Two-way Dispatched function in Sonic hedgehog shedding and transfer to high-density lipoproteins

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Running title: Disp-mediated Hedgehog solubilization

Summary

The Sonic hedgehog (Shh) signaling pathway controls embryonic development and tissue homeostasis after birth. This requires regulated solubilization of dual-lipidated, firmly plasma membrane-associated Shh precursors from producing cells. Although it is firmly established that the resistance-nodulation-division transporter Dispatched (Disp) drives this process, it is less clear how lipidated Shh solubilization from the plasma membrane is achieved. We previously showed that Disp enhances proteolytic Shh solubilization from its lipidated terminal peptide anchors. This process, called shedding, converts tightly membrane-associated hydrophobic Shh precursors into delipidated soluble proteins. We show here that Disp-mediated Shh shedding is modulated by a serum factor that we identify as high-density lipoprotein (HDL). In addition to serving as soluble sinks for free membrane cholesterol, HDLs also accept the cholesterol-modified Shh peptide from Disp. The cholesteroylated Shh peptide is required and sufficient for Disp-mediated transfer because mCherry linked to cholesteroylated peptides associates with HDL in a Disp-dependent manner, but an N-palmitoylated Shh variant that lacks C-cholesterol does not. Disp-mediated Shh transfer to HDL is finalized by proteolytic processing of the palmitoylated N-terminal membrane anchor. The resulting mono-lipidated Shh variant may help meet the demands for Hh activity regulation in different cell types and developing tissues.

Keywords

Signaling, Hedgehog, proteolysis, Dispatched, high-density lipoprotein, cholesterol.

Introduction

Hedgehog (Hh) ligands activate an evolutionarily conserved signaling pathway that provides instructional cues during tissue morphogenesis, which, if misregulated, contributes to developmental disorders and cancer. Hhs are unique in that they require autocatalytic covalent cholesteroylation of the C-terminus [1] and N-terminal palmitoylation by a separate Hh acyltransferase (Hhat) activity [2]. Both lipids firmly tether Hh to the plasma membrane of

producing cells to effectively prevent unregulated ligand release [3]. Signaling at distant target cells therefore requires the regulated solubilization of Hh from the membrane of ligand-producing cells, a process that is facilitated by vertebrate and invertebrate Dispatched (Disp) orthologs *in vitro* [4] and *in vivo* [5, 6, 7, 8]. Disp contains 12 transmembrane helices and 2 extracellular domains and belongs to the resistance-nodulation-division family of transmembrane efflux pumps. Normally, such pumps maintain cellular homeostasis and remove toxic compounds. In addition, Disp contains a conserved multipass transmembrane domain known as the sterol-sensing domain (SSD) that regulates the homeostasis of free or esterified cellular cholesterol in other SSD proteins [9]. This molecular architecture is in line with Disp extraction of free cholesterol from the plasma membrane to remove it from the cell [4]. In addition, given its established role in Hh/Shh release, Disp may also extract the C-terminal Shh sterol to solubilize Hh/Shh into the extracellular compartment. Both possibilities are currently intensely investigated.

Notably, Disp activity alone is insufficient to solubilize the vertebrate Hh family member Shh from the plasma membrane. A second required synergistic factor for maximum Shh signaling is the soluble extracellular glycoprotein Scube2 (Signal sequence, cubulin (CUB) domain, epidermal growth factor (EGF)-like protein 2) [9]. One way to explain Disp/Scube2 synergy is by Disp-mediated extraction of dual-lipidated Shh from the plasma membrane to hand it over to Scube2, a mechanism that depends on the C-terminal cysteine-rich and CUB domains of Scube2 [10, 11]. *In vitro* support for this mechanism comes from Disp and Scube2 co-immunoprecipitation with Shh [10, 11] and from structural data [12]. Other suggested carriers for Disp-extracted lipidated Hh/Shh include lipoproteins (LPPs) [13, 14, 15], exosomes as carriers of internalized and re-secreted Hhs [16, 17, 18], and micellous Hh assemblies [19]. Another suggested Shh release mode is Disp-regulated proteolytic processing (called shedding) from the plasma membrane [4] by two major plasma-membrane-associated sheddases called A Disintegrin and Metalloproteinase (ADAM) 10 and 17 [20, 21, 22]. The role of Scube2 in this model is to enhance shedding of both terminal lipidated Shh peptides in a CUB domain-dependent manner [23, 24, 25]. Consistent with this, CUB function in regulated shedding of other substrates often involves substrate recognition and induced structural changes in the substrate to boost turnover [25, 26, 27].

In this study, we systematically characterized the biochemical parameters of Disp-regulated Shh solubilization with all these models and parameters in mind. To this end, we used a unique bicistronic Hhat/Shh co-expression system to ensure that only dual-lipidated Shh as a substrate for Disp was produced and analyzed [23]. We further avoided any protein tags to ensure undisturbed Shh modification, secretion, and interaction with Disp and other potential pathway components. Finally, we used unbiased biochemical methods to analyze Shh solubilization, size and lipidation status under different experimental conditions. First, we confirmed by SDS-PAGE/immunoblotting and reverse-phase (RP)-HPLC that Disp and Scube2 synergistically enhance Shh shedding into the cell culture medium [23]. Notably, we also found that repeated careful washing of Disp- and Shh-expressing cells to remove all serum traces abolished Shh shedding, and that high serum amounts promoted the solubilization of a previously uncharacterized fully bioactive Shh variant that lacks its palmitoylated N-terminus but retains its cholesteroylated C-terminus. Size exclusion chromatography (SEC) analyses demonstrated co-elution of this novel C-cholesteroylated Shh variant with serum apolipoprotein A1 (ApoA1), the major protein component of the high-density lipoprotein (HDL) fraction in serum. Consistent with this observation, purified HDL restored Shh solubilization from Shh- and Disp-expressing washed cells. We also found that the most C-terminal cholesteroylated Shh peptide is sufficient

for Disp-mediated protein transfer to HDL. In contrast, palmitoylated N-terminal Shh membrane anchors are not transferred by Disp, but undergo shedding to complete Shh transfer to HDL. Our updated Hh shedding model therefore unifies previously disparate models of Disp-, sheddase-, and LPP-mediated Shh solubilization into one comprehensive system. This comprehensive system is fully in line with published *in vivo* Disp functions [5, 6, 7, 8, 28], *in vivo* Scube2 functions [29, 30, 31], *in vitro* roles of the Scube2 CUB domain [23, 25], the Disp structure [32, 33], and required N-terminal – but not C-terminal – shedding during Hh solubilization *in vivo* [34, 35, 36]. Our model is also in line with the established importance of Hh C-cholesterol for Hh association into "large punctate" structures that are visible by light microscopy [37], C-cholesterol-dependent Hh solubilization [10, 38], and established *in vivo* roles of LPPs in Hh biofunction [13, 14, 15]. We suggest that the purpose of Disp-regulated generation of different post-translationally modified soluble Shh forms, as shown in this study, is to fine-tune cellular responses in different Shh-receiving tissues at different developmental time points.

Results

Synergistic Disp and Scube2 function increases shedding of dual-lipidated Shh from the plasma membrane

To analyze Shh solubilization from the plasma membrane, we produced dual-lipidated, tightly plasma membrane-associated morphogens in Bosc23 control cells that express endogenous Disp [23] (called CRISPR non-targeting control (nt ctrl) cells in this study) and in Bosc23 cells made deficient in Disp function by CRISPR/Cas9 (Using previously characterized Disp$^{-/-}$ cells [4]; experimental outlines are described in Fig. S1). Shh biosynthesis in both cell lines starts with the covalent attachment of a cholesterol moiety to the C-terminus of the Shh signaling domain [39]. This reaction is autocatalytic and strictly coupled to the generation of 19 kDa Shh signaling domains from a 45 kDa precursor. Subsequent N-palmitoylation of cholesteroylated Shh/Hh requires a separate enzymatic activity encoded by Hh acyltransferase (Hhat) [40]. Because Bosc23 cells do not express endogenous Hhat [23], we minimized the production of non-palmitoylated Shh by using bicistronic mRNA that encodes the Shh precursor together with Hhat. Unlike Shh expression in the absence of Hhat, coupled Shh/Hhat expression ensures near-quantitative Shh N-palmitoylation in transfected Bosc23 cells [23]. SDS-PAGE/immunoblotting was then used to characterize dual-lipidated Shh extraction from the plasma membrane of both cell lines into serum-depleted media (Fig. 1A; Fig. S2 includes all transfection controls and loading controls). We confirmed that Scube2 enhances Shh solubilization from nt ctrl cells (arrowhead) and that Disp$^{-/-}$ cells do not solubilize Shh [4] (Fig. 1A'). We also confirmed that the electrophoretic mobility of most Shh released from nt ctrl cells was increased over that of the corresponding dual-lipidated cellular material on the same blot [4, 23, 24] (asterisk). RP-HPLC of the solubilized material shown in Fig. 1A demonstrated that the observed electrophoretic mobility increase was caused by proteolytic removal of both lipidated terminal peptides (called shedding throughout this manuscript, Fig. 1A''; see Fig. S2 for cellular dual lipidated or artificially produced monolipidated and unlipidated Shh standard proteins). Transgenic Disp expression in Disp$^{-/-}$ cells restored Shh shedding and solubilization, confirming the specificity of the assay (Fig. S2E) [4]. Strikingly, the same phenotypic reversal was achieved by the co-expression of the cholesterol pump Patched (Ptc) [41] that depletes the plasma membrane of cholesterol [4]. This

suggests an indirect "second messenger" role of plasma membrane cholesterol not only in the regulation of Smoothened downstream of Ptc [41, 42, 43], but also in the regulation of Shh shedding downstream of Disp (Fig. S2E) [4]. Note that alternative solubilized Shh variants (represented by the "upper" bands) were also present in the media. These variants, however, were always produced independent of Disp and Scube2 function. In contrast, Scube2 and Disp synergistically and specifically increased Shh shedding from the cell surface [4].

We also observed that solubilization of artificially produced, monolipidated Shh variants (^{C25S}Shh that lacks N-palmitate, Fig. 1B, B', and ShhN that lacks C-cholesterol, Fig. 1C, C', Fig. S2) was much less controlled, consistent with the observations of others [10, 11]. Release of both monolipidated Shh variants remained linked to shedding of the respective lipidated membrane termini, as shown by electrophoretic mobility shifts of soluble proteins (Fig. 1B,C, arrowheads) and their delipidation during solubilization (Fig. 1B'',C''). Non-lipidated ^{C25S}ShhN was always solubilized in a Disp- and Scube2-independent manner, as expected (Fig. 1D-D''). These results suggest that cell-surface shedding represents a "ground-state" from which dual-lipidated Shh is protected, but only until Disp and Scube2 render it prone to shedding. Our results also explain the complete conservation of dual N- and C-terminal Hh lipidation during biosynthesis by the prevention of unregulated protein release from producing cells. This also demonstrates the importance of coupled Shh/Hhat expression to reliably characterize Disp- and Scube2-regulated Shh solubilization *in vitro*.

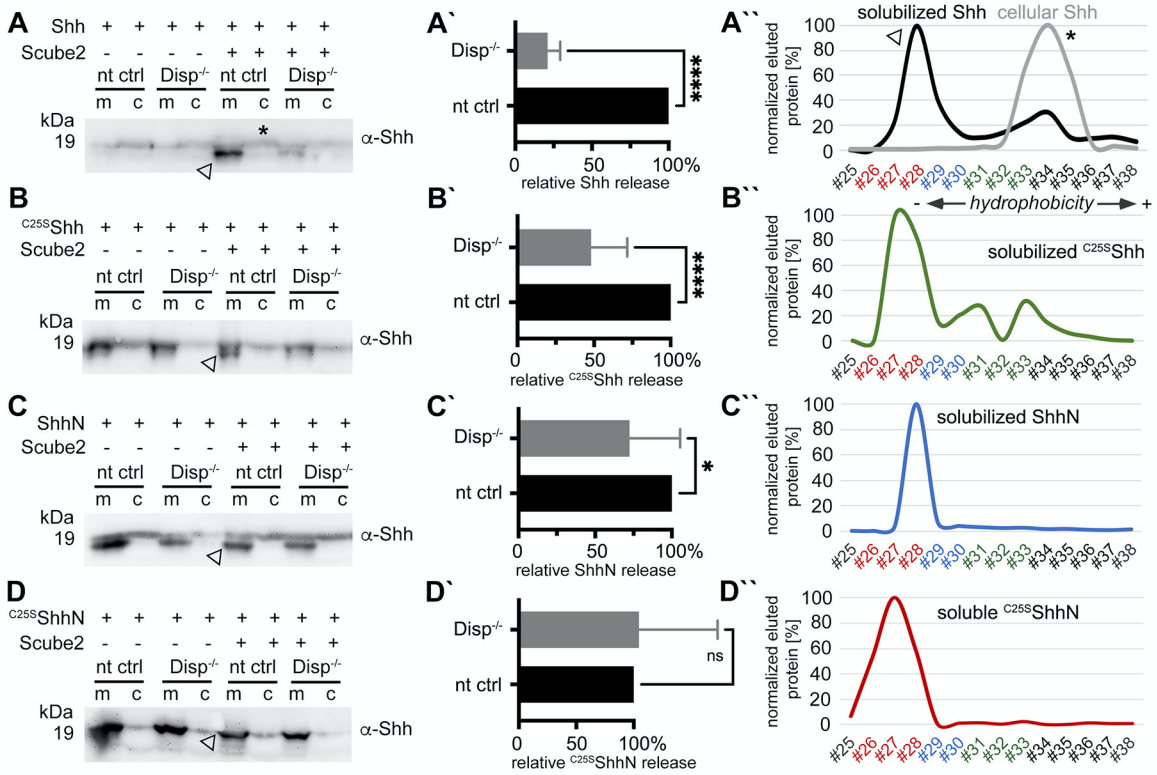

Figure 1: Disp and Scube2 enhance shedding of cell surface-associated dual-lipidated Shh into delipidated soluble forms. Media containing 10% serum were exchanged for serum-free DMEM 36 h

post-transfection and proteins solubilized for 6 h. Cells were not washed between media changes to leave residual serum traces in the assay. We refer to this experimental condition as "serum-depleted" throughout this work. **A)** Cap-dependent Shh translation and cap-independent Hhat translation from one bicistronic mRNA generated dual-lipidated, plasma membrane-associated proteins (asterisk in the cellular (c) fraction) in all transfected cells. Disp and Scube2 synergistically and specifically enhance dual-lipidated Shh precursor conversion into truncated soluble Shh (arrowhead). m: media, c: cell lysate. **A')** Quantification of relative Shh release from nt ctrl and Disp$^{-/-}$ cells in the presence of Scube2. Ratios of solubilized *versus* cellular Shh were determined and expressed relative to Shh solubilization from nt ctrl cells (set to 100%, black bar). **A'')** Reverse-phase HPLC analyses by using a C4 column revealed that specifically solubilized Shh (the same fraction indicated by the arrowhead in A, black line) was less hydrophobic than its cell surface-associated precursor (gray line, the asterisk denotes analysis of the same cellular fraction as shown in A). C4 column calibration and color-coding of fractions is explained in Fig. S2. **B-D)** Solubilization of non-palmitoylated ^{C25S}Shh, non-cholesteroylated ShhN and lipid-free control ^{C25S}ShhN under the same serum-depleted conditions. Arrowheads denote the Disp- and Scube2-solubilized material. **B'-D')** Quantifications of B-D, again from nt ctrl and Disp$^{-/-}$ cells in the presence of Scube2. **B''-D'')** Reverse-phase HPLC demonstrates similar elution of ^{C25S}Shh, ShhN, and non-lipidated ^{C25S}ShhN. This suggests that lipidated peptide termini were removed during ^{C25S}Shh and ShhN (as well as Shh, A'') release. Unpaired t-test, two-tailed. ****: $p<0.0001$, *: $p<0.016$, n.t.: 0.79. n=20 datasets (A'), n=13 datasets (B'), n=10 datasets (C'), and n=17 datasets (D'). See Table S1 for detailed statistical information.

Shh solubilization depends on cleavage-activated Disp and the presence of serum

It was recently shown that the prohormone convertase furin cleaves Disp at a conserved processing site to activate the protein [44]. On the basis of this activation mode, we hypothesized that furin inhibition should specifically impair Disp-regulated Shh shedding. To test this hypothesis, we added 50 μM peptidyl chloromethylketone (CMK, a competitive inhibitor of furin) to our solubilization assays. Indeed, CMK strongly reduced Shh shedding from the cell surface (Fig. 2A,B, Fig. S3A). During these assays, we also noted that repeated careful washing of cells to quantitively remove all serum traces strongly impaired Disp- and Scube2-mediated Shh solubilization into serum-free media (Fig. 2C, Fig. S3B). We derive two important conclusions from this latter observation. The first is that the minimal requirements of Na^+-driven, Disp-mediated Shh extraction and hand-over to Scube2 [45] are insufficient to explain Shh release. The second is that Shh self-assembly by the law of mass action [46] is also not supported, because this process should solubilize Shh regardless of the presence or absence of serum. In contrast, one function of Disp is to extract and transfer plasma membrane cholesterol to a soluble sink to transport it away from the cell [4, 47]. In vertebrates, HDLs represent soluble sinks for "free" peripheral cholesterol [48], and HDLs as well as the pharmacological cholesterol chelator methyl-β-cyclodextrin (mβcd) were previously shown to increase Shh shedding [4, 23, 49]. These findings suggest that the permissive factor lacking from our shedding assay was most likely a soluble cholesterol acceptor, such as HDL or a related serum LPP.

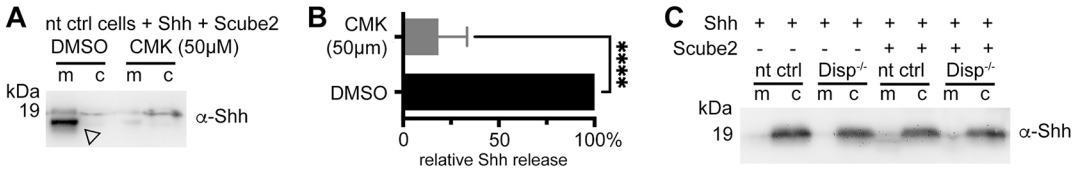

Figure 2: Shh shedding depends on Disp activation by furin and the presence of serum traces. A) nt ctrl cells were transfected with Shh and Scube2, and Shh solubilization was monitored in the presence or absence of 50 µM CMK furin inhibitor. **B)** Quantification of CMK-inhibited Shh shedding. Ratios of solubilized *versus* cellular Shh were determined and expressed relative to Shh solubilization in the absence of inhibitor (set to 100%, black bar). Unpaired t-test, two-tailed. ****: $p<0.0001$, n=9. See Table S1 for additional statistical information. **C)** Immunoblotted cellular (c) and medium (m) fractions of Shh expressing nt ctrl and Disp$^{-/-}$ cells in the complete absence of serum (referred to as "serum-free" conditions throughout this work). Note that Shh solubilization is completely abolished and that cellular precursors accumulate in the cells.

Increased serum concentrations shift shedding of both Shh termini to selective N-terminal shedding

To characterize serum-enhanced shedding in more detail, dual-lipidated Shh was expressed in nt ctrl cells and Disp$^{-/-}$ cells and solubilized into serum-depleted Dulbecco's Modified Eagle's Medium (DMEM) or into DMEM supplemented with 0.05%, 2%, and 10% fetal calf serum (FCS) (Figs. 3A-D; Fig. S3 shows transfection and loading controls). We observed that Scube2 enhances Shh shedding from Disp-expressing cells into serum-depleted media (Fig. 3A, arrowhead), but does not enhance Shh shedding from Disp$^{-/-}$ cells. RP-HPLC of the solubilized material confirmed that the observed increase in electrophoretic mobility was caused by the proteolytic removal of both terminal lipidated peptides (Fig. 3A'). Increased serum concentrations during Shh solubilization (0.05% FCS (Fig. 3B), 5% FCS (Fig. 3C), and 10% FCS (Fig. 3D)) did not appear to affect Disp- and Scube2-specific Shh shedding much (arrowheads). To our surprise, however, RP-HPLC of the solubilized materials shown in Fig. 3B-D revealed a gradual shift from dual Shh shedding (labeled [1] in Fig. 3A'-D') toward the solubilization of variant proteins with their C-terminal cholesteroylated peptide still intact (labeled [2] in Fig. 3B'-D', Fig. S4A-E). We note that dual-lipidated Shh was also present in serum-containing media (indicated by an asterisk in Fig. S4A-E). However, we found that relative amounts of this fraction increased strongly in the absence of Scube2 (Fig. S4F; here, cells required incubation for 24 h to compensate for low Shh solubilization) and in the absence of Disp$^{-/-}$ (Fig. S4G), while Scube2 expression in Disp$^{-/-}$ cells increased relative amounts of delipidated soluble Shh (Fig. S4H). Disp- and Scube2-independent solubilization of dual-lipidated Shh, as observed here (Fig. 1A), indicates that it is probably an overexpression artefact or caused by soluble plasma membrane remnants of apototic cells.

So far, our data supported that Scube2 increases N-terminal and C-terminal Shh shedding from Disp-expressing cells and that serum enhances this process, likely by providing a sink for membrane cholesterol transferred by Disp [4] (Fig. S4I). Moreover, high serum amounts seem to promote a second Disp solubilization mode in which Shh shedding is restricted to the palmitoylated N-peptide while the C-terminus remains intact. This latter finding raised the

interesting possibility that serum factors may accept or protect the cholesteroylated Shh C-terminus. Note that a filtrate of blood serum through the capillary walls, called interstital fluid, represents the microenvironment of tissues and cells *in vivo*, also of those that express and solubilize Shh during development [14]. Interstitial fluid is rich in proteins and lipoprotein particles of small mass from the serum [50]. Therefore, we suggest that Shh expression in serum presence resembles conditions in Shh expressing tissues *in vivo*, and may therefore be physiologically relevant.

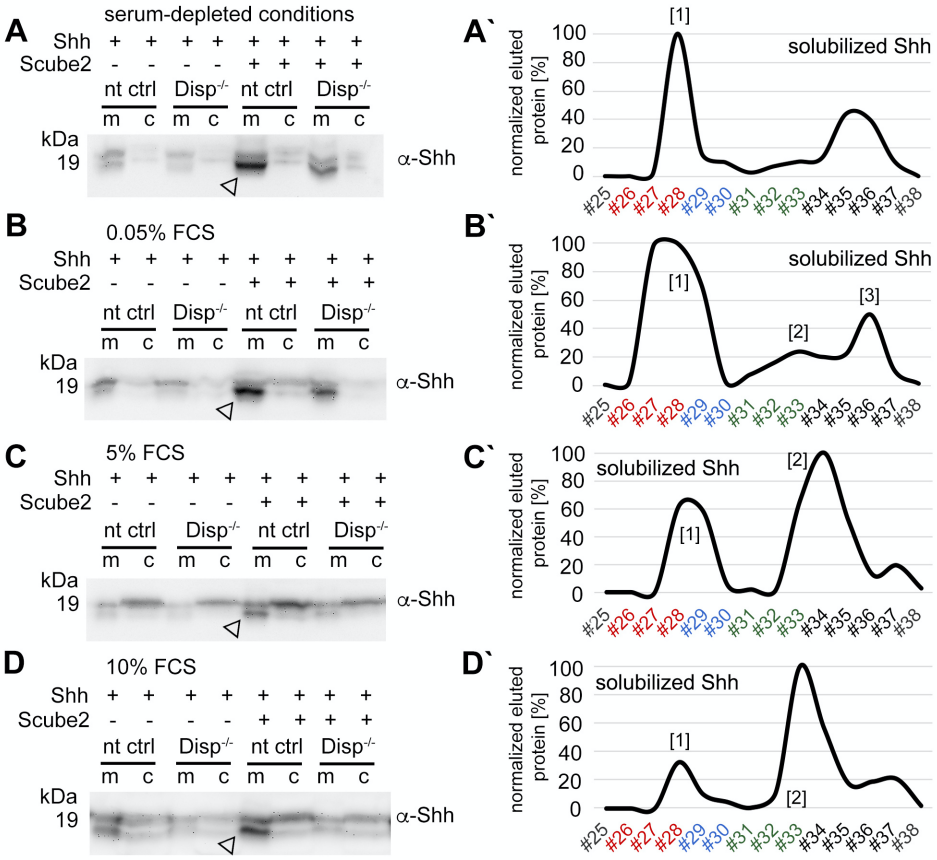

Figure 3: Dual-lipidated cell-surface Shh converts into delipidated soluble forms under low-serum and high-serum conditions. Media were changed for serum-free DMEM 36 h post-transfection (cells were not washed) or DMEM containing the indicated amounts of serum, and proteins were solubilized for 6 h (serum-depleted) or 24h (with 0.05%, 5% and 10% serum). **A)** Under serum-depleted conditions, Disp and Scube2 increase dual-lipidated Shh shedding into truncated soluble forms (arrowhead). m: media, c: cell lysate. **A')** RP-HPLC confirmed loss of both terminal lipidated Shh peptides during Disp- and Scube2-regulated shedding. **B-D)** The appearance of truncated Shh in serum-containing media remained dependent on Disp and Scube2 (arrowheads). **B'-D')** RP-HPLC revealed that increased serum amounts shift dual Shh shedding (low serum amounts, [1]) toward N-terminally restricted shedding and release of a cholesteroylated Shh form [2]. Low levels of dual-lipidated Shh are also detected [3]. See Fig. S4 for experimental details.

N-palmitate is not required for Shh-induced *in vitro* differentiation of C3H10T1/2 cells

Is this novel N-terminally shed Shh variant in serum-containing media functional? It is known that dual lipidation is required for unperturbed Hh biofunction *in vivo* [51, 52, 53, 54, 55], and dual-lipidated Shh expression *in vitro* generates soluble variants that are 10-30 times more bioactive than ^{C25S}Shh that does not undergo Hhat-catalyzed N-palmitoylation during its biosynthesis, yet undergoes unperturbed C-terminal cholesteroylation and secretion to the cell surface (Fig. 1B) [40, 55]. According to these published observations, the selectively N-terminally shed Shh variant described here should not be very active. To test this possibility, we used the multipotent fibroblastic C3H10T1/2 cell line as a reporter. We first verified multipotency of our C3H10T1/2 reporter cell line to differentiate into osteoblasts [56], chondrocytes [57] or adipocytes [58] (Fig. S5A). To this end, C3H10T1/2 cells were cultured for various time periods in the presence of adipogenic, chondrogenic and osteogenic supplements, and their responsiveness confirmed based on phenotype and the expression of cell surface markers. We then incubated C3H10T1/2 cells with R&D 8908-SH (Fig. 4A). R&D 8908-SH is commercially available, dual-lipidated Shh obtained by detergent-extraction from transfected cells and shares 91% sequence identity and both lipids with Indian hedgehog, an established osteogenic factor [59]. R&D 8908-SH induced alkaline phosphatase (Alp) expression and C3H10T1/2 differentiation into osteoblasts in a concentration-dependent manner [56]. We also incubated C3H10T1/2 cells with Shh expressed in the presence of serum and physiological release regulators Scube2 [30, 31] and Disp [5, 6] (Fig. 4A, inset). Surprisingly, we found that the activity of solubilized Shh was increased over similar amounts of R&D 8908-SH (Fig. 4A, inset), and ^{C25A}Shh expressed under the same conditions was equally active. Quantitative reverse transcription-polymerase chain reaction analysis (qPCR) of Ptc1, Ptc2 and Gli1 expression in Shh-treated C3H10T1/2 cells (Fig. 4B, Fig. S5B) confirmed this finding: After protein normalization (using 4ng R&D 8908-SH and 100µl Shh or ^{C25S}Shh), qPCR confirmed similar mRNA expression increases of Ptc1 and Gli1 under direct Shh, ^{C25S}Shh or R&D 8908-SH control (Fig. 4B). Ptc is known to be upregulated by Shh [60], and Gli1 is a zinc finger transcription factor that operates downstream of Ptc (mainly Ptc1 in vertebrates, a less characterized Ptc2 homolog is also expressed) and is also transcribed in a Shh-dependent manner [61]. Apparent differences between determined Ptc2 levels, as observed in this assay, can be explained by the very low Ptc2 expression levels in C3H10T1/2 cells that give rise to high margins of error (Ptc2 Cq~33 for Shh-treated cells, Ptc2 Cq~31 for ^{C25A}Shh-treated cells, Ptc2 Cq~37 and ~34 for R&D 8908-SH-treated cells). Key regulators for adipogenesis, osteogenesis, chondrogenesis and proliferation in Shh and ^{C25S}Shh-induced C3H10T1/2 cells were also similar (Fig. S5B,C). Taken together, our results demonstrate that N-palmitate is not essential for Shh signaling if proteins were produced and released in the presence of serum.

Soluble Shh/Hh and ApoA1-containing LPPs have similar sizes

A decade ago, it had already been shown that sterol-modified Hh/Shh is released in LPP-associated bioactive form – as wellas in desteroylated, non-associated form – in flies and mammals [14]. This suggested that serum LPPs in our assays may have promoted dual Shh shedding [4] (Fig. 1) but possibly also promoted C-cholesteroylated Shh assembly into soluble complexes. We used size exclusion chromatography (SEC) to test this hypothesis. SEC detects soluble Shh monomers (20 kDa) and covers the entire molecular weight range up to a MW cutoff of about 1 mDa. The molecular weight span of serum HDL ranges from 1.75×10^5 Da to 3.6×10^5 Da, corresponding to particle sizes of 5-11 nm. This small size range makes HDLs abundant

constituents of interstitial fluid and good candidates for Shh solubilization from cells and tissues. Larger complexes in mammalian serum include low-density lipoproteins (LDLs, MW 2.75×10^6 Da) and very low-density lipoproteins (VLDLs, MW $10\text{-}80\times10^6$ Da) that both elute in the void column volume. As shown in Fig. 4C, Shh in DMEM + 10% FCS elutes in fractions that cover the entire molecular weight range (black line). One prominent Shh peak is detected around 20 kDa – most likely representing the fully delipidated Shh fraction (Fig. 3, labeled [1]) – and a second peak is detected between 300 kDa and 600 kDa. Reprobing the same (stripped) blot with antibodies directed against ApoA1 (the major protein component of HDL) revealed an elution profile that overlapped with that of Shh (Fig. 4C, orange line). Of note, most Shh co-eluted with the largest HDL particles, suggesting that maturation of small early (discoidal) HDL into larger (spherical) HDL upon incorporation of cholesterol may be accompanied by gradual Shh transfer to the same particles. In contrast, Scube2 (Fig. 4C, blue line) co-eluted with smaller Shh multimers [46]. In order to determine which size fraction contained the most biologically active Shh, we analyzed C3H10T1/2 osteoblast precursor cell differentiation by using aliquots of eluted fractions from the same SEC run. Shh-induced C3H10T1/2 cells in a size-dependent activity distribution: Large Shh assemblies were strongly bioactive, smaller assemblies were also strongly bioactive, but monomeric Shh was only moderately active (Fig. 4C, green dashed line). We also found that *Drosophila* Hh expressed in S2 cells and solubilized into serum-containing medium showed a similar size distribution (Fig. 4D, black line) and that Hh assemblies co-eluted with serum ApoA1 as well (orange line). This suggests that the mechanism of serum-enhanced Hh assembly during solubilization is conserved. The possibility of Hh assembly as a consequence of heparan sulfate (HS) proteoglycan interactions [62] was excluded because site-directed mutagenesis of the HS binding Cardin-Weintraub motif did not change $Hh^{\Delta HS}$ size distribution in our assays (Fig. 4D, black dotted line). From these observations, we conclude that large Shh/Hh assemblies form by HDL association *in vitro*, which is in line with a previous study showing that *Drosophila* Hh copurifies with LPP and co-localizes with LPP in the developing wing epithelium *in vivo* [15]. The same study showed that reduced LPP levels in larvae impair Hh release and lead to Hh accumulation at the site of production, similar to the *in vitro* findings shown in Fig. 2C.

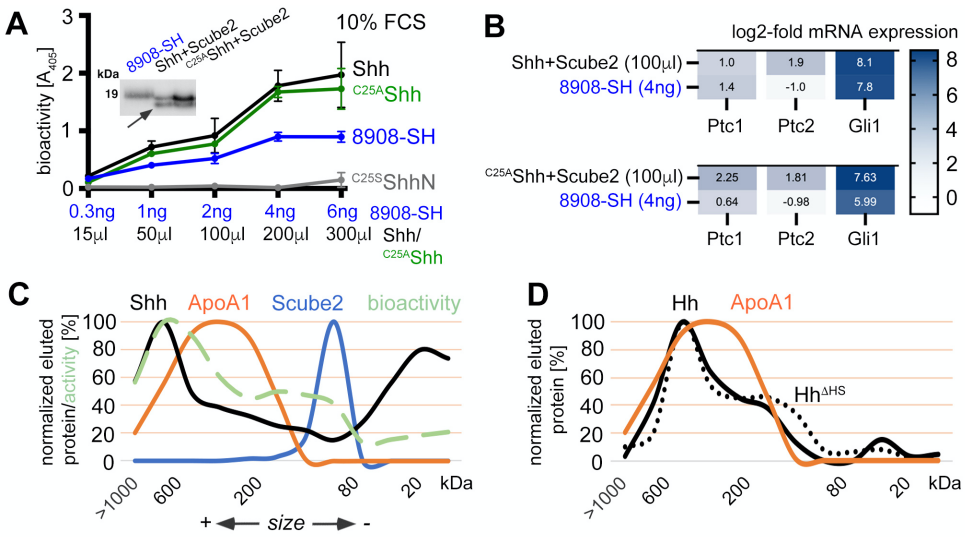

Figure 4: Activities and SEC of dual-lipidated Shh/Hh and depalmitoylated Shh variants solubilized into serum-containing media. **A)** Immunoblotted proteins (inset) were used for quantification and visualization of dually lipidated R&D 8908-SH, Shh, ^{C25A}Shh (this artificial variant has the cysteine palmitate acceptor changed for a non-accepting alanine) and unlipidated ^{C25S}ShhN. Data were normalized to mock-treated C3H10T1/2 cells. Shh, ^{C25A}Shh and ^{C25S}ShhN were solubilized from Scube2- and Disp-expressing cells into media containing 10% FCS. 0-4 ng of R&D 8908-SH induced C3H10T1/2 reporter differentiation in a concentration-dependent manner, as determined by Alp activity measured at 405nm (blue line). Similar amounts of solubilized Shh, as determined by immunoblotting, increased C3H10T1/2 osteogenesis over that of the dual-lipidated R&D 8908-SH standard (black line). Notably, ^{C25A}Shh was also highly bioactive if released into serum-containing medium and tested in this experimental system (green line). Inset: Immunoblot of proteins used in this assay. The arrow indicates proteolytic processing of the palmitoylated N-terminal peptide from Shh and ^{C25A}Shh, but not from R&D 8908-SH. Artificial unlipidated soluble ^{C25S}ShhN showed only little activity [63] (gray line). **B)** Similar induction of transcription of Hh target genes Ptc1 and Gli1 in C3H10T1/2 reporter cells. Cells were stimulated with 4ng R&D 8908-SH or similar amounts of Shh and ^{C25A}Shh. Shh and ^{C25A}Shh were both solubilized from Bosc23 cells into serum-containing media in the presence of Scube2 (100µl Shh/^{C25A}Shh+Scube2). **C)** SEC reveals substantial amounts of increased molecular weight Shh in media that contain 10% serum (black line). The increased molecular weight Shh eluted together with ApoA1 (orange line). In contrast, Scube2 was largely monomeric in solution (blue line). Levels of Shh-induced alkaline phosphatase activity in C3H10T1/2 cells were again measured as absorption at 405 nm, demonstrating the strongest C3H10T1/2 differentiation by eluted fractions containing large Shh assemblies. **D)** SEC of *Drosophila* Hh (black line) and of a variant lacking its HS binding site ($Hh^{\Delta HS}$, black dotted line). Both proteins were expressed under actin-Gal4/UAS-control from S2 cells and solubilized into media containing 10% FCS.

Disp function depends on HDL

To test whether soluble HDL is sufficient to increase Disp-mediated Shh solubilization, we next induced this process directly. To this end, we expressed Shh in nt ctrl cells and $Disp^{-/-}$ cells in the presence or absence of Scube2, washed the cells 36 h post transfection, and added serum-free DMEM or DMEM supplemented with 40 µg/mL purified human HDL [4]. We first confirmed that lack of serum factors strongly reduced Shh solubilization (Fig. 5A, labeled [1], Fig. S6A). RP-HPLC of the small amounts of released Shh confirmed removal of both lipidated Shh peptide anchors during solubilization (Fig. 5A', Fig. S6A'), and SEC confirmed that

proteins were monomeric (Fig. 5A''). In contrast, Shh solubilization from nt ctrl cells into DMEM supplemented with 40 µg/mL HDL was strongly increased (Fig. 5B,C, Fig. S6B). As previously observed for Shh expression in the presence of serum, RP-HPLC of HDL-solubilized Shh [2] revealed that its C-terminal cholesteroylated peptides were still intact (Fig. 5B', Fig. S6B'), and that the proteins were strongly bioactive (Fig. 5B''). Solubilized dual-lipidated Shh was also found, but its solubilization was independent of Scube2 and Disp function (Fig. S6B'',B'''), indicating unspecific release as observed before (Fig. 1). From the observed size shift and overlap with the ApoA1 elution profile in SEC – note that the observed Shh molecular weight range almost exactly matches that of HDL – we suggest that monolipidated truncated Shh associates with HDL in a Disp-regulated manner. This possibility is supported by the previous observation that Hh levels in the hemolymph of Disp-deficient fly larvae were strongly decreased [14]. We also tested whether HDL constitutes the only possible Shh acceptor, or whether LDL can also carry monolipidated morphogens. As shown in Fig. S6C,D, Shh transferred to HDL-supplemented serum-free DMEM, but not to LDL-supplemented DMEM.

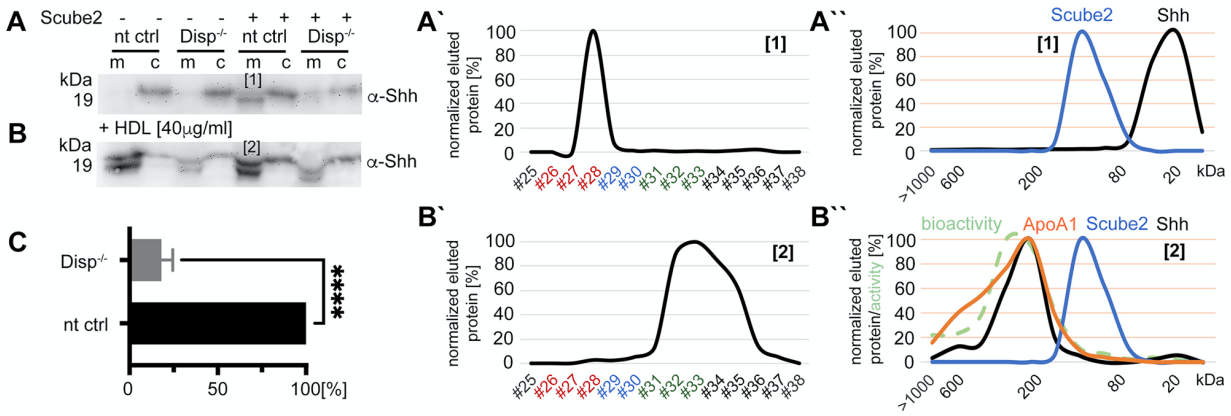

Figure 5: HDL increases N-processed Shh solubilization by Disp. Media were changed for serum-free DMEM 36 h post-transfection (and cells washed three times) or serum-free DMEM supplemented with 40 mg/mL HDL before proteins were solubilized for 6 h. **A)** Immunoblotted cellular (c) and medium (m) fractions of Shh expressing nt ctrl and Disp$^{-/-}$ cells in the complete absence of serum. Note that Shh solubilization is strongly reduced [1]. **A')** RP-HPLC of the material labeled [1] revealed complete delipidation during release. **A'')** SEC of the same delipidated material demonstrates that it is readily soluble and not associated with Scube2. **B)** Immunoblotted cellular (c) and medium (m) fractions of Shh expressing nt ctrl and Disp$^{-/-}$ cells in the presence of 40 µg/mL HDL. Shh shedding and solubilization are strongly increased by Disp [2] but not by Scube2. **B')** RP-HPLC of the material labeled [2] revealed that HDL shifts Shh shedding from dual processing (A', [1]) toward the release of cholesteroylated Shh. **B'')** SEC of the same material [2] (black line) shows a molecular weight increase that matches the molecular weight range of HDL (orange line). Again, the soluble Shh elution profile does not overlap with that of Scube2 (blue line). **C)** Quantification of HDL-induced Shh solubilization from nt ctrl cells and Disp$^{-/-}$ cells. Unpaired t-test, two-tailed. ****: $p<0.0001$, n=6. See Table S1 for additional statistical information.

The C-terminal cholesteroylated Shh peptide is required and sufficient for Disp-mediated export and association with HDL

We asked next whether both terminal lipids are required for Disp-mediated Shh export to HDL, or whether the C-cholesteroylated peptide is sufficient for the transfer. The latter possibility was suggested by N-palmitoylated peptide loss during solubilization from Disp-expressing cells in the presence of serum (Figs. 1-3) and by Disp-independent ShhN solubilization into serum-depleted medium (Fig. 1C) [64]. We expressed palmitoylated/non-cholesteroylated ShhN and non-palmitoylated/cholesteroylated ^{C25S}Shh and confirmed that palmitoylated ShhN solubilization from the plasma membrane was independent of Disp and HDL (Fig. 6A, [1] denotes material solubilized into serum-free DMEM, [2] denotes material solubilized by HDL; also see Fig. S7A,B). SEC detected only monomeric ShhN, regardless of the absence or presence of HDL (Fig. 6A',A''). These findings suggest that the N-terminal Shh palmitate is not a good substrate for Disp-mediated transfer to HDL. In contrast, only small amounts of monomeric ^{C25S}Shh were solubilized into serum-free media (Fig. 6B,B', labeled [3]), and HDL strongly increased soluble ^{C25S}Shh and its assembly into sizes similar to those of HDL (Fig. 6B'', labeled [4]; also see Fig. S7C,D). Supporting the importance of Shh cholesteroylation for its transfer to HDL, soluble ^{C25S}Shh remained lipidated (Fig. S7E). Together, our findings suggest that the C-terminal cholesterol moiety is required for Disp-mediated Shh transfer and HDL association. However, we also noted that a fraction of monolipidated ^{C25S}Shh solubilized in a Disp independent manner (Fig. 6B, asterisks; Fig. S7F, Fig. 6C, the green dotted line indicates the distribution of ^{C25S}Shh sizes if released from Disp$^{-/-}$ cells). We explain this finding by unspecific ^{C25S}Shh release as a consequence of protein overexpression in the presence of HDL and support this claim by a report showing that half-times for spontaneous cholesterol desorption from membranes of several hours are dramatically decreased if large proteins – such as Shh – are attached to a single anchor [3]. Once desorped, lipidated proteins either reintegrate into the plasma membrane or associate with lipophilic soluble competotors, as shown in Fig. S7G. This demonstrates that another important role of fully conserved dual N- and C-terminal Hh lipidation is to prevent non-enzymatic morphogen desorption from the plasma membrane.

Is the cholesteroylated peptide sufficient for Disp-mediated transfer to HDL, or does the globular Shh ectodomain participate in the transfer [45, 65]? To answer this question, we replaced most of the ^{C25S}Shh ectodomain with mCherry N-terminally flanked by the Shh secretion signal and C-terminally flanked by the Shh cholesterol transferase/autoprocessing domain. This strategy resulted in the secretion of cholesteroylated mCherry (Fig. S7H) to the cell surface and its association with the outer leaflet of the plasma membrane. As observed previously for Shh (Fig. 5), mCherry remained monomeric or formed small assemblies if released into serum-depleted media (Fig. 6D, dashed line), and HDL shifted mCherry elution into fractions that also contained ApoA1 (solid line). This result supports that HDL serves as a soluble acceptor for C-terminally cholesteroylated peptides. SEC also revealed that loss of Disp function decreased the relative amounts of HDL-associated material (solid line) and increased the relative amounts of solubilized monomeric protein (Fig. 6D, dotted line). Therefore, cholesteroylated peptides represent the critical determinant for Disp-mediated Shh transport to HDL. In contrast, Shh ectodomain interactions with Disp are not essential for the transfer. We supported physical ^{C25S}Shh interactions with HDL by the addition of EtOH or Triton X-100, both of which disassembled soluble high-molecular weight aggregates into monomers or small assemblies (Fig. 6E).

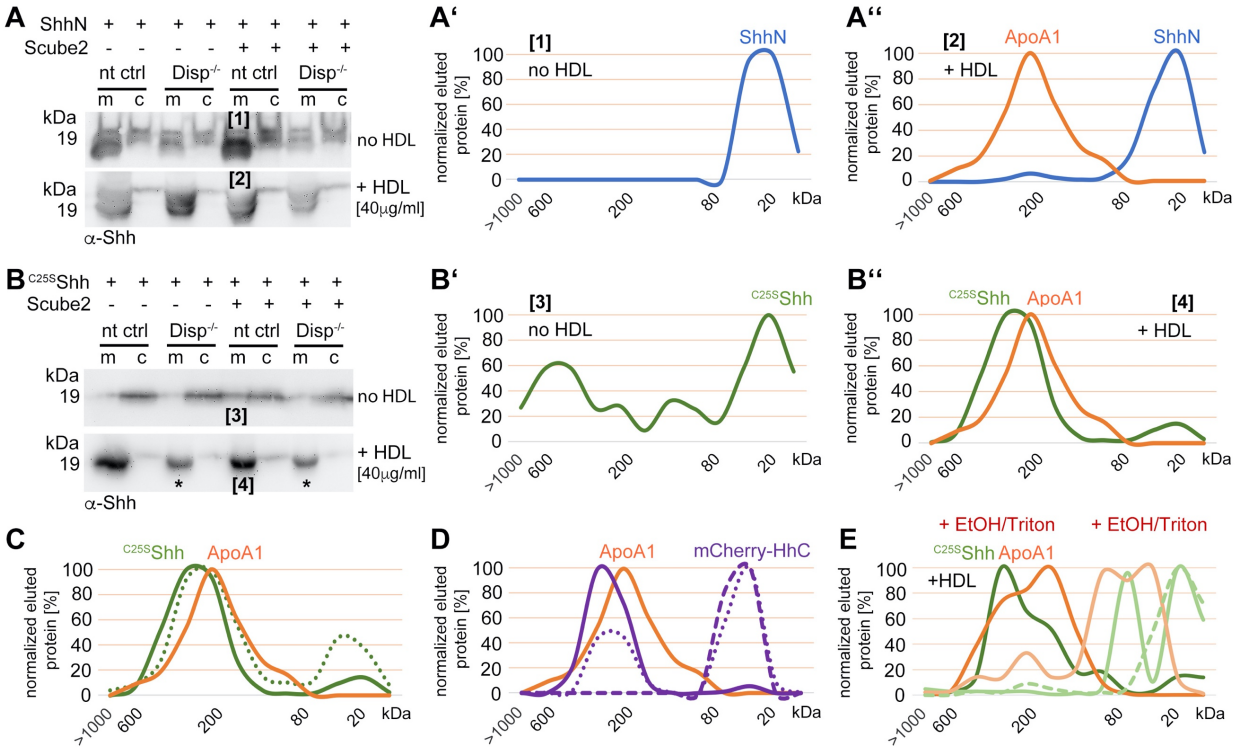

Figure 6: Cholesteroylated C-terminal peptides are required and sufficient for Disp-mediated protein transfer to HDL. A) Immunoblotted cellular (c) and medium (m) fractions of ShhN-expressing nt ctrl and Disp$^{-/-}$ cells. Shown is unspecific ShhN solubilization into serum-free media (top blot, labeled [1]) or into serum-free DMEM supplemented with HDL (bottom blot, labeled [2]). **A')** ShhN [1] expressed under serum-free conditions is solubilized in a monomeric state. **A'')** ShhN [2] expressed in the presence of HDL remained monomeric. **B)** Cholesteroylated ^{C25S}Shh solubilization into serum-free medium is strongly impaired (top blot, labeled [3]), but increases Disp-dependently in the presence of HDL (bottom blot, labeled [4]). Asterisks denote ^{C25S}Shh solubilized independent of Disp function. **B')** Most ^{C25S}Shh [3] in serum-free media is monomeric, but a small fraction also assembles into larger units. **B'')** ^{C25S}Shh [4] expressed in the presence of HDL increases in molecular weight to match the molecular weight range of HDL (orange line). **C)** SEC of ^{C25S}Shh solubilized from Disp-expressing cells (solid green line) or from Disp$^{-/-}$ cells (dotted line). **D)** SEC of cholesteroylated mCherry solubilized from nt ctrl cells (solid lines) or from Disp$^{-/-}$ cells (dotted lines) under the same conditions. Dashed lines indicate proteins solubilized under serum-free conditions. Note that most mCherry associates with HDL in a Disp-regulated manner. **E)** ^{C25S}Shh (green line) disassembles from HDL in 50% ethanol (bright green line) or in 0.1% Triton X-100 (bright green dashed line). HDL (orange line) disassembly under the same conditions is confirmed by ApoA1 size shift towards the monomeric 32 kDa protein (bright orange line).

Discussion

Dual Shh lipidation and Disp-regulated Shh solubilization are interdependent

It is firmly established that Hh solubilization and signaling depend on Disp co-expression in source cells. However, the additional involvement of many cell surface and soluble co-factors has complicated efforts to unravel the exact nature of Disp-mediated Hh solubilization. To address this problem, we transfected Disp-deficient and Disp-expressing source cells [4] with Shh and systematically modified Shh solubilization by a number of extracellular co-factors. Then, cell-surface-associated Shh upstream of Disp function and soluble Shh downstream of Disp function were analyzed together with their co-factors by using unbiased biochemical tools. The first important result of this approach is that both Shh lipids act together to prevent uncontrolled Shh solubilization from producing cells. In contrast, artificially produced ^{C25S}Shh that lacks N-palmitate and ShhN that lacks C-cholesterol are constitutively solubilized – either by non-enzymatic desorption or by unregulated shedding (Fig. 7A). The second important result is that Disp and Scube2 specifically and synergistically increase shedding of the dual-lipidated precursor. These two observations suggest that cell-surface shedding constitutes a "ground state" from which dual-lipidated Shh is protected by tight plasma membrane association of both lipids – but only until Disp and Scube2 render Shh prone to proteolytic processing of the terminal peptides. This concept is important for the reinterpretation of the observation that dual lipidation is essential for unperturbed Hh biofunction *in vivo* [51, 52, 53, 54, 55]. The current interpretation of these observations is that both Hh lipids contribute directly to Ptc receptor signaling for maximal signaling. Our results support an alternative mechanism acting upstream of Ptc: The selective artificial prevention of N- or C-terminal lipidation during Hh/Shh biosynthesis *in vivo* (achieved by the deletion of the N-terminal palmitate acceptor cysteine or of Hhat, or by prevented C-cholesteroylation [51, 52, 53, 54, 55]) may have rendered spatiotemporally controlled Hh/Shh solubilization into unregulated "leakage" of monolipidated precursors from producing cells and tissues (Fig. 1). The "non-availability" of dual-lipidated Shh for Disp-controlled spatio-temporal signaling may then have caused the observed Hh loss-of-function phenotypes [51, 52, 53, 54, 55]. Therefore, our release model does not contradict the established essential role of unimpaired dual Shh lipidation during biosynthesis for Hh activity regulation *in vivo*, but provides an alternative explanation for it. Support for our model of Disp- and Scube2-regulated Hh shedding and release on HDL include the following: Disp is required only in Hh-producing cells and not in receiving cells [64]; Hh signaling defects in Disp-deficient model organisms are caused by a defect in the deployment of cell-surface associated Shh [5, 6, 7]; and full Disp activity requires Scube2 [10]. Further support comes from our finding that de-palmitoylated Shh in serum-containing media is fully bioactive (Fig. 4A) and from the *in vivo* observation that de-palmitoylated Hh variants impair development to variable degrees but can also induce increased ectopic signaling [38, 55].

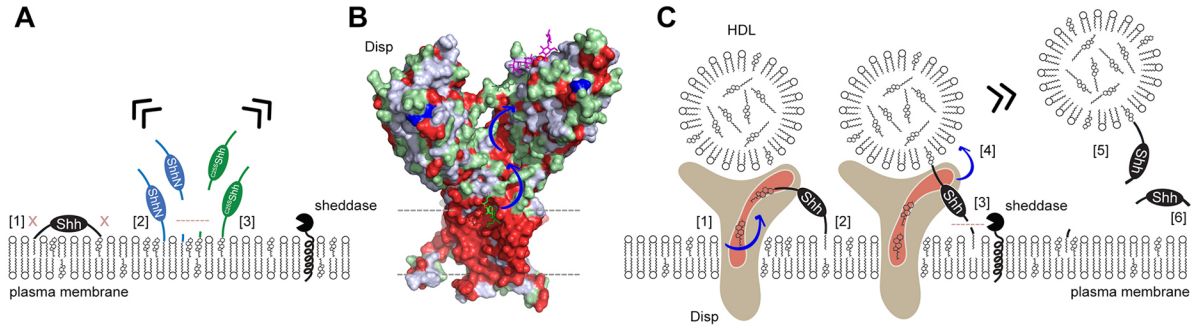

Figure 7: Model of two-way Disp-mediated Shh solubilization. A) Dual lipidation protects Shh from unregulated cell-surface shedding by tight plasma membrane association of both lipids (blocked shedding is indicated by an x in [1]). In contrast, monolipidated ShhN [2] and ^{C25S}Shh [3] are prone to unregulated membrane-proximal shedding (indicated by the dashed line) or non-enzymatic desorption. **B)** Representation of surface hydrophobicity of pdb:7RPH [45] (red) suggests an extended hydrophobic surface channel that may function as a "slide" for lipophiles extending from the plasma membrane (dashed lines) to a cavity of the second extracellular domain (blue arrows). An upward lifted sterol (green stick representation) at the start point of the hydrophobic track may represent an intermediate state of sterol extraction from the membrane, and a lipidic group modeled as the amphiphilic detergent lauryl maltose neopentyl glycol (violet stick structure) may represent the end point of the transfer [45] prior to hand-over to HDL. **C)** We suggest two sequences of Shh transfer events. In the first event [1], plasma membrane sterol is transferred through the Disp hydrophobic surface channel to HDL acceptors. This process resembles established reverse cholesterol transport. In the second event, if present, C-terminal cholesterol moieties of Shh can also be transferred [2]. This partial Shh extraction exposes the N-terminal cleavage site [3] and makes it prone to proteolytic processing (as is the case for ShhN in A). N-terminal Shh shedding may then release the protein from the plasma membrane [4] to complete the transfer [5]. In addition to, or competing with this process, cholesterol depletion of the plasma membrane (representing the first event, [1]) may trigger shedding of both terminal Shh peptides and the solubilization of monomeric proteins in an indirect manner [6]. See discussion for details.

Disp mediates Shh solubilization by two functionally related transfer modes

The second important result of our study is that the absence of all serum traces reduces or even abolishes Disp/Scube2-mediated Shh solubilization. This reveals that Disp and Scube2 are insufficient to solubilize dual-lipidated Shh and that a previously overlooked serum factor – that we identified as HDL – is also required. We showed previously that Disp knockout in cells impairs ^{3H}cholesterol efflux and increases cholesterol levels in membranes, and that this in turn inhibits Shh shedding in an indirect manner [4]. We also suggested that HDL promotes shedding by serving as a soluble sink for Disp-exported cholesterol [4]. Consistent with this possibility, Disp contains a sterol-sensing domain and an extended hydrophobic surface channel that may function as a "slide" for lipophiles. This slide may be powered by a transmembrane Na^+ flux [45], similar to the H^+ flux that drives related prokaryotic resistance-nodulation-division transporter export of small molecules [66].

We describe here that Disp can additionally transfer cholesteroylated Shh to soluble HDL acceptors in a direct manner (Fig. 7C). Both Disp functions confirm and support the established concept that the fly LPP lipophorin carries cholesteroylated Hh *in vivo* [13, 14, 15], yet that highly bioactive proteins that do not associate with LPPs, that lack the sterol, and that are secreted as dimers and monomers also exist [14]. We extend this established concept further by showing that the most C-terminal cholesteroylated Shh peptide is sufficient for direct Disp-mediated protein export to HDL because mCherry linked to this peptide also transfers to HDL in a Disp-dependent manner. This finding is also in line with a previous report demonstrating that C-cholesterol is required and sufficient for Disp-mediated protein export [10]. Because of their small size of 5-10 nm, HDLs are not only abundant in the circulation, but are also present in interstitial fluids, both in the adult and throughout development [14]. In contrast, larger LPPs such as LDLs and VLDLs are restricted in their distribution by vascular impermeability [50, 67]. The ratio of HDL to LDL in interstitial fluid has been estimated to average 50:1 [67]. These properties make HDL well-suited

not only for reverse cholesterol transport but also for the transport of cholesteroylated Hh/Shh cargo to its receptor Ptc – that has also been shown to act as a LPP receptor [68], just like Disp [4]. This would lead to the interesting concept that "Hh-free" LPP and "Hh-loaded" LPPs may compete for Ptc receptor binding, the former by promoting Ptc-mediated cholesterol export to suppress signaling and the latter by terminating cholesterol efflux to trigger Hh signaling [41]. Indeed, this fascinating mechanism of Ptc activity regulation has already been demonstrated *in vitro* and *in vivo* [14].

N-terminal shedding during Disp-mediated Hh export is also required *in vivo*

Our *in vitro*-derived concept of direct cholesteroylated Hh hand-over from Disp to LPPs and coupled N-terminal peptide shedding is supported by the *in vivo* finding that site-directed mutagenesis of the sheddase target site fully abolishes mutant Hh biofunction in the developing *Drosophila* wing and eye (see Fig. S8 for a detailed description of these previously published experiments [34, 35]). More importantly, the same mutated proteins suppress endogenous Hh biofunction in a dominant-negative manner [34, 35]. We can now explain both observations by mutated Hh transgenes having their C-terminal peptides readily associated with the extended Disp "slide" for lipophiles, but being unable to complete the transfer due to blocked N-terminal shedding and continued plasma membrane association of the Hh N-terminus (preventing step [3] and [4] to freeze [2] in Fig. 7C). As a consequence, the resulting Disp bottleneck would also slow down endogenous Hh release, explaining the observed dominant-negative developmental defects [34, 35] (Fig. S8C,D). This sequence of events is supported by reversed dominant-negative defects of the mutant protein upon additional removal of the N-terminal palmitate membrane anchor [34, 35]. The same site-directed mutagenesis approach on the Hh C-terminus, however, affects Hh biofunction only mildly and does not suppress endogenous Hh biofunction *in vivo*. Together, these findings suggest that most Hh solubilization *in vivo* does not require C-terminal Hh shedding, but requires direct cholesteroylated Hh hand-over from Disp to LPPs. During this process, only the palmitoylated N-terminus is shed to complete the transfer (Fig. 7C, Fig. S8). Our finding that cholesteroylated C-terminal peptides are sufficient for Disp-mediated transfer to HDL is also consistent with the *in vivo* observation that transgenes with 27 kDa green fluorescent protein (GFP) tags inserted between the 19 kDa Hh signaling domain and its C-terminal cholesteroylated peptide are bioactive in flies [69] and mice [70]. We also note that Disp specificity of C-cholesteroylated Hh and Hh-GFP release *in vivo* can be elegantly explained by Hh being the only known metazoan protein with a covalently linked cholesterol moiety. Finally, our finding that palmitoylated Shh N-termini are not extracted and relayed by Disp (but instead undergo shedding) is supported by the *in vivo* observation that transgenic expression of ShhN – the variant protein that is N-palmitoylated but lacks C-cholesterol – rescues many early embryonic lethal defects in Disp null mutant mice [64].

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Author contributions

Kristina Ehring: Investigation, Visualization, Formal analysis, **Sophia F. Ehlers:** Investigation, Visualization, Formal analysis. **Jurij Froese:** Investigation, Visualization, Formal analysis. **Fabian Gude:** Investigation, Visualization. **Janna Puschmann:** Investigation, Validation. **Kay Grobe:** Conceptualization, Supervision, Writing- Reviewing and Editing, Funding acquisition. **Competing interests:** Authors declare no competing interests. **Data and materials availability**: All data are available in the main text or the supplementary materials. Materials can be made available upon request.

Materials and Methods

Fly lines: The following fly lines were used: *En-Gal4e16E* (*En>*): *P(en2.4-GAL4)e16E*, FlyBaseID FBrf0098595 and *GMR-GAL4 (GMR>): GMR17G12 (GMR45433-GAL4): P(y[+t7.7]w[+mC]=GMR17G12-GAL4)attP2*, Bloomington stock #45433 (discontinued but available from our lab) were crossed with flies homozygous for *UAS-hh* [35] or variants thereof (previously published in [34, 35, 36]). Shh cDNA cloned into pUAST-attP was first expressed in *Drosophila* S2 cells to confirm correct protein processing and secretion. Transgenic flies were generated by using the landing site *51C1* by BestGene. Driver lines were crossed with flies homozygous for *UAS-hh* or variants thereof and kept at 25°C unless otherwise noted. Cassette exchange was mediated by germ-line-specific phiC31 integrase [71]. $w^{-};+/+;hh^{bar3}/hh^{AC}$ flies served as negative controls; $white^{1118}$ flies served as positive controls. Eye phenotypes were analyzed with a Nikon SMZ25 microscope.

Preparation of Drosophila *larval lysates: Drosophila* third-instar larvae were collected and transferred into a microcentrifuge to which 1 mL lysis buffer was added (phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100). Larvae were homogenized with a micropestle and the solution was cleared at 15,000 rpm for 15 min at 4°C. The supernatant was sterile filtered (45 µm) and transferred into a fresh microcentrifuge tube for SEC analysis. All processing was conducted at 4°C.

Cholesterol efflux assay. To conduct this assay, we followed a published protocol [72]. Briefly, $Disp^{-/-}$ cells and nt ctrl cells were seeded in 12-well plates at a final density of 0.2×10^6 cells per well in 0.9 mL DMEM containing 10% FCS and 100 µg/mL penicillin-streptomycin, and cells were incubated at 37°C, 5% CO_2. After 24 h, the medium was changed for DMEM containing 10% FCS, 100 µg/mL penicillin-streptomycin, and 0.5 µCi [^{3}H]-cholesterol (Perkin-Elmer, Foster City, USA) per well. After 2 days, media containing the [^{3}H]-cholesterol were removed, the cells were gently washed, and serum-free media with 0.1% BSA was added. After 18 h, cells were checked under the microscope for confluency and the medium exchanged for 250 µL serum-free medium or media containing 0.05%, 5%, and 10% FCS. After 3 h, cells and media were harvested

and transferred into scintillation vials, [^{3}H] signals were counted, and the amount of released [^{3}H]-cholesterol was expressed as the proportion of solubilized [^{3}H]-cholesterol detected in the media (minus the blank efflux) divided by the cellular [^{3}H]-cholesterol amounts after normalization for protein content.

Cell lines. The generation and validation of Disp1 knockout cells (Disp$^{-/-}$) and nt ctrl cells was previously described [4]. Disp$^{-/-}$, nt ctrl, and C3H10T1/2 reporter cells were maintained in DMEM supplemented with 10% FCS and 100 µg/mL penicillin-streptomycin.

Cloning of recombinant proteins. Shh expression constructs were generated from murine cDNA (NM_009170: nucleotides 1-1314, corresponding to amino acids 1-438; and ShhN: nucleotides 1-594, corresponding to amino acids 1-198) and human Hhat cDNA (NM_018194). Both cDNAs were cloned into pIRES (Clontech) for their coupled expression from bicistronic mRNA to achieve near-quantitative Shh palmitoylation [23]. ShhN (nucleotides 1-594, corresponding to amino acids 1-198) and Hhat were also cloned into pIRES. ^{C25S}Shh was generated by site-directed mutagenesis (Stratagene). Unlipidated ^{C25S}ShhN cDNA and non-palmitoylated ^{C25S}Shh cDNA (amino acids 1-438) were inserted into pcDNA3.1 (Invitrogen). Primer sequences can be provided upon request. Human Scube2 constructs were a kind gift from Ruey-Bing Yang (Academia Sinica, Taiwan). Murine V5-tagged Disp^{tg} was a kind gift from Stacey Ogden (St. Jude Children's Research Hospital, Memphis, USA). Murine Ptc^{tg} and Ptc$^{\Delta L2}$ were generated from Ptc Full Length (pcDNA-h-mmPtch1-FL, Addgene #120889). Ptc$^{\Delta L2}$ was generated by deletion of the second extracellular loop (L2) between transmembrane domains 7 and 8 (amino acids 794-997). Primer sequences can be provided upon request. For Shh-NanoLuc, NanoLuc (pNH-NanoLuc, Plasmid #173075, Addgene), flanked by one glycine residue on both sides, was inserted into murine Shh between amino acids 92N and 93T (corresponding to N91 and T92 in human Shh) by using Gibson assembly (HiFi Assembly Kit, NEB).

Protein detection. Bosc23 cells, nt ctrl cells, or Disp$^{-/-}$ cells were seeded into six-well plates and transfected with 1 µg Shh constructs together with 0.5 µg Scube2 or empty cDNA3.1 by using Polyfect (Qiagen). Where indicated, 0.5 µg Disp or Ptc encoding constructs were co-transfected. Cells were grown for 2 days at 37°C with 5% CO_2 in DMEM containing 10% FCS and penicillin-streptomycin (100 µg/mL). Where indicated, 50 µM peptidyl-CMK (Millipore 344930), an inhibitor of furin activity in DMSO, or DMSO alone was added to the media. Serum-containing media were aspirated and serum-free DMEM added for 6 h, harvested, and centrifuged at 300*g* for 10 min to remove debris. Supernatants were incubated with 10% trichloroacetic acid (TCA) for 30 min on ice, followed by centrifugation at 13,000*g* for 20 min to precipitate the proteins. Proteins solubilized into serum-containing media were pulled down using heparin-sepharose beads (Sigma). Cell lysates and corresponding supernatants were analyzed on the same reducing SDS polyacrylamide gel and detected by Western blot analysis by using goat-α-Shh antibodies (R&D Systems, C9C5), rabbit-α-GAPDH antibodies (Cell Signaling, GAPDH 14C10, #2118), or anti-β-actin antibodies (Sigma-Aldrich, A3854) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Flag-tagged Scube was detected by using polyclonal α-FLAG antibodies (Sigma, St. Louis, USA). GAPDH, β-actin (for cell lysates) or Ponceau S (for media)

served as a loading control. Note that the amounts of immunoblotted soluble and cellular Shh do not correlate inversely. This is because medium lanes represent all TCA-precipitated proteins or all proteins captured by heparin-pulldown in medium, while cells were directly lysed in SDS-buffer and only a small fraction (about 5%) were applied to the gel. As a consequence, a 100% increase in Shh solubilization will correlate to only 5% reduction in the amount of cell-surface associated Shh, and *vice versa*. Shh release was quantified by ImageJ and calculated as the ratio of total or processed (truncated) soluble Shh relative to the cellular Shh material. Relative Shh release from control cells (nt ctrl) was set to 100% and Shh release from Disp$^{-/-}$ cells was expressed relative to that value. This protocol was varied in three ways: For *serum-free* release, cells cultured in DMEM+10% FCS were carefully washed three times with serum-free DMEM before serum-free media were added for 6 h of protein release. For Shh release into *serum-depleted* medium, cells were not washed before the serum-free DMEM was added. For release into *serum-containing* media, DMEM containing the indicated amounts of serum were added for 6 h. For mCherry visualization at the surface of Bosc23 cells, cells were incubated with polyclonal anti-mCherry antibodies (Invitrogen PA5-34974) under non-permeabilizing conditions, and mCherry was visualized by secondary anti-rabbit IgG (Dianova) by using a Zeiss LSM700 confocal microscope.

Shh release in the presence of HDL or LDL. nt ctrl or Disp$^{-/-}$ cells were transfected with pIRES for coupled Shh and Hhat expression, together with Scube2 cDNA as described earlier. Two days after transfection, cells were washed twice with serum-free DMEM and additionally incubated for 1 h in serum-free DMEM. This extensive washing was intended to quantitatively remove serum LPPs. Serum-free DMEM was then discarded and cells were incubated in serum-free DMEM containing 80 µg/mL human HDL (Millipore, LP3, MW 175,000-360,000) or LDL (Millipore, LP2, MW 2,300,000 Da) at 80 µg/mL or at similar molarity (630 µg/mL) for 6 h. Increased Shh release was observed for HDL concentrations ranging from 40 µg/mL to 120 µg/mL, higher HDL concentrations were not tested. For cell debris removal, supernatants were centrifuged for 10 min at 300*g*. For subsequent Shh purification, supernatants were incubated with 5 µg/mL anti-Shh antibody DSHB 5E1 for 2 h at 4°C, followed by the addition of 5 mg protein A beads (Sigma, P1406) in PBS and incubated at 4°C overnight. Immunoprecipitates were collected by centrifugation at 300*g* for 5 min and subjected to reducing SDS-PAGE followed by immunoblot analysis. Shh release was quantified by first determining the ratios of soluble Shh signals detected in 5E1-Protein A pulldown samples relative to cellular Shh signals. Shh release from nt ctrl and Disp$^{-/-}$ cells were next compared, with nt ctrl release set to 100%. Were indicated, ^{C25S}Shh/HDL assemblies were dissolved in 50% ethanol or 0.1% Triton X-100 for 2 min before SEC analysis.

Size exclusion chromatography (SEC) chromatography. Shh size distribution in the presence or absence of soluble carriers was confirmed by SEC analysis with a Superdex200 10/300 GL column (GE Healthcare, Chalfornt St. Giles, UK) equilibrated with PBS at 4°C fast protein liquid chromatography (Äkta Protein Purifier (GE Healthcare)). Eluted fractions were TCA-precipitated, resolved by 15% SDS-PAGE, and immunoblotted. Signals were quantified with ImageJ. When indicated, eluted fractions were split and one half used for Shh activity determination.

Shh bioactivity assay. SEC fractions from Shh expressed into serum-containing media or DMEM supplemented with HDL were sterile filtered, FCS was added to the fractions at 10% and mixed 1:1 with DMEM supplemented with 10% FCS and 100 µg/mL antibiotics, and the mixture was added to C3H10 T1/2 cells. Cells were harvested 6 days after osteoblast differentiation was induced and lysed in 1% Triton X-100 in PBS, and osteoblast-specific alkaline phosphatase activity was measured at 405 nm by using 120 mM p-nitrophenolphosphate (Sigma) in 0.1 M Tris buffer (pH 8.5). Values measured in mock-treated C3H10 T1/2 cells served as negative controls and were subtracted from the measured values.

Reverse-phase high performance liquid chromatography (RP-HPLC). Bosc23 cells were transfected with expression plasmids for dual-lipidated Shh, unlipidated ^{C25A}ShhN control protein, cholesteroylated (non-palmitoylated) ^{C25A}Shh, and palmitoylated ShhN. Two days after transfection, cells were lysed in radioimmunoprecipitation assay buffer containing complete protease inhibitor cocktail (Roche, Basel, Switzerland) on ice and ultracentrifuged, and the soluble whole-cell extract was acetone precipitated. Protein precipitates were resuspended in 35 µL of (1,1,1,3,3,3) hexafluoro-2-propanol and solubilized with 70 µL of 70% formic acid, followed by sonication. RP-HPLC was performed on a C4-300 column (Tosoh, Tokyo, Japan) and an Äkta Basic P900 Protein Purifier. To elute the samples, we used a 0%-70% acetonitrile/water gradient with 0.1% trifluoroacetic acid at room temperature for 30 min. Eluted samples were vacuum dried, resolubilized in reducing sample buffer, and analyzed by SDS-PAGE and immunoblotting. Proteins expressed into the media were analyzed in the same way. Signals were quantified with ImageJ and normalized to the highest protein amount detected in each run. Where indicated, Disp$^{-/-}$ cells were incubated for 24 h instead of the standard 6 h for protein expression before media harvest and TCA precipitation to compensate for the very low Shh release rate from these cells.

Bioanalytical and statistical analysis. All statistical analyses were performed in GraphPad Prism. Applied statistical tests, post hoc tests, and number of independently performed experiments are stated in the figure legends. A p-value of < 0.05 was considered statistically significant. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, and ****: $p<0.0001$ in all assays. Error bars represent the standard deviations of the means. Standard deviations as shown for Shh protein expression and release from nt ctrl cells on Western blots represent their variations from the average value (set to 100%) detected on the same blot.

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