during the course of this work. In addition we specially thank Dr. Thomas Kornberg for helpful suggestions regarding our manuscript.

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