

Inhibition of tetrameric Patched1 by Sonic Hedgehog through an asymmetric paradigm

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Abstract

The Hedgehog (Hh) pathway controls embryonic development and postnatal tissue maintenance and regeneration. Inhibition of oligomeric Hh receptor Patched (Ptch) by the secreted and post-translationally modified ligand Hh relieves suppression of the signaling cascades. Here, we report the cryo-EM structure of tetrameric Ptch1 in complex with palmitoylated N-terminal signaling domain of human Sonic hedgehog (ShhN_p). The structure shows that four Ptch1 protomers are organized as a loose dimer of dimers and each dimer binds to one ShhN_p through two distinct inhibitory interfaces. Ptch1-A binds to ShhN_p through the well-characterized Ca²⁺-mediated interface on the globular domain of ShhN_p, and Ptch1-B primarily interacts with the N-terminal peptide and the palmitoyl moiety. Map comparison reveals that the cholesteryl moiety of native ShhN occupies a recently identified extracellular steroid binding pocket in Ptch1-B. Our structure elucidates the tetrameric assembly of Ptch1 and suggests asymmetric mode of actions of the Hh ligands for inhibiting the potential cholesterol transport activity of Ptch1.

Introduction

The Hedgehog (Hh) precursor undergoes autocatalytic processing, and the resulting N-terminal signaling domain is modified with N-terminal palmitoylation and C-terminal cholesterylation¹⁻³. Binding of the processed Hh ligand to the surface receptor Patched (Ptch) relieves suppression of the downstream G-protein coupled receptor Smoothed (Smo) and subsequently activates the Hh signaling cascade that controls embryogenesis and tissue homeostasis⁴⁻⁶. Deficient Hh signaling can lead to birth defects, whereas abnormal Hh signaling activation is associated with various human cancers⁷⁻⁹. Small molecule modulators have been identified to target this pathway, and inhibitors of the Hh signaling have been explored as potential therapeutics for cancer treatment^{10,11}.

Lacking direct interaction between the Hh receptor Ptch and the downstream Smo¹²⁻¹⁴, Ptch was suggested to inhibit Smo through an indirect mechanism, possibly by limiting the availability of modulatory ligand(s) to Smo¹⁵. Ptch shares sequence homology with the prokaryotic resistance-nodulation-division (RND) family transporters, exemplified by the bacterial proton-driven multidrug resistance exporter AcrB¹⁶. This phylogenetic evidence led to a model suggesting that Ptch may act as a transporter for the ligands, antagonists or agonists, of Smo.

The physiological importance of Ptch is underscored by its association with basal cell carcinoma, the most common cancer in humans^{17,18}. The structure of Ptch1 was recently reported by several groups including ours¹⁹⁻²¹. The 1447 amino acids in human Ptch1 fold to a tripartite architecture containing two extracellular domains (ECD1 and

ECD2), a 12- transmembrane helix (TM) transmembrane domain (TMD) that exhibits a two-fold pseudo-symmetry, and intracellular domains that were unresolved in all the reported structures.

Distinct from most bacterial RND transporters whose structures were resolved as trimers²², all the physiologically relevant Ptch1 structures were in monomeric form, despite Ptch1 was shown to be an oligomer in physiological condition²³. The oligomerization of Ptch1 is mediated by the intracellular middle-loop domain (MLD) and C-terminal domain (CTD)²³. It was reported that the CTD of *Drosophila* Ptc formed a trimer²⁴. In contrast, another mammalian RND homolog human Niemann-Pick type C1 (NPC1) appears to be a monomer^{25,26}. The oligomeric state of Ptch1 therefore requires further investigation.

In our cryo-EM structure of the monomeric human Ptch1 (the C-terminal half of CTD truncated) in complex with an unmodified N-terminal domain of Sonic Hh (ShhN), ShhN engages its concave side to bind to Ptch1 through extensive polar interactions. Ensuing biochemical and structural characterizations reveal that formation of this interface requires binding of steroid to both the sterol-sensing domain (SSD) and an extracellular steroid binding site (ESBS) enclosed by ECD1 and ECD2¹⁹. We suggested that ShhN may relieve Smo inhibition by preventing conformational changes of Ptch1 that are required for its transport activity. Two weeks later, Qi *et al* published the structure of native lipid-modified ShhN (hereafter designated ShhN_n) bound to a monomeric mutant Ptch1* (both MLD and CTD truncated)²¹. While the N-terminal

palmitoyl moiety and the ensuing fragment of ShhN_n bind to a pocket enclosed by ECD and TMD of Ptch1*, the globular protein domain of l ShhN_n only has very limited contact Ptch1* through its convex side. Considering the oligomerization of Ptch1, binding and inhibition of oligomeric Ptch1 by ShhN is thereby more complex and remains to be investigated.

Results

Complex of Ptch1 and palmitoylated ShhN obtained by co-expression

We have obtained an optimal human Ptch1 construct (residues 1-1305) that had markedly improved overexpression level and solution behavior compared to the full-length Ptch1¹⁹. The major species of this Ptch1 construct existed in an oligomeric form upon size-exclusion chromatography (SEC), although the minor monomeric form was used for cryo-EM analysis in our previous study. Cryo-samples made from the oligomeric peaks were highly heterogeneous, impeding structural determination to high resolution¹⁹. To overcome the heterogeneity of oligomeric Ptch1, we made several attempts, including screening of detergents and buffer conditions, engineering of protein with distinct boundaries and chemical cross-linking. Among all the endeavors, when glycol-diosgenin (GDN, Anatrace) was used for protein extraction and purification, the cryo-sample became amenable for cryo-EM analysis.

The details for protein generation can be found in Methods. Briefly, the human Ptch1 (residues 1-1305) with N-terminal FLAG tag and C-terminal His₁₀ tag was co-expressed with untagged human ShhN (residues 1-197) in human embryonic kidney

(HEK) 293F cells (Fig. 1a). After tandem affinity purification, the complex was eluted from SEC mainly in an oligomeric form with the elution volume similar to Ptch1 alone, consistent with the blue native PAGE (BN-PAGE) analysis (Fig. 1b). The signal peptide of ShhN expressed in HEK 293F cells was expected to be removed, and the resulting segment containing residues 24-197 should be palmitoylated but without C-terminal cholesterylation³. Mass spectrometric analysis confirmed the palmitoylation at Cys24 of ShhN in the co-expressed complex. We will refer to this protein as ShhN_p.

Structural determination of tetrameric Ptch1 in complex with ShhN_p

Protocols for grid preparation, cryo-EM data acquisition, and structural determination of oligomeric Ptch1 in complex with ShhN_p are described in details in Fig. 1c, Extended Data Figs. 1, 2, and Methods. After cycles of 3D classification, two major maps were obtained at 6.8 Å and 6.5 Å resolutions out of 39,503 and 25,510 selected particles, respectively (Fig. 1d, Extended Data Figs. 1, 2). The resolutions were sufficient to resolve the secondary structure features for the majority of ECD and TMD (Extended Data Fig. 2e). Blobs of densities were observed on the intracellular side of the detergent micelles, likely belonging to the intracellular domains (Fig. 1d). After application of adapted mask to ECDs, the resolutions of ECDs of the two maps were increased to 4.6 Å and 4.3 Å, respectively (Extended Data Figs. 1, 2b).

The two 3D reconstructions of Ptch1-ShhN_p complex revealed a similar 4:2 assembly (Fig. 1d). The four Ptch1 molecules are organized in two slightly different tetrameric forms, although both are dimer of dimers. One ShhN_p molecule is positioned

in the middle of each dimer and simultaneously interacts with the extracellular domains from two Ptch1 protomers (Fig. 1d). The two dimers have a relative rotation along an axis perpendicular to the membrane in the two distinct tetrameric assemblies (Fig. 1d and Extended Data Fig. 2d). The two dimers loosely contact each other through ECD2 in both tetrameric assemblies without any interaction in the TMDs (Fig. 1d and Extended Data Fig. 2c). The weak interactions may lead to structural flexibilities that limited the resolution of the cryo-EM reconstruction of the tetramer.

Structure of the 2:1 Ptch1-ShhN_p complex

Considering that Ptch1 dimer with one ShhN_p appears to be the basic unit within each 4:2 complex, we further applied adapted mask on one dimer to reduce the heterogeneity caused by the distinct dimer of dimers. The resulting EM map for the 2:1 Ptch1-ShhN_p complex out of 171,590 selected particles reached the resolution of 3.6 Å according to the gold-standard Fourier shell correlation (FSC) 0.143 criterion (Figs 2a, 2b and Extended Data Fig. 1). The map was well resolved for the majority of two Ptch1 protomers and one ShhN_p molecule (Fig. 2a and Extended Data Fig. 3). A total of 2,153 residues was built with 2,110 side chains assigned (Extended Data Table 1). The intracellular segments remained poorly resolved likely due to their flexibility. Consistent with the previous report that the intracellular domains are responsible for the oligomerization of Ptch1²³, the two Ptch1 protomers have no contact in the resolved structure (Fig. 2c).

One ShhN_p simultaneously recognizes two Ptch1 through distinct interfaces (Fig. 2c). To facilitate illustration, we named the two Ptch1 protomers A and B. Ptch1-A

interacts with the pseudo-active site groove (the Ca²⁺-mediated interface) of ShhN_p corresponding to the complex resolved by us ¹⁹. Ptch1-B mainly accommodates the N-terminal fragment and palmitoyl moiety with limited contact with the globular domain of ShhN_p, corresponding to the one in the Ptch1*-ShhN_n complex ²¹ (Fig. 2d). The transmembrane domains of the two Ptch1 protomers stand in parallel within the membrane plane with the SSDs sandwiched inside (Fig. 2e).

The asymmetric binding of one ShhN_p with two Ptch1

Superimposing the structures of the 2:1 Ptch1-ShhN_p with our previous 1:1 Ptch1-ShhN (PDB code 6DMY) relative to Ptch1-A reveals nearly no change in Ptch1 (Fig. 3a). The interface between ShhN_p and Ptch1-A remains nearly identical to what we have described previously ¹⁹, hence will no longer be illustrated here.

Structural superimposition of our 2:1 complex with Ptch1*- ShhN_n (PDB code 6D4J) revealed nearly identical structures for Ptch1, while ShhN_p moved slightly away from Ptch1-B likely due to its interaction with Ptch1-A (Extended Data Fig. 4). Examination of the deposited EM map for Ptch1*-ShhN_n (EMDB code EMD-7796) showed that the local resolution for the interface mediated by the N-terminal segment of ShhN_n and Ptch1* was ~ 5 Å, unable to support reliable analysis for detailed interactions. The local resolution in our current EM map enabled detailed analysis of the interactions between Ptch1-B and the N-terminal segment of ShhN_p (Fig. 3b).

The N-terminal peptide (N15: ₂₄CGPGRGFGKRRHPKK₃₈) and the palmitoyl

moiety of ShhN_p inserts into a tunnel above TMD that is enclosed by ECD1 and ECD2 of Ptch1-B (Figs. 3b, 3c). The N15 segment mainly interacts with ECD1 of Ptch1-B through extensive polar interactions (Fig. 3b). The positively charged Cardin-Weintraub (CW) motif (residues 32-38) of ShhN_p, which is important for binding to heparan sulfate proteoglycans (HSPGs) and Hh transport²⁷, dominates the polar interactions with Ptch1 (Fig. 3b). The palmitate binding pocket is formed by a number of hydrophobic residues from the TMD-ECD connecting elements, including the Linker1, Neck helix 2, Linker7 and Neck helix 8, and TMs 4/6/10/12 (Fig. 3c). The limited contact between the convex side of ShhN_p and Ptch1-B involves the E loop and ensuing helix α 3 on ECD1, in part overlapping with the interface between Ptch1-A and ShhN_p (Fig. 3d).

The cholesteryl moiety of native ShhN inserts into the ESBS of Ptch1*-B

During the preparation of this manuscript, a structure of monomeric Ptch1* in complex with ShhN_n at 2:1 stoichiometric ratio was published²⁸. Although Ptch1* is a monomeric mutant, one ShhN_n can still bind to two Ptch1*. Structural comparison of the two 2:1 complexes reveals nearly identical architecture, except the two Ptch1* molecules move towards each other relative to the ones in our Ptch1-ShhN_p complex (Extended Data Fig. 5), possibly owing to the lack of stabilization by the intracellular domains in Ptch1*.

When the maps of the two 2:1 complexes were scrutinized, an extra EM density was founded in that of the Ptch1*-ShhN_n complex (EMDB code EMD-8955) (Fig. 4a). This density, which was not structurally assigned by the authors, is contiguous with the C-terminus of ShhN_n and protrudes into a binding cavity corresponding to the ESBS of

Ptch1^{*}-B (Fig. 4a). We tentatively built a structural model for this C-terminal segment (C7: ₁₉₁VAAKSGG₁₉₇) and it became evident that the end density likely belongs to the cholesteryl moiety of ShhN_n (Fig. 4b). The cholesteryl moiety is embedded in a cavity mainly enclosed by ECD1, where nearly 20 hydrophobic residues of ECD1 form the contour of the pocket (Fig. 4c). In support of cholesteryl moiety binding to the ESBS, two cholesterol-like densities were also observed in the similar positions of two ESBS in our structure (Extended Data Fig. 3e)

The different oligomeric organizations of Ptch1 and bacterial RND transporters

The twelve TMs of Ptch1 protomer exhibit identical fold with those in the bacterial RND transporters, although their oligomeric organizations are different (Extended Data Fig. 6). Both the TMDs and ECDs of trimeric AcrB, the best characterized bacterial RND transporter, have extensive inter-protomer interactions to support trimer formation²⁹. Such organization provides structural basis for the coupled rotating mechanism among the three protomers during substrate transport^{30,31}. In contrast, tetramerization of Ptch1 is mediated by the intracellular domains lacking interaction among the TMDs and ECDs.

Such architectural differences suggest that Ptch1 may not undergo the similar rotating mechanism as the bacterial RND transporters and the four Ptch1 protomers likely operate independently of each other. Supporting this notion, deletion of the intracellular domains led to monomerization of Ptch1, which nevertheless remained functional in cultured cell signaling assays²³. ShhN binding can result in both Ptch1 inhibition and internalization from the primary cilia¹⁴. The tetrameric assembly of Ptch1 may influence

the internalization efficiency, a caveat to be investigated.

Discussion

Cholesterol was identified as an endogenous Smo agonist that can induce an active conformation of Smo³²⁻³⁴. Several studies have suggested that Ptch may act as a cholesterol exporter as a means to suppress Smo^{20,35}. Consistent with this speculation, five cholesterol-like densities were observed in our current EM reconstruction, four occupying the previously identified binding sites on SSD and ESBS in both Ptch1 protomers and an extra one between SSD and ESBS in Ptch1-A, the corresponding site in Ptch1-B is occupied by the palmitoyl moiety (Extended Data Fig. 7a). A tunnel connecting the SSD and ESBS was observed in our previous structure¹⁹. In the present 2:1 complex, a similar tunnel stretches throughout the ECDs of Ptch1-A, but not Ptch1-B, that may represent the cholesterol transport path (Extended Data Fig. 7b). Nevertheless, there still lacks killer experiment to directly demonstrate the cholesterol transport activity by Ptch1. In addition, an unidentified extracellular cholesterol carrier may be required to take the cholesterol expelled by Ptch1.

The asymmetric binding interfaces between one ShhN_p and two Ptch1 reveal two distinct inhibitory mechanisms for the two Ptch1 protomers by one ShhN_p. In both cases, ShhN binding would block the cholesterol transport activity of Ptch1. Inhibition of Ptch1-A was mediated by the globular domain of ShhN_p through a Ca²⁺-mediated interface. We previously proposed that ShhN binding to ECD1 and ECD2 of Ptch1 could prevent the conformational changes of Ptch1 that are required for its transport activity¹⁹. Inhibition

of Ptch1-B was mainly mediated by the palmitoylated N15 peptide, which blocks the tunnel connecting SSD and ESBS (Extended Data Fig. 7b). The structure provides a nice explanation for the recent study that a palmitoylated N-terminal 22-residue peptide could partially activate Hh signaling by binding to Ptch1³⁶. An unexpected discovery is the insertion of the cholesteryl moiety into ESBS of Ptch1-B, which will naturally block binding of cholesterol to this site, further block the transport of cholesterol along this path. No substantial conformational changes were observed between the TMDs of Ptch1 alone and the two Ptch1 molecules in Ptch1-ShhN_p complex (Extended Data Fig. 7c), suggesting that ShhN_p association do not affect cholesterol binding to TMD.

The palmitoyl and cholesteryl moieties render the Hh ligands as hydrophobic morphogens and several factors have been described as Hh chaperons to ensure its solubility, such as Hh itself to form soluble multimeric Hh^{37,38}, lipoprotein particles^{39,40}, and Scube proteins^{41,42}. The structures suggest that Ptch1 binding could shield both the hydrophobic lipid moieties of ShhN from the aqueous environment (Fig. 5).

In sum, the structure of tetrameric Ptch1 in complex with palmitoylated ShhN at a 4:2 stoichiometric ratio further completes the molecular picture for the interplay between Ptch1 and Shh, and sets an important framework for future investigation of Hh signaling (Fig. 5). It is noted that the asymmetric binding between the surface receptor and the ligand at a 2:1 stoichiometric ratio has been discovered in several cases, such as growth factor receptor⁴³, erythropoietin receptor⁴⁴ and netrin-1 receptor⁴⁵. Such asymmetric signaling mechanism may be essential or more efficient in the signal transduction from

ligands to the receptors. Considering the common dimeric or high-order oligomeric form of surface receptors, the substoichiometric ratio and asymmetric modes of actions between ligands and receptors may represent a paradigm that is more general than currently known.

METHODS

Protein expression and purification

The cDNA of human Ptch1 (Uniprot: Q13635) (residues 1-1305) was cloned into the pCAG vector with an amino-terminal FLAG tag and a carboxy-terminal His₁₀ tag. The cDNA of human Shh (Uniprot: Q15465) N-terminal domain (ShhN, residues 1-197) was cloned into the no-tag pCAG vector. HEK 293F suspension cells were cultured in Freestyle 293 medium (Thermo Fisher Scientific) at 37 °C supplied with 5% CO₂ and 80% humidity. When the HEK 293F cell density reached 2.0×10⁶ cells per ml, the cells were transiently transfected with the expression plasmids and polyethylenimines (PEIs) (Polysciences). For the Ptch1 alone, approximately 1 mg Ptch1 plasmids were pre-mixed with 3 mg PEIs in 50 ml fresh medium for 15-30 min before application. For the Ptch1-ShhN complex, approximately 1 mg Ptch1 and 1 mg ShhN plasmids were pre-mixed with 6 mg PEIs in 50 ml fresh medium for 15-30 min before application. For transfection, 50 ml mixture was added to one-liter cell culture and incubated for 15-30 min. Transfected cells were cultured for 48 h before harvest. For the purification of Ptch1 alone or its complex with ShhN, the HEK 293F cells were collected and resuspended in the buffer containing 25 mM Tris pH 8.0, 150 mM NaCl and protease inhibitor cocktails (Amresco). After sonication on ice, the membrane fraction was solubilized at 4 °C for 2 hours with 1%

(w/v) GDN (Anatrace). After centrifugation at 20,000 g for 1 h, the supernatant was collected and applied to anti-Flag M2 affinity resin (Sigma). The resin was rinsed with the wash buffer (W1 buffer) containing 25 mM Tris pH 8.0, 150 mM NaCl, and 0.02% GDN. The protein was eluted with the W1 buffer plus 200 μ g/ml FLAG peptide. The eluent was then applied to the nickel affinity resin (Ni-NTA, Qiagen). After three times of rinsing with W1 buffer plus 20 mM imidazole, the protein was eluted from the nickel resin with W1 buffer plus 250 mM imidazole. The eluent was then concentrated and further purified by size-exclusion chromatography (SEC, Superose® 6 10/300 GL, GE Healthcare) in the buffer containing 25 mM Tris pH 8.0, 150 mM NaCl, and 0.02% GDN. The peak fractions for the oligomeric and monomeric Ptch1 or its complex with ShhN were separately collected.

Blue Native PAGE (BN-PAGE)

Chromatographically purified Ptch1 and Ptch1-ShhN_p samples were mixed with 4 \times loading buffer and Coomassie G-250 additive and then subjected to 3-12% NativePAGE™ Novex Bis-Tris gel (Invitrogen) for native electrophoresis at 4°C. The electrophoresis was conducted at 150V constant for 60 minutes, and then increase voltage to 250V constant for another 75 minutes. After electrophoresis, the gel was transferred to a container for fixation in fixing solution (40% methanol, 10% acetic acid), and then for staining (0.02% Coomassie R-250 in 30% methanol and 10% acetic acid) and destaining (8% acetic acid). All the procedures were performed according to the manufacturer's protocol.

Cryo-EM sample preparation and data collection

The cryo grids were prepared using Thermo Fisher Vitrobot Mark IV. The Quantifoil R1.2/1.3 Cu grids were firstly glow-discharged with air for 40 s at medium level in Plasma Cleaner (HARRICK PLASMA, PDC-32G-2). Then aliquots of 3.5 μ l purified Ptch1-ShhN complex (concentrated to approximately 15 mg/ml) were applied to glow-discharged grids. After being blotted with filter paper for 3.5 s, the grids were plunged into liquid ethane cooled with liquid nitrogen. A total of 4,003 micrograph stacks were automatically collected with SerialEM on Titan Krios at 300 kV equipped with K2 Summit direct electron detector (Gatan), Quantum energy filter (Gatan) and Cs corrector (Thermo Fisher), at a nominal magnification of 105,000 \times with defocus values from -2.0 μ m to -1.2 μ m. Each stack was exposed in super-resolution mode for 5.6 s with an exposing time of 0.175 s per frame, resulting in 32 frames per stack. The total dose rate was about 50 $e^-/\text{\AA}^2$ for each stack. The stacks were motion corrected with MotionCor2⁴⁶ and binned 2 fold, resulting in a pixel size of 1.114 $\text{\AA}/\text{pixel}$, meanwhile dose weighting was performed⁴⁷. The defocus values were estimated with Gctf⁴⁸.

Cryo-EM data processing

A total of 1,226,114 particles were automatically picked with RELION 2.0⁴⁹. After 2D classification, a total of 448,682 particles were selected and subject to a guided multi-reference classification procedure. The references, two good and two bad, were generated with limited particles in advance. A total of 266,572 particles selected from multi-references 3D classification were subjected to local search 3D classification with adapted mask on the flexible half (to obtain a complete map) and performed five parallel runs at

the same time. Then, a total of 107,265 particles were selected from good classes and subjected to seven parallel runs of local search 3D classification without adapted mask. Two distinct classes were selected, Class I with 39,503 particles and Class II with 25,510 particles, yielding 3D reconstitutions with 6.8 Å and 6.5 Å, respectively. Lastly, ECD masks were applied to increase the local resolution to 4.6 Å and 4.3 Å for these two classes, respectively.

To increase the resolution of stable half, the 266,572 particles selected from multi-references 3D classification were subjected to a global angular search 3D classification with one class and 40 iteration. The outputs of the 30th-40th iterations were subjected to local angular search 3D classification with three classes separately. A total of 171,590 particles were selected by combining the good classes of the local angular search 3D classification, yielding a 3D reconstruction with an overall resolution of 3.6 Å after 3D auto-refinement with an adapted mask on the stable half.

All 2D classification, 3D classification, and 3D auto-refinement were performed with RELION 2.0. Resolutions were estimated with the gold-standard Fourier shell correlation 0.143 criterion⁵⁰ with high-resolution noise substitution.

Model building and refinement

Firstly, the map at 3.6 Å was used to build a 2:1 complex structure of Ptch1 and ShhN. Two previously reported 1:1 complex structures (PDB code 6DMY and 6D4J) served as initial models to be docked into the map with Chimera, following by manual adjustment

in Coot to generate the final structure. Then, two 2:1 structures were fitted into the maps of Class I or Class II to generate two complex structures at 4:2 stoichiometry.

All structure refinements were carried out by PHENIX⁵¹ in real space with secondary structure and geometry restraints. Overfitting of the models was monitored by refining the model against one of the two independent half maps and testing the refined model against the other map⁵².

Author Contributions. N.Y. and X.G. conceived the project. X.G., H.Q., P.C., M.H., and S.G. performed the experiments. All authors contributed to data analysis. N.Y. and X.G. wrote the manuscript.

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Figures

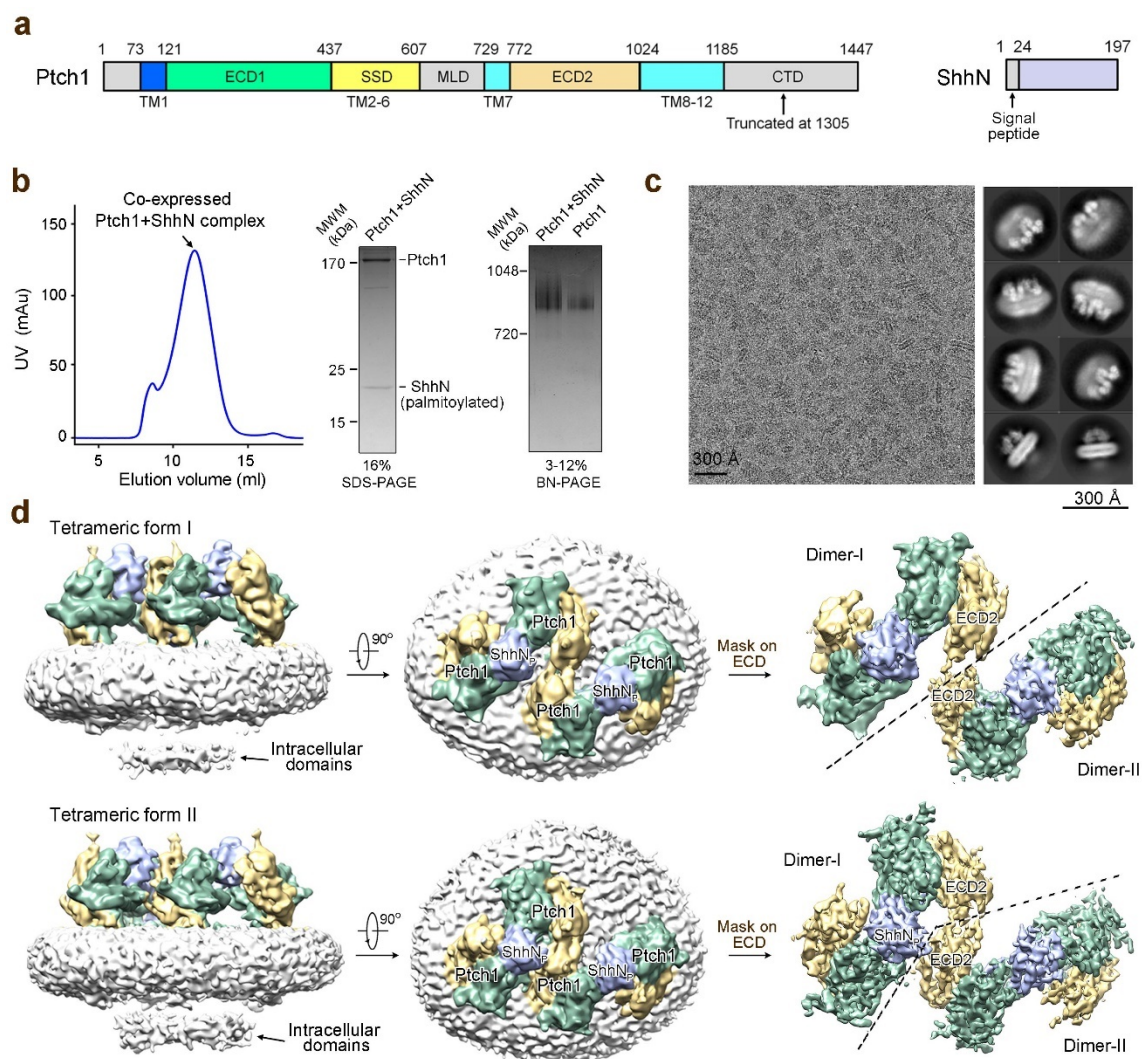


Fig. 1 | Structural determination of tetrameric Ptch1 in complex with palmitoylated ShhN (ShhN_p). **a**, Schematic illustration of the domain organization of Ptch1 and the constructs of Ptch1 and ShhN used for recombinant co-expression. TM: transmembrane segment; ECD: extracellular domain; MLD: middle-loop domain; CTD: C-terminal domain. **b**, Purification of co-expressed Ptch1 (residues 1-1305) and ShhN (residues 1-197). Shown here is a representative size exclusion chromatography (SEC) of the complex. The peak fractions were subjected to SDS-

PAGE and blue native (BN)-PAGE. Ptch1 alone migrates at similar position as the complex on BN-PAGE. **c**, Representative cryo-EM micrograph and 2D class averages. **d**, Two three-dimensional reconstructions of the complex. Side and extracellular views are shown for both reconstructions. With adapted mask on ECD, improved density maps for the ECD are shown on the right. The maps were generated in Chimera ⁵³.

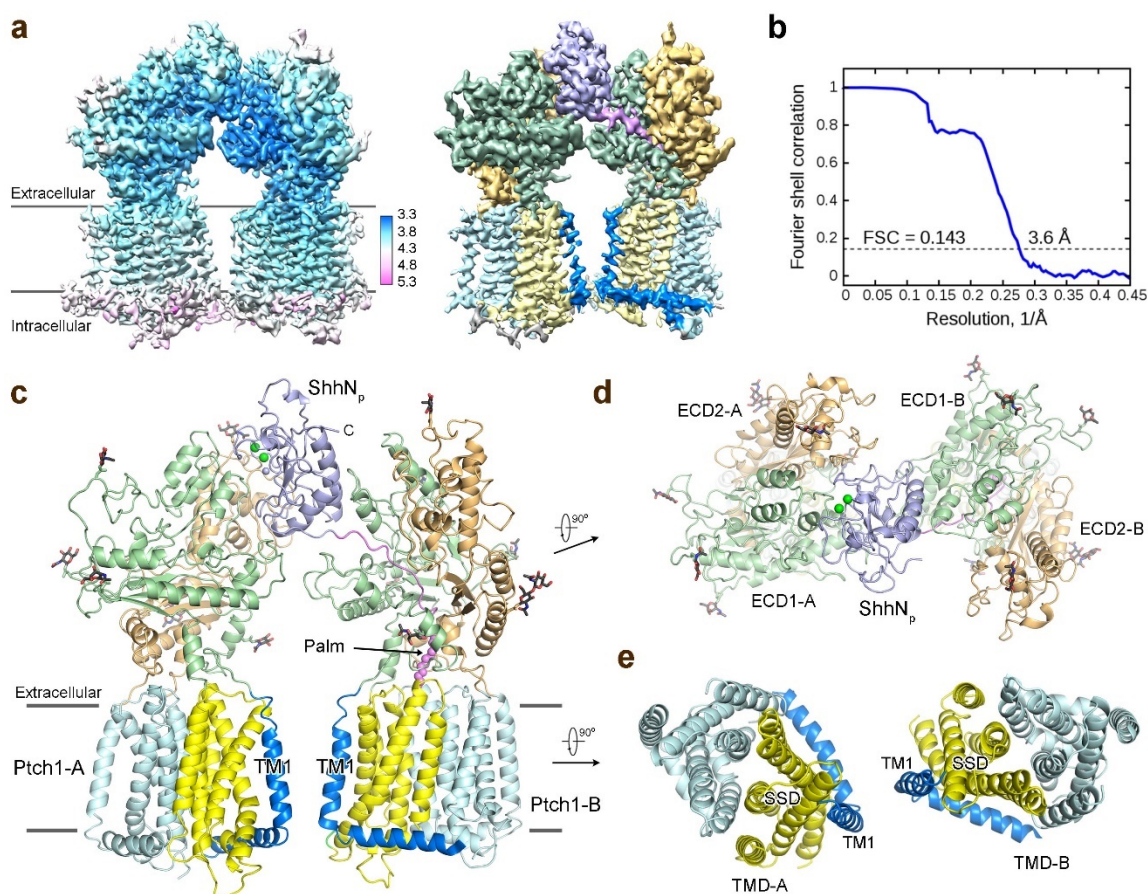


Fig. 2 | Structure of the 2:1 Ptch1-ShhN_p complex. **a**, Cryo-EM map for the 2:1 Ptch1-ShhN_p complex. Higher resolution was achieved after applying adapted mask to dimeric Ptch1 with one ShhN_p. *Left panel*: local resolution map calculated using RELION 2.0. *Right panel*: domain-colored map with the same color code for our previous report¹⁹. **b**, The gold-standard Fourier shell correlation curve for the cryo-EM map. **c**, Overall structure of one ShhN_p bound to two Ptch1. The N-terminal palmitoyl moiety is highlighted as violet sphere. The Ca²⁺ and Zn²⁺ ions are shown as green and purple spheres, respectively. The glycosyl moieties are shown as black sticks. **d**, ECDs and ShhN_p viewed from extracellular view. **e**, Transmembrane domains shown in the extracellular view. All structure figures were prepared in PyMol⁵⁴.

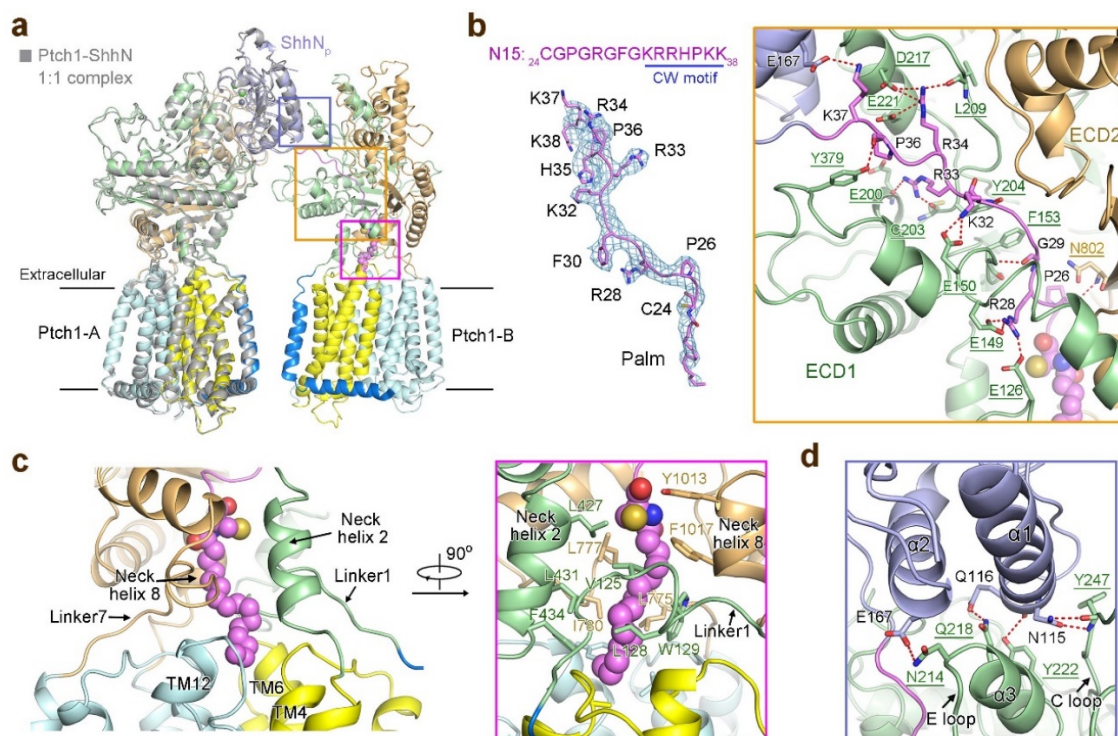


Fig. 3 | Asymmetric binding of one ShhN_p with two Ptch1. **a**, Structural comparison of 2:1 Ptch1-ShhN_p with our previously reported 1:1 Ptch1-ShhN complexes. The 2:1 Ptch1-ShhN_p complex was domain colored. The 1:1 Ptch1-ShhN complex (PDB code 6DMY) was colored grey. The superimposition was made relative to Ptch1-A molecule. **b**, The N-terminal peptide (N15) and palmitoyl moiety of ShhN_p, which are well-resolved in the 3.6 Å EM map for the 2:1 complex, interact extensively with ECD1 of Ptch1-B. The map, shown as blue mesh, was contoured at 6 σ . The potential electrostatic interactions are represented by dashed red lines. **c**, The palmitate binding pocket in Ptch1-B. The hydrophobic residues in TMD-ECD connecting elements (including the Linker1, Neck helix 2, Linker7 and Neck helix 8) and hydrophobic surface residues in TM4/6/10/12 together constitute the binding pocket for the palmitate. The coordinating residues are shown as sticks and the palmitoyl moiety is shown as violet sphere. **d**, The secondary interface between ShhN_p and Ptch1-B. The globular domain of ShhN_p interacts with ECD1 of Ptch1-B through limited polar interactions.

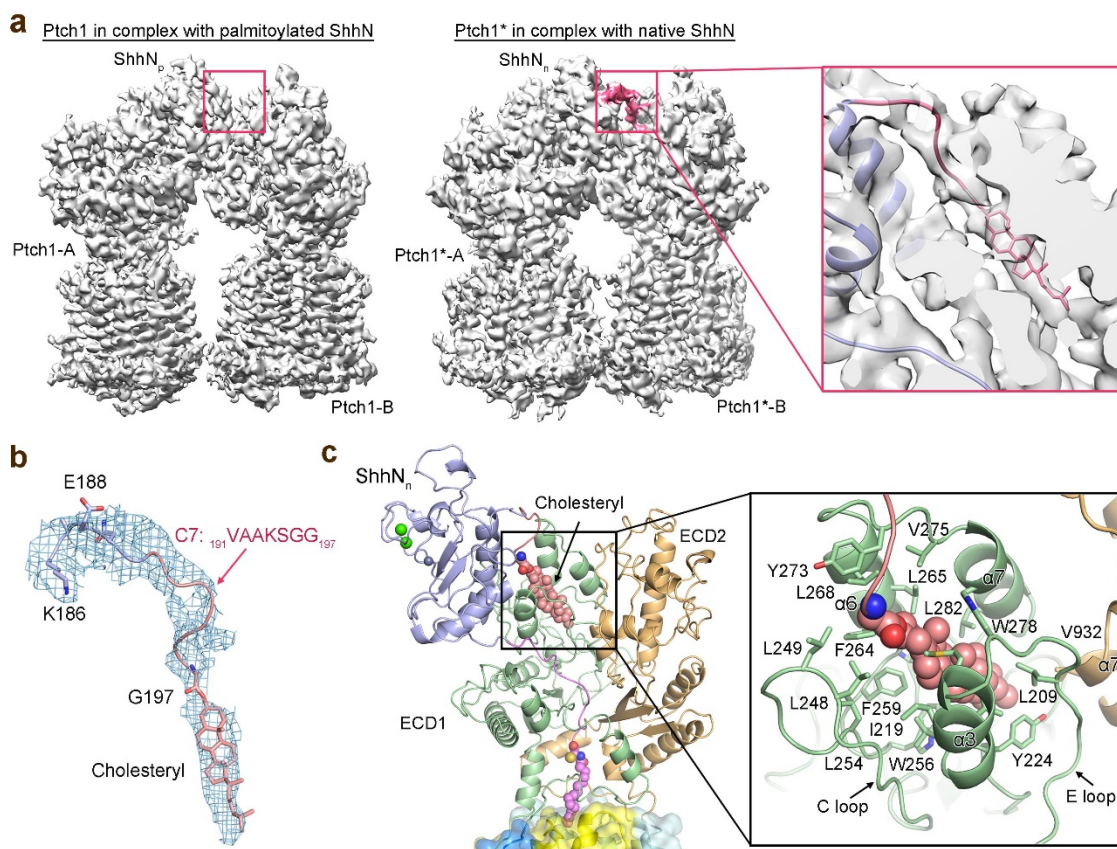


Fig. 4 | The cholesterol moiety of native ShhN (ShhN_n) inserts into the extracellular steroid binding site (ESBS) of Ptch1*-B. **a**, Comparison of the EM maps for our Ptch1-ShhN_p and the recently published Ptch1*-ShhN_n (EMDB code EMD-8955) reveals an extra density of ShhN_n in the Ptch1*-ShhN_n map that protrudes into ESBS of Ptch1*-B. The extra density is highlighted in pink. A zoomed-up cut-open view of the C-terminus of ShhN_n (ribbon representation) into ECD1 is shown on the right. **b**, Density corresponding to the C-terminal peptide (C7) and cholesterol moiety of ShhN_n contoured at 3σ. **c**, The accommodation of the cholesterol moiety by ESBS. As described previously, ESBS is mainly constituted by helices α3/α6/α7, E loop and C loop of ECD1. The hydrophobic residues that constitute the pocket are shown as sticks. The cholesterol moiety is shown as pink sphere.

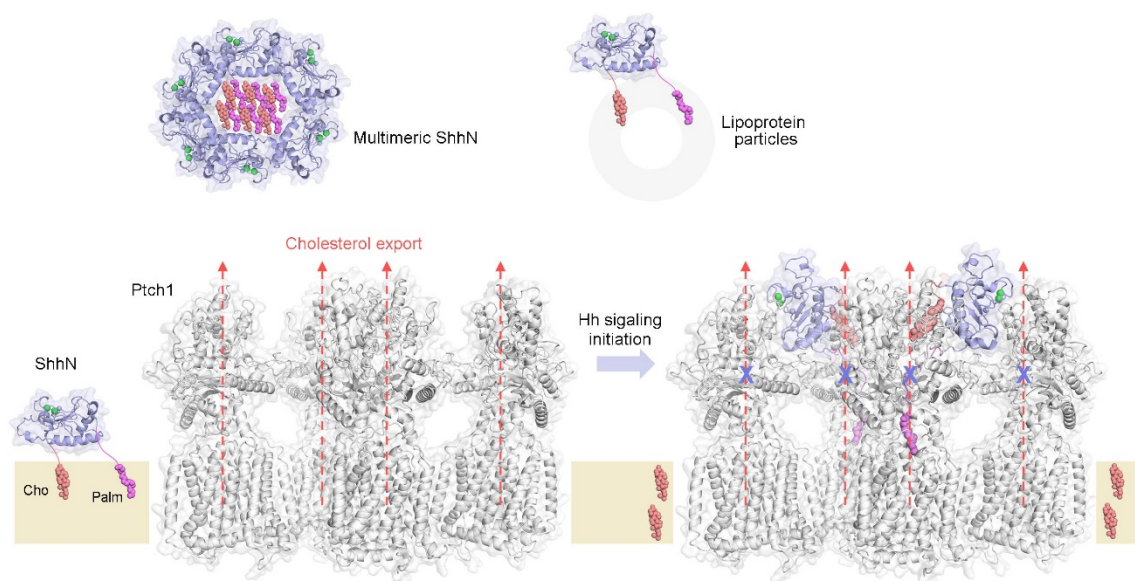


Fig. 5 | Model for the inhibition of tetrameric Ptch1 by lipid-modified ShhN.

The palmitoyl and cholesteryl modified ShhN signaling domain becomes hydrophobic that can be attached to the membrane. Multimerization of the modified ShhN or interaction with lipoprotein particles keep the morphogen soluble. In the absence of Hh ligands, Ptch1 may export the cholesterol, the agonist to Smo, out of the lipid bilayer as a means to suppress Smo. Lipid-modified ShhN inhibits the cholesterol transport activity of Ptch1 using two asymmetric modes, resulting in elevated cholesterol in the membrane that leads to Smo activation.