An Emerging Role of Sonic hedgehog Shedding as a Modulator of Heparan sulfate Interactions

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Running title: Analysis of the N-terminal Sonic hedgehog processing site

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Background: Sonic hedgehog is released from expressing cells by proteolytic cleavage (shedding) of lipidated N-terminal peptides

Results: The heparan sulfate (HS)-binding Cardin-Weintraub motif represents one N-terminal protease cleavage site in vitro and in vivo

Conclusions: This results in impaired HS-binding of solubilized proteins

Significance: Shedding may facilitate Sonic hedgehog diffusion through the HS-rich extracellular matrix

Summary: Major developmental morphogens of the Hedgehog (Hh) family act at short range and long range to direct cell fate decisions in vertebrate and invertebrate tissues. To this end, Hhs are released from local sources and act at a distance on target cells that express the Hh receptor Patched (Ptc). However, morphogen secretion and spreading are not passive processes, because all Hhs are synthesized as dually (N- and C-terminally) lipidated proteins that firmly tether to the surface of producing cells. On the cell surface, Hhs associate with each other and with heparan sulfate (HS) proteoglycans. This raises the question of how Hh solubilization and spreading is achieved.

We recently discovered that Sonic hedgehog (Shh) is solubilized by proteolytic processing (shedding) of lipidated peptide termini in vitro. Because unprocessed N-termini block Ptc-receptor binding sites in the cluster, we further suggested that their proteolytic removal is required for simultaneous Shh activation. In this work, we confirm inactivity of unprocessed protein clusters and demonstrate restored biological Shh function upon distortion or removal of N-terminal amino acids and peptides. We further show that N-terminal Shh processing targets and inactivates the HS-binding CW-motif, resulting in soluble Shh clusters with their HS-binding capacities strongly reduced. This may explain the ability of Shh to diffuse through the HS-containing extracellular matrix, whereas other HS-binding proteins are quickly immobilized. Our in vitro-findings are supported by the presence of CW-processed Shh in murine brain samples,
providing first in vivo evidence for Shh shedding and subsequent solubilization of N-terminally truncated proteins.

Introduction:

The proteins of the Hedgehog (Hh) family are powerful morphogens that control growth and patterning at various developmental stages. In vertebrates, the function of Sonic Hedgehog (Shh) - one of the three members of the Hh protein family (Shh, Indian Hh and Desert Hh) - has been thoroughly characterized (reviewed by (1)). Shh is essential for patterning of the ventral neural tube (2), for specifying vertebrate digit identities (3,4) and for the control of axon guidance in the developing nervous system (5). Given these various functions, it is not surprising that down regulation of Hh signaling leads to severe developmental abnormalities. In the adult, Hh pathway activation is involved in maintaining the stem cell niche, including the cancer stem cell niche (6), and Hh activity up regulation contributes to the formation and progression of various cancers (6-8). Thus, tight control of Hh secretion and spreading is essential and its molecular characterization required to better understand how Hh signals elicit dose-dependent responses in temporally and spatially specific manner.

The Hh spreading mechanism is especially intriguing, because all Hh family members are released from producing cells despite being synthesized as dually lipid-modified, insoluble molecules (9). Both in vertebrates and in Drosophila melanogaster, Hh synthesis starts with precursor proteins that undergo a series of post-translational modifications (10). Following cleavage of an N-terminal signal peptide, Hh proteins undergo intein-related processing that involves internal cleavage between residues G198-C199 (mouse Shh nomenclature) (11-14) and covalent attachment of a cholesteryl adduct to G198 of the Shh signaling domain (11,15-17). This modification results in protein multimerization on the cell surface and subsequent release of the protein in multimeric form (18). The second lipid adduct that modifies Hh proteins is palmitic acid, which attaches to the conserved N-terminal cysteine (C25 in murine Shh) via the primary amine exposed after signal peptide cleavage (19). This unusual N-acylation is catalyzed by the product of the skinny hedgehog (Ski) gene (also designated sightless, central missing, raspberry or Hh acyltransferase (Hhat) (20-23)). Although this hydrophobic modification would be expected to further decrease solubility (and thus morphogen diffusion), palmitoylation is absolutely required for Hh biological activity: Unpalmitoylated multimeric proteins are 10x-30x less active than N-palmitoylated wild type forms (21,24). The questions of how Hh lipidation and activity are linked, and how dually lipid-modified Hhs are released from producing cells and spread through the extracellular compartment are currently under intense debate. So far, Hh release has been explained by lipid-dependent micelle formation, co-transport with lipoprotein particles, or ectodomain solubilization via proteolytic removal of both terminal, lipidated peptides (18,25-27)(summarized in Fig. 1).

In the latter scenario, dually lipidated Shh multimers (from hereon called ShhNp, standing for Shh N-terminal processed signaling domains) are released from transfected cells via ADAM (A Disintegrin And Metalloprotease)-mediated ectodomain shedding (18,25). Shedding, however, does not only remove both lipidated peptide anchors, but also activates ShhNp ectodomains in the process. Initially, on the cell surface, ShhNp N-terminal peptides interact with adjacent ShhNp zinc-coordination sites in the cluster, thereby blocking Patched (Ptc)-receptor binding to these sites (Fig. 2A) (18,28-30). This renders the surface-tethered molecule inactive. By removing N-terminal acylated peptides, ADAMs expose Ptc-binding zinc-
coordination sites and thereby couple ShhNp release with its biological activation. The established role of N-palmitoylation for the biological activity of secreted Hhs (21) is therefore indirect: N-acylation merely serves to anchor inhibitory N-terminal peptides to the cell membrane as a prerequisite for their subsequent sheddase-mediated removal. Thus, any absence of N-palmitoylation (in the respective acyltransferase-deficient mutant, or by site directed mutagenesis of the acceptor cysteine residue into a serine (C25S mutagenesis in mouse ShhNp C25S)) restricts sheddase activity towards the C-terminus and results in the release of inactive protein clusters with their zinc-coordination sites (the Ptc binding sites) still blocked. The indirect role of N-acylation for ShhNp function is strongly supported by restored biological activity of otherwise inactive ShhNp C25S upon N-terminal deletion mutagenesis (artificial truncation of the unpalmitoylated protein to mimic processing) (18). This suggests that N-palmitate does not directly contribute to receptor binding on receiving cells, at least in vitro.

In this work, we further support these findings. First, we show that in addition to N-terminal deletion mutagenesis, insertional mutagenesis as well as forced proteolytic processing restore biological activities of otherwise inactive ShhNp C25S (31). We also demonstrate that the N-terminal Cardin-Weintraub (CW) motif (32,33) is processed during sheddase-mediated ShhNp release in vitro and in vivo. Because this motif contributes to ShhNp/HS interactions (30,34,35), CW-processing strongly reduces HS-binding of truncated proteins. On the basis of these findings, we suggest that sheddase-mediated ShhNp release is coupled to functional morphogen activation and impaired HS binding of solubilized clusters, resulting in their facilitated diffusion into the HS-rich extracellular compartment.

Materials and Methods:

Mice: Endogenous murine (m)ShhNp was isolated from forebrains of 8 week old C57/B6 mice (n=6). Brains were separately homogenized in 20mM Tris/HCl pH 8.0, 137mM NaCl and 10% glycerol, and subjected to ultracentrifugation. mShhNp in supernatants was then analyzed by heparin and heparan sulfate affinity chromatography (below) in three independent experiments. Additionally, All 6 cleared homogenates were concentrated via heparin-agarose pulldown or 5E1-immunoprecipitation (also described below). Precipitates were washed and binding of Shh-specific antibodies 5E1 and αCW analyzed by immunoblotting. All experimental procedures were done in accordance with Society for Neuroscience and European Union guidelines.

Cloning and expression of recombinant proteins. Shh constructs were generated from murine cDNA (NM_009170) using primers carrying desired point mutations or deletions by PCR. PCR products (nucleotides 1-1314, corresponding to amino acids 1-438) were ligated into pDrive (Qiagen), sequenced, and subsequently released and religated into pcDNA3.1 (Invitrogen) for the expression of secreted, lipidated 19kDa ShhNp in Bosc23 cells or HEK293 cells. PCR products (nucleotides 1-594, corresponding to amino acid 1-198 of murine Shh) were also cloned into pcDNA3.1 for expression of unlipidated 19kDa ShhN in Bosc23 cells. Where indicated, a hemagglutinin (HA) tag was inserted between N-terminal amino acids G32 and K33 by site-directed mutagenesis (Stratagene), resulting in internally HA-tagged ShhNp. ShhNp C25S (C25 is required for N-terminal palmitoylation during release (18)) was generated by site directed mutagenesis (Stratagene). N-terminally truncated forms and ShhNp5AA or ShhNp·ACW (both lacking HS-binding Cardin-Weintraub function) were likewise generated by site-directed mutagenesis and sequenced. Primer sequences can be provided upon request.
Constructs used in this study are presented in Supplement Fig. 1.

**Cell culture and protein analysis.** Human Bosc23 or HEK293 cells were cultured in DMEM (PAA) with 10% fetal calf serum (FCS) and 100µg/ml penicillin/streptomycin and were transfected using PolyFect (Qiagen). Cells were then kept for 36 hours before the medium was harvested. In most assays, mutated or wild type ShhNp were secreted into serum-containing or serum-free media without any additional treatment. Proteins secreted into serum-containing media were enriched by heparin-sepharose (Sigma-Aldrich) pulldown overnight. Where indicated, Methyl-β-cyclodextrin (Sigma-Aldrich) was used at 300µg/ml and 600µg/ml in serum-free DMEM. Proteins secreted into serum-free media were Trichloroacetic acid (TCA)-precipitated. All proteins were analyzed by 15% SDS-PAGE, followed by Western blotting using PVDF-membranes. Immunodetection was conducted using polyclonal αShhN (goat IgG; R&D Systems), monoclonal 5E1 (DSHB, University of Iowa, USA)(36) or polyclonal αCW-antibodies directed against the HS-binding Cardin-Weintraub sequence (rabbit IgG, Cell Signaling) for primary protein detection. Visualization was performed after incubation with peroxidase-conjugated donkey-α-goat, α-mouse or α-rabbit IgG (Dianova) followed by chemiluminescent detection (Pierce). For radiolabeling, 1mCi [9,19(n)-3H] palmitic acid was added to Shh-transfected HEK293 cells cultured in 35mm dishes for 28 hours under serum-free conditions prior to ShhNp release overnight. 3H-palmitic acid-labeled cells as well as the harvested media were subjected to heparin sepharose pulldown to improve the signal to noise ratio. After SDS-PAGE, gels were immunoblotted and the same blot analyzed by autoradiography.

**Chromatography.** Proteins were purified by FPLC (Äkta Protein Purifier (Pharmacia)) at 4°C. Gel filtration analysis was performed using a Superdex200 10/300 GL column (Pharmacia) equilibrated with PBS at 4°C. Eluted fractions were TCA-precipitated, resolved by 15% SDS-PAGE and immunoblotted. Signals were quantified using ImageJ. To determine HS-binding of endogenous and recombinant forms of Shh, mouse brain lysates or supernatants of Shh-transfected Bosc23 cells were subjected to HS affinity chromatography (Äkta Protein Purifier). HS columns were generated as follows: Embryonic day (E)18 mouse embryos were digested over night with 2mg/ml pronase in 320mM NaCl, 100mM sodium acetate (pH 5.5) at 40°C, diluted 1:3 in water and applied to 2.5ml DEAE sephacel columns. GAGs were applied to PD-10 (Sephadex G25) columns (Pharmacia). GAGs were lyophilized, again purified on DEAE as described above, applied to PD-10 columns and again lyophilized. Via the peptides attached to the HS chains, samples were coupled to an NHS-activated sepharose column according to the manufacturers protocol (Pharmacia). Proteins were applied to the column in the absence of salt and bound material was eluted with a linear NaCl gradient from 0-1M in 0.1M sodium acetate buffer (pH 6.0). Fractions were TCA-precipitated, resolved by 15% SDS-PAGE and immunoblotted. The same E18 HS-column was used for all experiments shown in this work.

**Shh reporter assays.** C3H10T1/2 cells (37) were grown in DMEM supplemented with 10% FCS and antibiotics. 24h after seeding, Shh-conditioned media were mixed 1:1 with DMEM containing 10%FCS and antibiotics, and applied to C3H10T1/2 cells in 15-mm plates. To some assays, 2.5µg/ml 5E1 function blocking antibodies were added (36). Generally, due to variable expression levels, mutant and wild type proteins required adjustment to comparable levels before induction of C3H10T1/2 differentiation. For adjustment, proteins were detected by immunoblotting, and detected bands were quantified by ImageJ. Cells were lysed 5-6 days after induction (20mM Hepes, 150mM NaCl, 0.5% TritonX-100, pH 7.4) and
osteoblast-specific AP activity was measured at 405nm after addition of 120mM p-nitrophenolphosphate (Sigma) in 0.1M glycine buffer, pH 9.5. Assays were performed in triplicate.

Chondrocytes were isolated from the cranial third of 17-day old chick embryo sterna by overnight digestion with collagenase and cultured in agarose suspension cultures under serum-free conditions. Cells were suspended in 0.5% low melting agarose in DMEM and allowed to sediment on the culture dishes which were pre-coated with 1% high melting agarose in water. Cells were grown at densities of 2x10^6 cells/ml in DMEM mixed 1:1 with conditioned, serum-free medium from Shh expressing cells. Media also contained 60µg/ml β-aminopropionitrile fumarate, 25µg/ml sodium ascorbate, 1mM cysteine, 1mM pyruvate, 100units/ml penicillin, and 100µg/ml streptomycin. After 14 days in culture, newly synthesized chondrocyte proteins were metabolically labeled with 1µCi/ml of 14Cproline (250Ci/mmol, NEN Life Science products) for 24h. Collagens were isolated after limited digestion with pepsin and were analyzed by SDS-PAGE followed by fluorography (38).

5E1 immunoprecipitation. 10µg monoclonal 5E1 antibodies were coupled to 2.5mg ProteinA-sepharose beads (Sigma) per IP. 500µl-1ml lysate or medium were incubated overnight on a rotator and analyzed by SDS-PAGE and immunoblotting. For pulldown controls, 40µl heparin-sepharose beads were added to 500µl-1ml Shh-containing media and incubated overnight on a rotator. Experiments were performed in triplicate.

Immunohistochemical detection. sAP-ShhN and mutant forms generated by site-directed mutagenesis were cloned into gWIZ-SEAP (Gene Therapy Systems, San Diego), expressed in Bosc23 cells and secreted into the medium. Conditioned media were normalized for sAP activity and applied to 4% paraformaldehyde (PFA)-fixed frozen embryonic day (E)12 mouse embryo sections overnight, followed by three washes with PBS. sAP-ShhN bound to HS was directly visualized by addition of NBT-BCIP (Roche). Control slides were heparinase I-III digested (IBEX, Montreal, Canada) (50mM Hepes, 100mM NaCl, 1mM CaCl2, 5µg BSA/ml, pH 7.0) at room temperature overnight to confirm Shh/HS specificity. sAP-ShhN was also incubated in the presence of 1M NaCl to prove Shh/HS specificity.

Statistical analysis. All statistical analysis was performed in Prism using Student’s t-test (Two-tailed, unpaired, CI 95%). All error estimates are standard deviations of the mean.

Molecular modeling. The crystal structure of human Shh (PDB: 3M1N)(39) was displayed employing the PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC.

Results:

Isolation of N-terminally truncated ShhNp from mouse brain

In our model of Shh release, sheddase-mediated processing of inhibitory N-terminal peptides is directly linked to biological activation of solubilized protein clusters. Unprocessed N-palmitoylated peptides (Fig. 2A, black ribbon) otherwise interact with zinc-coordination sites of adjacent molecules in the cluster (yellow molecule, zinc is shown as a black sphere). Because Shh zinc-coordination sites represent binding sites for the Shh receptor Ptc (colored in orange)(28,29), unprocessed N-termini prevent receptor-binding and thereby render the protein inactive. In this scenario, palmitate facilitates shedding via membrane-proximal positioning of N-terminal inhibitory peptides as a prerequisite for processing, and also prevents solubilization of any incompletely activated (partially processed) morphogen clusters via their firm attachment to the cell membrane.
To confirm these in vitro derived data, we aimed to detect ShhNp processing in vivo. To this end, brain samples from 8-week old C57/B16 mice were lysed in detergent-free buffer and centrifuged to deplete membrane-tethered murine (m)ShhNp. We then assessed binding of the soluble protein to physiologically relevant HS (Fig. 2B). To this end, HS derived from embryonic day (E)18 C57/B16 mouse embryos was coupled to a HiTrap column and protein binding analyzed by FPLC affinity chromatography. The soluble fraction was also subjected to heparin affinity chromatography. Recombinant ShhN and mutant ShhNΔCW lacking the HS-binding CW-motif served as positive and negative controls, respectively. Consistent with previous observations, control ShhN eluted from HS at 0.6M NaCl and from heparin at 1M NaCl (30). In contrast, brain-derived 5E1-reactive mShhNp (40) did not bind to HS but eluted from heparin at 0-0.75M NaCl. In this regard, endogenous mShhNp resembled ShhNΔCW which also binds heparin but not HS (30,35). This suggested that endogenous, mouse brain-derived mShhNp lacks functional CW-residues required for HS-binding of the solubilized morphogen.

This finding prompted us to directly confirm loss of N-terminal CW-residues during release. Murine brain samples were homogenized in detergent-free buffer and subjected to ultracentrifugation. Soluble mShhNp in supernatants was then incubated with agarose-linked heparin in the presence of 0.5M NaCl to reduce unspecific interactions, or immunoprecipitated using ProteinA sepharose-coupled 5E1 antibodies (Supplement Fig. 2A). Like Ptc, monoclonal 5E1 binds the Shh zinc-coordination site (Fig. 2A, 5E1 epitope in red), residue histidine 180 being bound by both, the receptor and the antibody (Fig. 2A, pink) (31). 5E1 binding thus competes with Ptc binding (thereby inhibiting Shh signaling)(36), and ShhNp binding of the antibody and the receptor is blocked by unprocessed N-terminal peptides (18). As a consequence, 5E1 binds N-terminally truncated ShhNp but not the unprocessed precursor. For subsequent Shh detection, we employed SDS-PAGE and PVDF-immunoblotting using αCW-antibodies raised against the N-terminal peptide immunogen K33RRHPKK39 (the CW-motif). The same blot was then stripped and incubated with 5E1 antibodies. Both antibody specificities were confirmed using recombinant Shh controls (Supplement Fig. 2B, C). Consistent with impaired HS-binding shown in Fig. 2B, 5E1-reactive endogenous proteins were clearly detected in the immunoprecipitated material, but not after heparin-pulldown (Fig. 2C). Notably, 5E1-immunoreactive mShhNp on the same (stripped) blot lacked αCW-antibody reactivity, in contrast to recombinant control proteins. Thus, results shown in Figs. 2B and 2C suggest N-terminal processing of solubilized mShhNp in vivo.

As an approximation for the N-terminal mShhNp processing site, we next determined minimum requirements for αCW-antibody binding. To this end, we employed artificially truncated forms of recombinant ShhN that lacked variable numbers of N-terminal amino acids, and determined that amino acids R35HPKK39 were required and sufficient for αCW-antibody binding to soluble proteins (Fig. 2D, residues are colored blue in A, and Supplement Fig. 2C). The observed lack of mShhNp αCW-antibody reactivity thus suggests processing of endogenous proteins at arginine (R)35 or downstream residues.

Notably, proteolytic CW-processing is consistent with two published findings: 1) Site-directed exchange of all five basic CW-residues for alanines (ShhNp5xA) increases morphogen processing (30), indicating cleavage susceptibility of this site in the absence of bound HS. 2) Automated Edman degradation of E. coli-expressed Shh revealed two potential CW-cleavage sites,
C\textsuperscript{25}GPGRFGK \downarrow \text{R\textsuperscript{34}}RHPKKLTPLAY… (CW-residues underlined, \downarrow denotes the processing site) and C\textsuperscript{25}GPGRFGKR\text{RRHPK} \downarrow \text{K\textsuperscript{39}}LTPLAY… (25). Importantly, N-terminal CW-residues are conserved in all vertebrate Hh family members, raising the possibility that this site represents a general sheddase target site. Unfortunately, multiple attempts to N-terminally sequence soluble proteins from \textit{in vivo} sources or transfected cells \textit{in vitro} failed due to insufficient Shh recovery after purification (data not shown).

**N-terminal ShhNp\textsuperscript{C25S} truncation restores 5E1 binding**

Therefore, we established alternative strategies to identify N-terminal ShhNp processing sites \textit{in vitro}. One strategy involved recombinant expression of unpalmitoylated (and thus, N-terminally unprocessed) ShhNp\textsuperscript{C25S} in addition to engineered variants that lacked variable numbers of N-terminal amino acids (Figs. 3A, B). The underlying idea was that expression of 5' truncated cDNA lacking codons for Shh amino acids 26-38 would mimic N-terminal processing, and thus restore 5E1-binding of otherwise unreactive ShhNp\textsuperscript{C25S}. To this end, secreted proteins were either 5E1 immunoprecipitated or pulled down using heparin-sepharose beads that bind all forms (30). We indeed detected strong heparin binding of all proteins (Fig. 3C, top blot, left), but found that ShhNp\textsuperscript{C25S}, ShhNp\textsuperscript{C25S,A26-35} and ShhNp\textsuperscript{C25S,A26-36} were not 5E1-immunoprecipitated (Fig. 3C, bottom blot, left). In contrast, 5E1-binding of ShhNp\textsuperscript{C25S,A26-37}, ShhNp\textsuperscript{C25S,A26-38}, ShhNp\textsuperscript{C25S,A26-33} and ShhNp\textsuperscript{C25S,A26-34} was fully restored, as it was comparable to ShhNp\textsuperscript{5A} serving as a positive control (30). Further analysis confirmed blocked 5E1-binding \textit{in trans}, because 5E1-binding of monomeric ShhN\textsuperscript{C25S} and truncated monomers ShhN\textsuperscript{C25S,A26-31}, ShhN\textsuperscript{C25S,A26-32}, ShhN\textsuperscript{C25S,A26-33} and ShhN\textsuperscript{C25S,A26-38} was comparable (Fig. 3C, right).

**N-terminal ShhNp\textsuperscript{C25S} truncation restores Ptc-binding**

Thus, to rule out false positive results due to the possible release of monomeric truncated ShhNp\textsuperscript{C25S} variants, we confirmed their unimpaired multimerization by gel filtration (Fig. 4A). Unimpaired multimerization of all forms allowed us to subsequently conduct biological tests as a read-out for their Ptc-binding capacities. To this end, we took advantage of Shh-dependent C3H10T1/2 osteoblast precursor cell differentiation as a sensitive bioassay (37). As shown in Fig. 4B, wild-type control ShhNp strongly induced C3H10T1/2 differentiation into alkaline phosphatase (AP)-producing osteoblasts. In contrast, negative control media obtained from mock-transfected Bosc23 cells as well as full-length ShhNp\textsuperscript{C25S} were inactive, as expected. In agreement with restored 5E1 binding shown before, we observed variable C3H10T1/2 differentiation induced by truncated ShhNp\textsuperscript{C25S} variants: Here, ShhNp\textsuperscript{C25S,A26-33} and ShhNp\textsuperscript{C25S,A26-34} showed strongest relative activities (control ShhNp activity was set to 100%, Fig. 4B). ShhNp\textsuperscript{C25S,A26-34} also induced Gli1-dependent Firefly luciferase expression in Light-2 cells (41) and led to Shh-dependent differentiation of primary chondrocytes (38) (Supplement Fig. 3). However, we noticed that induced C3H10T1/2 and Light 2 activities varied considerably, possibly due to the presence of serum factors or unspecified effects of N-terminal serine 25 replacing the palmitate acceptor cysteine. Yet, variable but restored biological activities observed in all three assays indicate preferred processing at, or close to, CW-residues R34/R35 during ShhNp release \textit{in vitro}.

**Forced N-terminal processing also restores ShhNp\textsuperscript{C25S} bioactivity**

A more robust test for processing-dependent Shh biofunction involved incubation of transfected HEK293 cells with 300\textmu g/ml and 600\textmu g/ml methyl-\textbeta-
cyclodextrin (MβCD), a cyclic oligosaccharide that depletes cholesterol from living cells. Under serum-free conditions, MβCD strongly increases ShhNp release (25) in agreement with MβCD acting as a sheddase stimulator. Fig. 5A shows N-terminally processed ShhNp (double asterisks) generated from the cell-tethered precursor protein (single asterisk) (42-47). MβCD-induced shedding targeted CW-residue R35 or downstream residues, as indicated by reduced αCW-antibody reactivity of the bottom (processed) band (same stripped blot, double asterisks denote the processed form). This observation confirms ShhNp release via MβCD-activated sheddases, because any possible direct extraction of molecules via their cholesteryl moiety should have left their molecular weight and αCW-antibody reactivity unchanged.

MβCD-treatment of ShhNpC25S-transfected HEK293 cells also resulted in increased morphogen solubilization (via stimulated processing of membrane-linked C-termini), as expected. Notably, this treatment also resulted in N-terminal protein processing (Fig. 5A, double asterisks), in contrast to normal culture conditions that always produced soluble intact ShhNpC25S (Fig. 5A, left). Based on our model of coupled N-terminal processing and activation (Figs. 3C, 4B), we expected increased bioactivity of MβCD-released ShhNpC25S over the unprocessed form (18,21,24,27,48-50). As shown in Fig. 5B, released ShhNpC25S in the presence of 300µg/ml MβCD indeed stimulated C3H10T1/2 cells (1.1±0.2 arbitrary units (ShhNpC25S) versus 1.34±0.06 arbitrary units (ShhNp), p=0.1919, relative activity ratio 1.3:1, n=4). In contrast, ShhNpC25S expressed under normal conditions (in the presence of FCS, no MβCD) failed to significantly differentiate C3H10T1/2 cells (0.32±0.001 arbitrary units versus 0.97±0.018 arbitrary units obtained for ShhNp, p≤0.0001, n=4 (Fig. 5B)). Correction for the mock value (0.24 arbitrary units) resulted in ShhNp/ShhNpC25S activity ratio of 10:1. 1.55±0.23 arbitrary units were obtained after incubation with 600µg/ml MβCD (ShhNpC25S) compared to 2.1±0.14 arbitrary units for the wild type, p=0.062, n=4 (ShhNp/ShhNpC25S activity ratio: 1.44:1). Control MβCD-treatment of mock-transfected HEK293 cells did not induce C3H10T1/2 differentiation. We draw two conclusions from this experiment: First, restored ShhNpC25S bioactivity demonstrates that N-palmitate is not directly required for ShhNp signaling on receiving cells, consistent with previous in vitro findings (Fig. 4B). Second, restored bioactivity of N-processed ShhNpC25S is consistent with restored 5E1-binding and signaling pathway activation shown in Figs. 3C, 4B and Supplement Fig. 3, confirming coupled ShhNp N-terminal truncation and functional activation.

**Insertional mutagenesis also restores ShhNpC25S bioactivity**

Additional support for regulatory roles of N-terminal peptides was obtained by insertional mutagenesis. Hemagglutinin (HA)-tagged proteins (N-terminal peptide sequence C/S25GPGRGF32YPYDVPDYAK33RRHPKK39 (italicized letters represent the tag, Supplemental Fig. 1)) were expressed and the soluble proteins tested for their biological activities (18). As a control, media from untransfected Bosc23 cells failed to induce C3H10T1/2 differentiation (mock), and ShhNpC25S activity was ~30x reduced if compared to untagged wildtype proteins (2.8±0.11 arbitrary units (ShhNp) versus 0.18±0.016 (ShhNpC25S), p=0.0001, n=4)(Fig. 6A). These findings confirm our previous results (Fig. 5B) and those of others (19,51). In contrast, we noticed that biological activities of HA-tagged ShhNpC25S were only ~2x reduced if compared to HA-tagged wildtype proteins under the same experimental conditions (1.62±0.05 arbitrary units (ShhNpC25S) versus 0.81±0.04 (ShhNp), p=0.041, n=4).
(ShhNpHA) versus 0.85±0.03 (HA-tagged ShhNpC25S), p=0.0001, n=4). Co-incubation with Shh-neutralizing 5E1 antibodies resulted in strong inhibition of both observed activities, demonstrating specificity of the assay. We confirmed this result by Hh-dependent chondrocyte differentiation under serum-free conditions (38). In this bioassay, primary chick chondrocytes were incubated with ShhNp-conditioned media, and Collagen X-production was used as a readout for Hh-induced hypertrophic differentiation (Fig. 6B). Insulin-like growth factor I (IGF-I), ShhNp and ShhNpHA served as positive controls. In contrast, untagged ShhNpC25S and ShhNpC25S:5xA (Lacking N-palmitoylation in addition to a functional CW-motif) were always inactive, serving as negative controls. Notably, HA-tagged ShhNpC25S induced prehypertrophic chondrocyte differentiation into Collagen X-producing hypertrophic chondrocytes, and HA-tagged ShhNpC25S:5xA was also active. These observations confirm Shh activity regulation by N-terminal peptides, and that N-palmitate is not directly required for Shh biofunction on receiving cells. We hypothesized that the inserted 9 amino-acid HA-tag disturbed peptide/protein contacts, resulting in facilitated Ptc-binding of unprocessed clusters. To confirm this possibility, heparin-sepharose pulldown and 5E1-immunoprecipitation of ShhNpC25S and HA-tagged ShhNpC25S:5xA were conducted as described above (Fig. 3C), and PVDF-immunoanalyzed; the same blot was then subjected to autoradiography to detect C25-linked 3H-palmitic acid. As shown in Fig. 7B, cell-tethered 19kDa proteins (top bands, single asterisk) were labeled and largely unprocessed, consistent with their 3H-palmitic acid signals. These bands represented the dually lipidated morphogens. In contrast, increased electrophoretic mobility ShhNp5xA protein signals lacked 3H-palmitic acid (Fig. 7B bottom, double asterisks), thus representing the N-terminally processed proteins (Fig. 3C). Notably, in this assay, ShhNp processing was strongly reduced if compared to ShhNp5xA. Because CW-residues associate with cell-surface HS prior to release (52), this observation suggests that HS-binding may (down)regulate sheddase-mediated processing and release, possibly via blockade of susceptible CW-residues.

Interestingly, we observed that processed ShhNp5xA was inactive in C3H10T1/2 cells (Fig. 7C: 0.27±0.05 arbitrary units, p=0.57 compared to ShhNpC25S, 0.3±0.01 arbitrary units, n=5). In
contrast, ShhNp was always active (1.1±0.08 arbitrary units, n=5). As a possible explanation, we hypothesized that ShhNpC25S could have been processed at a “non-permissive” site different from the wild type site (Fig. 4B). We employed two alternative strategies to test this idea: First, molecular weight analysis of unprocessed versus processed ShhNp5xA using Bio1D software revealed a ~1400Da size shift, corresponding to N-terminal residues C25GPRGFGRGKRRHP37 (1405Da). This suggested peptide cleavage between P37 and K38. Second, we immunoblotted N-truncated ShhNpC25S mutants (Fig. 3B,C) together with ShhNp5xA. As shown in Fig. 7D, the processed ShhNp5xA protein (double asterisks) corresponded in size with ShhNpC25SΔ26-38, confirming the processing site. K38 processing is consistent with robust 5E1-reactivity of the protein (Fig. 3C) but low bioactivity of corresponding ShhNpC25S truncated mutants in C3H10T1/2 cells (Figs. 4B, 7C). In contrast, R34/R35 processing was linked to ShhNp release (Fig. 7E, top blot), consistent with impaired αCW-binding of processed proteins (Fig. 7E, bottom blot), strong 5E1-binding of corresponding ShhNpC25SΔ26-34 (Fig. 3C), and restored ShhNpC25SΔ26-34 and ShhNpC25SΔ26-35 biological activities in C3H10T1/2 cells (Fig. 4B). Based on these findings and those of others (25), we suggest cleavage of CW-residues R34/R35 as a requirement for ShhNp solubilization and activation.

**HS binding of CW-processed Shh is reduced**

Because HS/Shh-interactions are CW-dependent, as shown in this work and elsewhere (30,33,35), and because HSPGs are critically involved in Shh biology (53-58), we aimed to confirm that CW-processing at or beyond residue 35 reduces protein binding to HS (Fig. 2B). This would provide an explanation for the ability of solubilized ShhNp to travel long distance in the HS-containing extracellular compartment (56,59-62), effectively escaping HS-mediated protein immobilization and internalization.

To test this possibility, we first compared binding of processed and unprocessed proteins to FPLC-column-coupled E18-derived HS (Fig. 2B)(30). We first compared ionic interactions of ShhNpC25S (the N-terminally unprocessed multimer) with the unprocessed, alkaline phosphatase (sAP) tagged monomeric protein (sAP-ShhN). Both proteins eluted at 0.6M NaCl, demonstrating comparable HS-binding of monomeric and multimeric unprocessed proteins (Fig. 8A). Control sAP did not bind HS, demonstrating specificity of the assay. Next, comparable AP-activities of sAP, sAP-ShhN and CW-truncated sAP-ShhNA25-35 were applied to the same E18 HS-column, and sAP-activities in the flowthrough were monitored to confirm saturation of HS binding sites. Here, in notable contrast to strong sAP-ShhN binding to HS (30), sAP-ShhNA25-35 did not bind to E18 HS. This confirmed CW-dependent Shh/HS binding in vitro, and that CW-truncation impairs Shh association with physiologically relevant forms of HS, such as embryonic HS.

An embryonic tissue known to be patterned by Shh is the developing neural tube. To directly test this tissue, E12 embryo sections were incubated with equal amounts of the same sAP, sAP-ShhN and sAP-ShhNA25-35 proteins used above. As a readout for Shh binding to tissue HS, sAP activity was detected colorimetrically (Fig. 8B)(34). sAP-ShhN yielded a strong signal, and negative controls sAP and sAP-ShhNp5xA, HS digestion with Heparinase I-III (H-ase) prior to sAP-ShhN incubation or sAP-ShhN incubation in the presence of 1M NaCl demonstrated CW-dependent, specific HS binding of the protein. As expected, sAP-ShhNA25-35 failed to bind HS on tissue sections. Based on this and previous results, we conclude that HS/ShhN interactions depend on the CW-motif (30,34,35), and that CW-processing during release reduces HS-
binding of solubilized proteins. Conversion of HS-associated cell-surface clusters into soluble unreactive forms may thus underlie unimpaired extracellular ShhNp diffusion.

Discussion:

How morphogen gradients arise has attracted much controversy. So far, micelle formation and lipoprotein-mediated transport, morphogen transport by transcytosis (the sequential endocytosis and exocytosis of bound ligands (63)) and transport via “bucket brigade” (receptor-bound morphogen on one cell moves by being handed over to receptors on adjacent cells (64)) have been suggested. One major argument against extracellular Hh morphogen diffusion, which would represent another possible mechanism, was its apparent incompatibility with dual ShhNp lipidation and hydrophobicity. However, as confirmed in this work, ShhNp shedding represents a mechanism to overcome this problem. Another major argument against morphogen diffusion was that cell surface receptors or non-receptors, such as HSPGs, would strongly impair extracellular movement of “sticky” molecules. Indeed, extracellular glypicans represent a major group of Hh-binding HSPGs that modulate morphogen activity, mobility and stability (53,57,58,65-67). Here, negatively charged HS chains represent the main direct interactors between HSPGs and highly conserved CW-motifs present in all Hhs (30,33,35). The resulting Hh/HS interactions are essential for Hh multimerization on producing cells (52), however, similar interactions in the HSPG-rich extracellular matrix would be expected to prevent long-range diffusion of soluble proteins (64,68).

In this work, we suggest that CW-processing during release resolves this problem via conversion of HS-binding surface proteins into unreactive soluble clusters. This mechanism would allow for unimpaired HSPG/ShhNp interactions on the cell surface, yet facilitate protein diffusion upon removal of essential CW-amino acids. Therefore, we postulate that cell-surface shedding serves three functions in Hh biology: it is required for the solubilization of cell surface-linked morphogen clusters, their biological activation during release, and their associated reduction of HS-binding. We are aware of the possibility that N-terminal peptides may get cleaved at different CW-amino acids during release, possibly resulting in variable HS-binding of alternatively truncated soluble proteins. In addition to variable HS-binding, N-terminal Shh processing at different sites may also predetermine biological responses of receiving cells (Fig. 4 and Supplement Fig. 3) at the molecular level. This is indicated by alternative transcriptional activities of responding C3H10T1/2 cells after stimulation with differently truncated ShhNp, as shown in Supplement Fig. 4. Thus, alternative N-terminal processing may represent a new regulator for the various biological responses that Shh can elicit. Interestingly, and consistent with this idea, N-terminal proteolysis also specifically regulates biological activities of secreted Wnt morphogens. Wnt family members are essential for embryogenesis, pathogenesis and regeneration, and like Hhs, palmitoylated Wnt proteins initially locate to the cell surface and require solubilization to reach their targets. In this process, Wnts can undergo proteolysis-induced processing of eight N-terminal amino acids, resulting in Wnt oxidation-oligomerization and functional inactivation (69). Therefore, Wnts and Shh represent two examples for the emerging theme of functional morphogen control by proteolytic removal of regulatory N-terminal peptides.

In contrast to proteolysis-induced Wnt inactivation, however, removal of ShhNp N-termini activates the oligomerized protein (40). This is supported by restored ShhNp C2SS biofunction and 5E1-binding after deletion mutagenesis, as shown in this work. Furthermore, enhanced N-terminal processing
in the presence of MβCD is associated with increased electrophoretic mobility, associated loss of αCW-reactivity and biological activation of truncated ShhNp and ShhNpC25S. Notably, these observations may be reflected by recently demonstrated bioactivity of HEK293-expressed, 5E1-immunoprecipitated lower molecular weight ShhNp, and the lack of such an activity in the remaining higher molecular weight protein (Fig. 6 in (70)). Because we and others showed that higher molecular weight ShhNp is palmitoylated, in contrast to increased electrophoretic mobility ShhNp (the bottom band) which is not (Fig. 7B) (70), we conclude that N-acylation, although essential for ShhNp biological activity per se, is not directly involved in ShhNp signaling on receiving cells. This confutes the third argument against sheddase-dependent morphogen diffusion: the assumed direct role of N-palmitate for Hh bioactivity.

Instead, we suggest that N-palmitate ensures quantitative N-terminal ShhNp processing as a prerequisite for complete activation of all solubilized molecules, as incompletely processed clusters will remain tethered to the cell surface. As a consequence, protein concentrations at any position in the responsive field correlate with their biological activities (their Ptc-binding capacities). In contrast, in the absence of quantitative N-terminal processing, biological activities of solubilized clusters would vary, depending on the relative number of processed N-terminal peptides. As a result, protein concentrations at any position in the field would not reliably correlate with Shh signaling activities. This, in turn, would affect morphogen-dependent tissue patterning in unpredictable ways. In the most extreme case, e.g., in acyltransferase-deficient mutants, processing would be restricted to C-terminal peptides in the cluster, resulting in the solubilization of inactive proteins and complete decoupling of protein/activity gradients. This essential “cleavage/activation control” function may explain the unusual attachment of palmitate via an amide bond to the α-amino group of all Hhs, whereas palmitate in most other proteins is thioester (S)-linked (71). S-linked palmitate, however, is susceptible to enzymatic deacylation by palmitoyl-protein thioesterases which would result in a situation comparable to that in acyltransferase-deficient mutants. In contrast, amide-linked palmitate is resistant to thioesterase activity and thus restricts possible modes of Hh release to shedding, ensuring the release of fully activated proteins. As discussed above, N-palmitoylation may simultaneously ensure quantitative CW-processing and inactivation required for unimpaired ShhNp diffusion through the matrix. Endogenous expression of 5E1-reactive but αCW-antibody and HS-unreactive mShhNp in the in vivo mouse brain strongly supports this idea.

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Hybridoma Bank maintained by the University of Iowa, Iowa City, USA. The excellent technical and organizational assistance of Sabine Kupich is gratefully acknowledged.
Fig. 1: Proposed models for Hh release from producing cells. Human and mouse Shh signaling domains differ by only one amino acid, and their pdb-structures are highly homologous. This allowed us to combine murine Shh (pdb:1VHH, amino acids 39-195) and human Shh (pdb: 3M1N, amino acids 25-190) to visualize both terminal, extended peptides (top left). Both terminal peptides are lipidated in vitro and in vivo, resulting in ShhNp tethering to cell membranes and morphogen multimerization via electrostatic protein-protein interactions. Classic models of Hh solubilization suggest detachment of lipidated morphogens from the membrane via micelle formation (A) or association of lipidated proteins with the phospholipid monolayer of lipoprotein particles (B). Alternatively, membrane-proximal cleavage of lipidated extended peptides by A disintegrin and Metalloprotease (ADAM) sheddases results in the solubilization of truncated, biologically active ShhNp multimers in vitro (C). P: N-terminally linked palmitic acid, C: C-terminally linked cholesterol. Drawing not to scale.
Fig. 2: Endogenous secreted murine (m)ShhNp is N-terminally processed. A) Intermolecular interactions observed in the human ShhN crystal structure (pdb:3M1N)(18,39). The N-terminal peptide of one molecule (black ribbon) overlaps 5E1- and Ptc-binding sites of the adjacent molecule in the cluster (in yellow). 5E1-binding depends on Shh residues T125, D147, R153, S177, A179 and H180 (shown in red) (31). Ptc-binding residues are shown in orange, residue H180 bound by both, 5E1 and Ptc, in pink. The αCW-antibody-reactive CW-peptide R35HPKK39 is colored blue. Polyclonal αShhN binds to multiple, undefined epitopes. N: N-termini (C25). C: C-terminus B) Ionic heparin- and E18-HS interactions of mouse brain-derived mShhNp and recombinant wildtype (ShhN) and CW-deficient (ShhNΔCW) proteins. For HS-affinity chromatography, E18 embryonic HS was coupled to a FPLC column, proteins applied, and bound proteins eluted with a linear (0-1.5M) NaCl gradient. All proteins bound to heparin, in contrast, only ShhN bound to HS. ShhNΔCW and mShhNp were both detected in the flowthrough (ft), suggesting that the endogenous protein lacked functional HS-binding CW-residues. C) mShhNp and recombinant control proteins were concentrated by heparin-agarose pulldown in the presence of 0.5M NaCl to suppress weak ionic interactions, or 5E1-immunoprecipitation. As shown in B, heparin bound the recombinant protein but not endogenous mShhNp, again indicating loss of basic amino acids. Consistent with this, immunoprecipitated mShhNp was 5E1 reactive but lacked αCW-antibody reactivity in all assays (n=6). One representative experiment is shown. rec c: Recombinant control Shh confirmed specificity of both antibodies on the same (stripped) blot. D) Mapping of the αCW binding site. Truncated proteins (N-terminal amino acids are indicated, C25 and S25 represent full-length forms) were expressed and concentrated by heparin-pulldown, followed by immunoblotting. All truncated proteins were expressed at high levels, as indicated by polyclonal αShhN reactivity. However, αCW-specific antibodies failed to bind all proteins truncated beyond residue R35 on the same (stripped) blot, confirming that endogenous mShhNp was likely processed at or C-terminal of this residue.
Fig. 3: ShhNp^{C25S} N-terminal truncation restores 5E1 binding. A) Intermolecular interactions observed in the human Shh crystal structure. The 5E1-binding site is shown in red, the Ptc-binding site in orange, residue H180 bound by 5E1 and Ptc in pink. N-terminal amino acids blocking these sites are labelled. B) All mutant proteins lack N-terminal C25, preventing palmitoylation, and also variable numbers of N-terminal amino acids. Red: Ski/Hhat acyltransferase recognition motif. CW: Cardin-Weintraub motif (blue). C) ShhNp^{C25S} and N-terminally truncated forms were expressed in Bosc23 cells and soluble proteins either 5E1-immunoprecipitated or concentrated by heparin-sepharose pulldown. Proteins were immunoblotted and detected by αShhN antibodies. Consistent with a previous report, 5E1 failed to immunoprecipitate ShhNp^{C25S} (18). Consecutive N-terminal truncations, however, resulted in restored 5E1 binding, especially of ShhNp^{C25S,Δ26-37}, ShhNp^{C25S,Δ26-38}, ShhNp^{C25S,Δ26-33} and ShhNp^{C25S,Δ26-34}. Proteolytically processed ShhNp^{5xA} served as a positive control (30). Right: Corresponding monomeric proteins (ShhN) were N-terminally truncated and analyzed as described above (B, right). Comparable 5E1-binding of truncated and untruncated monomers confirms Shh clustering as a prerequisite for protein inactivation in trans.
Fig. 4: Restored biological activity of N-terminally truncated, palmitoylation-deficient ShhNp\(^{C25S}\) variants. All mutant proteins lacked N-terminal C25, preventing palmitoylation, in addition to consecutive N-terminal peptides (Fig. 3B). Comparable amounts of mutated proteins were employed in all following assays, as determined by immunoblotting. A) Comparable 600kDa and 100-300kDa multimerization was detected by gel filtration of ShhNp\(^{C25S}\) and compound mutant proteins (ShhNp\(^{C25S;\Delta26-31}\) to ShhNp\(^{C25S;\Delta26-38}\)). Elution profiles are expressed relative to the highest protein level in a given run, which was set to 100%. B) C3H10T1/2 osteoblast precursor cells were incubated with single and compound mutant proteins, and the relative amount of Shh-induced AP-production was determined as a biological readout. Medium obtained from mock-transfected Bosc23 cells and ShhNp\(^{C25S}\)-conditioned medium were used as negative controls, and ShhNp conditioned medium as a positive control (set to 100%, other values were expressed relative to ShhNp activity). In this assay, ShhNp\(^{C25S;\Delta26-33}\) and ShhNp\(^{C25S;\Delta26-34}\) showed activities comparable to that of the wild-type (103%±20% and 99.5%±11% relative to the wild-type control, p=0.9 and 0.94, n=6). All truncated ShhNp\(^{C25S}\) double mutants showed significantly increased biological activities if compared to non-truncated ShhNp\(^{C25S}\) (p<0.05 in all forms). *** denote significant ShhNp activity if compared to ShhNp\(^{C25S}\) (p<0.01). n.s.: not significant (p>0.05).
Fig. 5: Forced ShhNpC25S N-terminal processing and activation by MβCD. A) 40 hours after HEK293 cells were transfected with full-length Shh cDNA or cDNA encoding for the palmitoylation-deficient protein, cells were washed and serum-free DMEM with or without 300µg/ml and 600µg/ml methyl-β-cyclodextrin (MβCD) was added. After 4 hours, the medium was harvested, subjected to centrifugation and TCA-precipitation, followed by SDS-PAGE and Western-blot analysis using αShhN- and αCW-antibodies. Cellular protein expression levels (cell lysates) are shown on top. Right: MβCD treatment resulted in forced shedding and increased release of N-terminally truncated ShhNp and ShhNpC25S into supernatants. Reduced αCW-reactivity of processed proteins indicates cleavage at or in close vicinity to CW-residue R35 (bottom blot). Results from the same (stripped) blot are shown. Left: Control expression of N-terminally truncated ShhNp (double asterisks) and unprocessed ShhNpC25S (asterisk) in the presence of 10% FCS and without MβCD. B) C3H10T1/2 osteoblast precursor cells were incubated with aliquots of the same ShhNp and ShhNpC25S-conditioned media analyzed above, and relative amounts of Shh-induced AP-activity were determined. Medium from mock-transfected HEK293 cells was used as a control. *** denote significance between biological activities of ShhNp and unprocessed ShhNpC25S expressed without MβCD (p<0.01). In contrast, the observed difference between MβCD-released ShhNp and ShhNpC25S was insignificant (n.s., p>0.05).
Fig. 6: ShhNp<sup>C25S</sup> insertional mutagenesis restores 5E1-binding and biological activity. A) C3H10T1/2 osteoblast precursor cells were incubated with comparable amounts of untagged and HA-tagged wild-type and unpalmitoylated ShhNp<sup>C25S</sup> produced in DMEM+10% FCS. The relative amounts of Shh-induced AP-activity were determined as a readout for C3H10T1/2 differentiation and hence for biological activity of the proteins. Medium obtained from mock-transfected Bosc23 cells served as a negative control. For ShhNp and ShhNp<sup>C25S</sup>, differences in biological activity were always significant (ShhNp<sup>C25S</sup>: 0.18±0.017 arbitrary units versus ShhNp: 2.815±0.11 arbitrary units, p<0.0001, n=4, ShhNp/ShhNp<sup>C25S</sup> activity ratio: ~30x). The relative activity of HA-tagged ShhNpC25S, however, was greatly increased if compared to HA-tagged ShhNp (HA-tagged ShhNpC25S: 0.85±0.03 arbitrary units versus HA-tagged ShhNp: 1.62±0.05 arbitrary units, p=0.0086, n=4, ShhNp/ShhNpC25S activity ratio: ~2x). *** denote significance (p<0.01) B) Serum-free conditioned media were added to primary chick chondrocytes (38). Differentiation of these cells was monitored by <sup>14</sup>C proline incorporation into Collagen II (α<sub>1</sub>(II)) and Collagen X (α<sub>1</sub>(X)) after 14 days in culture, as analyzed by <sup>14</sup>C-autoradiography. Chondrocyte hypertrophy (as indicated by α<sub>1</sub>(X) production) was induced by HA-tagged ShhNp<sup>C25S</sup> in the presence or absence of the CW-motif (ShhNp<sup>5x</sup>A), but not by untagged ShhNp<sup>C25S</sup>. Insulin-like growth factor I (IGF-I) and ShhNp served as positive controls, media of mock-transfected Bosc23 cells as negative control. C) Secreted proteins were 5E1-immunoprecipitated or concentrated by heparin-sepharose pull-down and immunoblotted. ShhNp<sup>C25S</sup> was 5E1-unreactive (negative control), truncated ShhNp<sup>C25S;Δ26-38</sup> was 5E1-reactive (positive control as shown in Fig. 3C)). Full-length HA-ShhNp<sup>C25S</sup> was 5E1-immunoprecipitated, indicating that the HA-tag disturbed the location of inhibitory peptides in the cluster.
Fig. 7: CW-mutagenesis abolishes HS-binding and increases ShhNp processing. A) Deletion of all five basic CW-residues fully abolishes ShhNp<sup>5xA</sup> binding to E18 embryo-derived HS. Averaged elution profiles of 3 independent ShhNp and ShhNp<sup>5xA</sup> analyses on the same HS-coupled affinity column are shown. Error estimates are standard deviations of the mean. B) Immunoblot (top) and autoradiograph (bottom) of [9,10(n)-<sup>3</sup>H] palmitic acid-labeled, full-length ShhNp and ShhNp<sup>5xA</sup> in cell lysates (c) and media (m). Full-length 19kDa ShhNp (asterisk) was detected in lysates and media by Western blotting and <sup>3</sup>H-autoradiography. Unprocessed, radiolabeled ShhNp<sup>5xA</sup> was also detected in the cell lysate. In contrast, truncated soluble ShhNp and ShhNp<sup>5xA</sup> (double asterisks) derived from the labeled cell-bound forms lacked <sup>3</sup>H palmitic acid, indicating N-terminal processing. Note that relative processing of ShhNp<sup>5xA</sup> was increased if compared to the wild-type protein. The presence of unprocessed soluble ShhNp in media is due to extensive cell death caused by serum-free culture conditions required for labeling. C) C3H10T1/2 osteoblast precursor cell differentiation is significantly induced by processed ShhNp, but not by unprocessed ShhNp<sup>C25S</sup> (1.1±0.08 arbitrary units versus 0.3±0.01 arbitrary units, n=6, p=0.0023). In this assay, ShhNp<sup>5xA</sup> was inactive despite its N-terminal processing (0.27±0.05 arbitrary units, p=0.57 compared to ShhNp<sup>C25S</sup>). ** denote significance, n.s: not significant (p>0.05). D) Size comparison of processed ShhNp<sup>5xA</sup> with ShhNp<sup>C25S</sup> and N-terminally truncated proteins. Comparable size of ShhNp<sup>C25S/A26-38</sup> demonstrates cleavage at or close to position 37 (arrow). E) Analysis of ShhNp, as described in D. In contrast to ShhNp<sup>5xA</sup>, ShhNp is cleaved at or in proximity to position 34/35 (arrow), as confirmed by reduced αCW-antibody-reactivity of processed proteins.
Fig. 8: CW-truncated ShhN does not bind to HS. A) Ionic E18-HS interactions of mono- and multimeric proteins. HS-binding of unprocessed multimeric ShhN\textsuperscript{C25S} was comparable to that of alkaline-phosphatase (sAP)-tagged sAP-ShhN, the latter representing the directly secreted, unlipidated monomer. Equal amounts of sAP control protein and CW-truncated sAP-ShhN\textsuperscript{Δ26-35}, as determined by comparable AP-activity in both samples, were applied to the same E18-HS column, and sAP activities in the flowthrough (ft) confirmed saturation of Shh binding sites on the column. In contrast to sAP-ShhN, no sAP and sAP-ShhN\textsuperscript{Δ26-35} elution could be observed, demonstrating strongly impaired HS-binding of the N-terminally truncated protein. B) sAP-ShhN ligand binding to horizontal embryonic neural tube (dashed line) sections. PFA-fixed E12 mouse embryos were sectioned and treated with equal amounts of sAP and sAP-ShhN\textsuperscript{5xA} control proteins, sAP-ShhN\textsuperscript{Δ25-35}, wild-type sAP-ShhN, and sAP-ShhN in the presence of 1M NaCl or Heparinase (H-ase) I-III to remove cell surface HS. Bound sAP-ShhN was detected by its sAP-activity upon incubation with NBT-BCIP. In contrast, the truncated CW-mutant did not bind to HS.
An emerging role of Sonic Hedgehog shedding as a modulator of heparan sulfate interactions.

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