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Supplementary Information for

Hedgehog pathway activation through nanobody-mediated conformational blockade of the Patched sterol conduit

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Supplementary Information Text

Materials and Methods

Cell culture Sf9 and 293T cells were maintained in culture according to previously published conditions (1). 293-Freestyle cells were maintained in suspension culture in an 8% CO_2 incubator equipped with a shaking platform, using Freestyle 293 expression medium (Life Technologies) supplemented with 1% fetal bovine serum (Gemini Bio). Baculovirus production in Sf9 cells and infection of suspension 293 cultures with recombinant baculovirus (BacMam expression) was performed as previously described (1).

Molecular cloning All constructs were cloned with Gibson assembly. For BacMam expression, PTCH1 variants were cloned into pVLAD6 vector. For yeast selection, Ptch1-C and Ptch1-C-NNQ variants were used. Ptch1-C is mouse PTCH1 truncated at amino acid 1173, deleted at 619-711 and altered at C1167Y. Use of Ptch1-C for selection minimized the possibility of getting nanobodies that bind to PTCH1 intracellular domain, due to extensive deletion of the intracellular sequence. For structural determination and cell biology experiments, Ptch1-B as reported earlier was used. For luciferase assay and cell surface binding experiments, PTCH1 variants were cloned into pcDNA-h (pcDNA3 vector with the neomycin resistance cassette removed).

Yeast display selection The synthetic nanobody library was grown in SDCAA media at 30 C to a cell density of ~$1x10^8$/ml. Cells covering about 10 times the initial diversity ($5x10^8$ diversity, $5x10^9$ cells) were transferred into SGCAA media at 20C to induce expression of nanobody on cell surface. For selection, $7.5x10^9$ cells were pelleted by centrifugation and resuspended in selection buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 0.1% DDM, 0.02% CHS). The cells were then incubated with 100 nM 1D4-tagged Ptch1-C NNQ, spun down and washed with selection buffer, and then with FITC-labeled 1D4 antibody, then 100 µL anti-FITC MACS beads. After loading the beads-bound cells onto the magnetic manifold and washed extensively with selection buffer, the bound cells were eluted, cultured in SDCAA media and induced for nanobody expression in SGCAA media. A second round of selection was then performed on these cells, first with the Alexa647 labeled 1D4 antibody alone to counter-select antibody-binding cells and then with 100 nM 1D4 tagged Ptch1-C NNQ. The selected cells were grown in SDCAA and induced with SGCAA again and then incubated with 100 nM Myc-tagged Ptch1-C and 100 nM 1D4-tagged Ptch1-C-NNQ and stained with anti-Myc Alexa 647 and anti-1D4 FITC and cells showing stronger FITC signal on FACS were selected. The same FACS selection was repeated and the selected cells were grown and dilution-plated. Plasmid was prepared from single colonies and sequenced after rolling cycle amplification (RCA). 15 unique sequences were retrieved from 24 colonies. Yeast cells harboring these nanobody sequences were then tested for binding to anti-1D4 antibody and to Ptch1-C-NNQ. Three out of 15, Clone #4, #9 and #15, bind to 1D4 antibody directly. Clone 4 was used as a control nanobody in activity characterizations. The rest of the sequences were cloned into pET26b vectors for expression and purification from *E. coli*.

Nanobody purification pET26b vectors containing nanobody sequences were transformed into *E. coli* BL21 (DE3) strain. The bacteria were grown in Terrific broth media at 37 °C to OD600 of 0.8, and then induced with 0.2 mM IPTG and transferred to 20 °C. After overnight expression, the cells were harvested by centrifugation at 8,000 g. The cell pellet was resuspended in SET buffer (500 mM sucrose, 0.5 mM EDTA, pH 8.0, 200 mM Tris, pH 8.0) at a ratio of 5 ml buffer /1 g pellet. After stirring for 30 min at room temperature, two volumes of water was added. After stirring for an addition 45 min, $MgCl_2$ was added to 2 mM and benzonase at 1:100,000. After 5 min incubation, NaCl was added to 150 mM, imidazole to 20 mM and the whole mixture was centrifuged at 20,000 g for 15 min at 4 C. The supernatant was then loaded onto a Ni-NTA column, washed with ice-cold buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole) and then eluted in 20 mM HEPES pH 7.5, 150 mM NaCl, 250 mM imidazole. The eluted protein was then dialyzed overnight in 20 mM HEPES pH 7.5, 150 mM NaCl at 4 °C. All of the initial hits except for clone 13 could be expressed and purified. Clone 13 was then excluded from analysis.

Affinity maturation The first round affinity maturation library was made with error-prone PCR. Nanobody clone 17, 20 and 23 were chosen as the starting point of this selection. 10 ng plasmid

containing the nanobody sequence was used as the template (equivalent to ~1 ng DNA of nanobody sequence) and PCR amplified with Mutazyme kit. The PCR product was gel-purified and 10ng was then used as the template for the next round of PCR. A total of 4 rounds of PCR were performed. The final product was then amplified with Phusion polymerase to obtain sufficient amounts for yeast transformation. A total of ~100 µg DNA was purified for each parental sequence using ~2 µg of the error-prone PCR product. The DNA fragments were then transformed into yeast along with pYDS2.0 plasmid backbone. DNA from 3 different parental sequence, and a mixture of the three were electroporated separately into yeast cells, but the cells were pooled in YPD for recovery after electroporation. Serial dilution and plating gave an estimate of $1x10^9$ independent transformant for this library. The transformed yeast cells were then grown in YPD media with 100 µg/ml nourseothricin sulfate, and then induced in YPG media with the same antibiotic. The yeast cells were enriched for PTCH1 binding by MACS selection using concentrations of 1D4-tagged Ptch1-C NNQ at 100 nM, 5 nM, 0.8 nM. Then cells expressing nanobody were incubated with Ptch1-C NNQ at 0.6 nM. After washing in selection buffer, the cells were incubated with the parental 17, 20, 23 nanobody proteins at 1 µM each for 170 min at room temperature. The cells were then stained with FITC-labeled HA antibody to mark nanobody expression levels and Alexa 647-labeled anti-1D4 antibody to mark PTCH1 binding. Cells that maintain high PTCH1 binding were selected from FACS. 64 clones were sequenced to identify repeating changes.

The second round of affinity maturation was performed with a library targeting the complementarity determining regions (CDRs) using the one-pot mutagenesis method (2). A pool of DNA oligos with NNK substituting each codon in the CDR regions was used for one-pot mutagenesis of the CDRs so that theoretically all 20 amino acids at each position were represented in this library. The DNA product from one-pot mutagenesis was then amplified with Q5 polymerase and purified with gel extraction. A final product ~5 µg DNA was used for yeast transformation. The transformed cells were grown in YPD media containing 100 µg/ml nourseothricin sulfate and induced in YPG media containing the same antibiotic. The cells were then incubated with 10 nM protein C-tagged Ptch1-C, washed in selection buffer and then incubated with 1µM 23T (purified nanobody protein with the consensus sequence from the 1st round of affinity maturation) for one day. The cells were then stained with FITC-labeled HA and Alexa 647 labeled anti-protein C antibody and the PTCH1-high cells were selected in FACS. The cells were grown in YPD and induced again. The same FACS selection procedure was repeated to further purify the population. The nanobody sequences from the plasmids prepared from the initial yeast library and the final selected library were then amplified with Q5 polymerase and sent for amplicon sequencing at MGH sequencing core.

PTCH1 purification Purification of PTCH1 was performed as previously described with minor changes. Suspension 293 cells were grown to a density of 1.2 – 1.6 x 10^6/ml, supplemented with 10 mM sodium butyrate, and infected with high-titer Ptch1-SBP baculoviruses for 40-48 hr. Cell pellets were stored at -80°C. Pellets were thawed into hypotonic buffer (20 mM HEPES pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 0.25 M sucrose) supplemented with protease inhibitors and benzonase. Crude membranes were pelleted with centrifugation (100,000 x g, 30 min., 4°C). The pellet was resuspended in lysis buffer (300 mM NaCl, 20 mM HEPES pH 7.5, 2mg/ml iodoacetamide, 1% DDM / 0.2% CHS) with protease inhibitors and solubilized for 1 hour at 4°C with gentle rotation. After centrifugation (100,000 x g, 30 min., 4°C), the supernatant was incubated with streptavidin-agarose affinity resin in batch mode for 2-3 hours at 4°C with gentle rotation. The resin was packed into a disposable column, and washed with 20-30 column volumes of buffer (20 mM HEPES pH 7.5, 300 mM NaCl, 0.03% DDM / 0.006% CHS). Protein was eluted in the same buffer supplemented with 2.5 mM biotin.

Cryo-EM data acquisition Eluted Ptch1-B protein was mixed at 1:1.1 ratio with TI23 and then loaded onto Superdex 200 column pre-equilibrated with SEC buffer (20 mM HEPES, pH 8, 150 mM NaCl, 0.02% GDN). The peak fractions were collected and concentrated with an Amicon filter with molecular weight cutoff of 100 kDa to A280 ~4.5. 2.5 µL sample was applied to a glow-discharged quantifoil grid on a vitrobot. The sample chamber was kept at 100% relative humidity. The grid was blotted for 10s and plunged into liquid ethane bath cooled by liquid nitrogen.

The cryo grids were imaged on a Titan Krios 2 electron microscope operated at 300 kV. Images were taken on the pre-GIF K2 camera in dose fractionation mode, at nominal maginification of 22.5k, corresponding to a pixel size of 1.059 Å (0.5295 Å per super-resolution pixel). The dose rate was ~8e/pix/sec with a total exposure time was 12s at a frame rate of 0.2s/frame. Fully automated data collection was performed with SerialEM, with a defocus range of -1 µm to -3 µm. Gain reference was taken at the beginning of the data collection and was applied later in data processing.

Image processing A total of 7,046 movie stacks were collected. The movie stacks were corrected by gain reference, binned by 2, and corrected for beam-induced motion with MotionCor2. CTF was determined with CTFFIND4 from the motion-corrected sums without dose-weighting using a wrapper provided in cryoSPARC2. Dose-weighted sums were used for all the following steps of processing. Particles were autopicked cryoSPARC2. Particles corresponding to protein molecules were selected from 2D classification. These particles were then reconstructed ab initio, and then classified with heterogeneous refinement into 3 classes, using two copies of the map generated from the last step plus one junk map as the initial models. The best class was chosen for homogeneous refinement and then non-uniform refinement to obtain a map at 4.1 Å. The particles were then analyzed with the 3D variability analysis tool and the two extremes of the first eigenvector were used as the basis for further 3D classification. The final 3D class was refined with non-uniform refinement to a resolution of 3.7 Å. The particle stack was then exported to cisTEM using the scripts in pyEM. After one iteration of local refinement with a mask excluding the detergent micelle, a map was reported at 3.4 Å. The final map after sharpening was used for model building.

Protein model building Nanobody TI23 structure was generated with rosettaCM using 4mqtB and 5m30F as the template structures. The generated structure and the previously determined PTCH1 structure (6mg8) were docked into the cryo-EM map and refined in phenix.real_space_refine with morphing. The refined model was then edited manually in coot, to add in residues that are now resolved in the new structure, and the small molecules. The constraints for small molecules were generated on the PRODRG server. The entire structure was then refined in phenix.real_space_refine.

FACS-based ShhN binding assay 293 cells were transiently transfected with GFP-tagged Ptch1 constructs. After 24 hours, cells were dissociated using 10 mM EDTA, washed with HPBS 0.5 mM Ca^{2+}, and pelleted by centrifugation. Cells were then resuspended in binding buffer (HPBS, 0.5 mM Ca^{2+}, 0.5 mg/ml BSA) and incubated with purified ShhN-biotin (1:400 dilution) for 30 minutes at 4° C. Cells were then washed three times in binding buffer by centrifugation and subsequently incubated with Alexa Fluor 647 streptavidin conjugate (Invitrogen) for 15 minutes at 4° C. Cells were then washed three times by centrifugation in wash buffer (binding buffer plus 1 mM biotin) and the percentages of cells bound by ShhN were quantified by flow cytometry after gating for PTCH1-GFP expression (BD FACSAria II, Stanford Stem Cell Institute FACS Core).

Gli-dependent luciferase assay The luciferase assay was performed in $Ptch1^{-/-}$ MEFs, as previously described(3). $Ptch1^{-/-}$ MEFs were seeded into 24-well plates and then transfected with various plasmids along with a mixture containing 8xGli firefly luciferase and SV40-renilla luciferase plasmids. For each well, 2ng (0.4%) plasmid encoding Ptch1-B variants, or 5ng (1%) plasmid encoding full-length PTCH1 was used. When cells were confluent, they were shifted to DMEM with 0.5% serum containing ShhN-conditioned medium or control medium and incubated for 48 hr. Luciferase activity was then measured using a Berthold Centro XS3 luminometer. The ShhN conditioned medium was prepared from 293 cells transfected with a plasmid expressing the amino signaling domain of Shh. In brief, 293 cells were transfected with the ShhN expression plasmid with lipofectamine 2000. Twelve hours after transfection, culture medium was replaced with 2% FBS low-serum medium. The conditioned medium was then collected 48hours after medium change, and used at 1:10 for the luciferase assays.

Cellular cholesterol measurement The Perfringolysin O D4 domain (a.a. 391–500) and mutants were expressed as His_6–tagged proteins in *E. coli* BL21 RIL codon plus (Stratagene) cells and purified using the His_6–affinity resin (GenScript). These proteins were labeled at the single Cys site (C459) by a solvatochromic fluorophore to generate ratiometric sensors. $Ptch1^{-/-}$ MEFs were seeded into 50 mm round glass–bottom plates (MatTek) and grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% (v/v) fatal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (Life Technologies). After attachment to the culture vessels (~24hr), cells were transiently transfected with plasmids encoding Ptch1-B variants using the jetPRIME transfection reagent (Polyplus Transfection) according to the manufacturer's protocol. 1 μg plasmid was used for each transfection. Cholesterol in the inner (IPM) leaflets of the plasma membrane was quantified using cholesterol sensors as described previously (4) with some modification. Specifically, the Y415A/D434W/A463W (YDA) mutant of the D4 domain labeled with (2Z,3*E*)-3-((acryloyloxy)imino)-2-((7-(diethylamino)-9,9-dimethyl-9*H*-fluoren-2-yl)methylene)-2,3-dihydro-1*H*-inden-1-one (WCR) was delivered into the cells by microinjection for quantification of IPM cholesterol ($[Chol]_i$). All sensor calibration, microscopy measurements, and ratiometric imaging data analysis were performed as described (4-6).

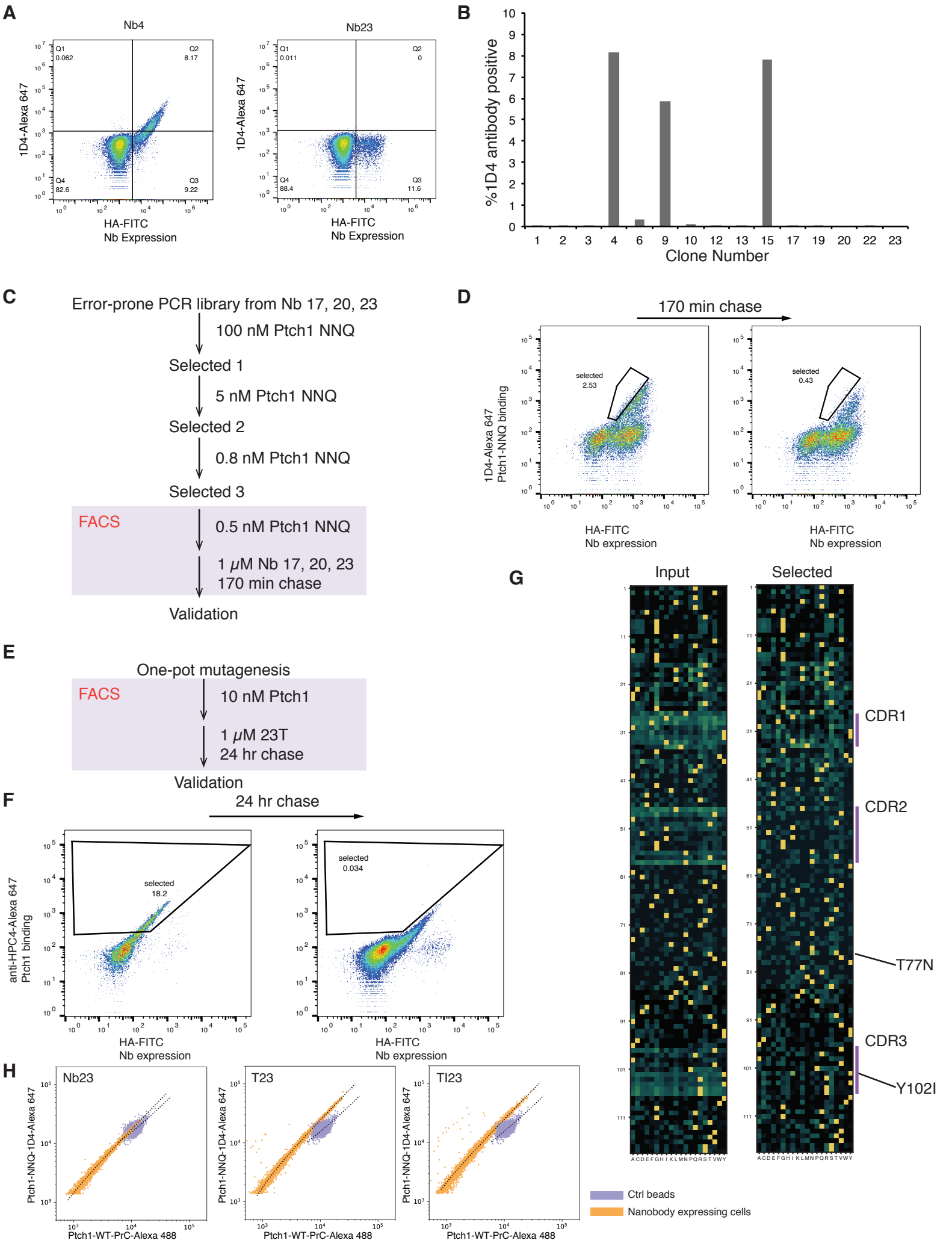
Mice All procedures were performed under Institutional Animal Care and Use Committee (IACUC)-approved protocol at Stanford University. Wild-type FVB/NCrl (207) mice were purchased from Charles River. Male mice at seven week-old age were randomly assigned to groups of predetermined sample size. All experiments with direct comparisons were performed in parallel to minimize variability. Hedgehog agonist SAG21k was delivered by osmotic pump (Alzet) over the course of two weeks at a dose of 2mg/kg/day.

Adeno-associated virus (AAV) production The backbones of all AAV plasmids were based on pAAV-EF1a-Cre (Addgene, 55636) with poly(A) signal replaced with bGH. Nanobody sequences were cloned into the vector for expression in infected cells. AAVs were generated in HEK 293T cells and purified by iodixanol (Optiprep, Sigma; D1556) step gradients as described (7). Virus titers were measured by quantifying DNase I–resistant viral genome with qPCR using a linearized viral genome plasmid as the standard. Purified virus was intravenously injected into anesthetized mice at 1 × 10^11 vg per mouse or other specifically indicated titer through the retroorbital sinus.

Histology Animals were euthanized and dorsal skin was excised for RNA extraction. Mice were then perfused with PBS and 4% paraformaldehyde (PFA) in PBS, and tongues and dorsal skin were post-fixed in 4% PFA for 24 hours. Tongues were processed for *in situ* hybridization according to RNAScope multiplex fluorescence kit (ACD systems) using mouse Gli1 probe (311001), followed by immunostaining as described (8). Immumofluorescence imaging was performed on laser scanning confocal microscopes (Zeiss LSM 800). Skin was processed for standard H&E staining by Animal Histology Service at Stanford University.

RNA extraction and qRT-PCR Skin samples were homogenized and extracted for RNA using TRIzol, followed by RNeasy Mini Kit (QIAGEN) and DNase Set (QIAGEN). *Gli1* and *Hprt1* levels were determined by one-step quantitative reverse transcriptase PCR (qRT–PCR) on an ABI 7900HT instrument using SuperScript III Platinum One-Step System with TaqMan Gene Expression Assays (Gli1, Mm00494654_m1; Hprt, Mm00446968_m1; Thermo Fisher). Normalized expression levels relative to control group were compared using ordinary one-way ANOVA tests with Dunnett's multiple comparison correction.

Fig. S1. Selection of nanobody. (A) Yeast cells expressing the initial clones were stained with the antibody used during FACS to ensure that the nanobody binds directly to PTCH1 protein. As summarized in B, Clones 4, 9 and 15 showed strong binding to the antibody and are thus false-positive clones during the selection. All the other clones were then purified and tested for activity on cells except for clone 13, which could not be expressed or purified from bacteria. (C) Flow chart of the first round of affinity maturation. Nanobody sequences from clone 17, 20 and 23 were mutagenized with error-prone PCR and transformed into yeast. After enriching for PTCH1 binding clones with MACS, the yeast cells are selected in FACS. In the final FACS steps, the cells were first incubated with PTCH1 to allow the nanobodies to bind and after wash, the cells were incubated with the parent nanobody proteins, to compete PTCH1 off the cell surface. FACS plots before and after the competitive chase are shown in D. The cells that retain binding to PTCH1 were selected by FACS. (E) Flow chart of the second round of affinity maturation. The sequence was mutagenized with one-pot mutagenesis and transformed into yeast. Yeast cells expressing the nanobody were selected in FACS with a similar competitive chase. The FACS plots before and after the competition were shown in F. (G) The amino acid sequences of the round 2 affinity maturation library were determined with MiSeq and are plotted here. The selection enriched for T77N and Y102I variants. (H) Yeast cells expressing Nb23, T23, or TI23 preferentially bind to PTCH1-WT over PTCH1-NNQ. OneComp beads that bind to all antibodies equally well were used as a control.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Q2
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Q4
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Q3
9.22
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Q2
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Q4
88.4
Q3
11.6
1D4-Alexa 647
HA-FITC
Nb Expression
B
%1D4 antibody positive
Clone Number
1 2 3 4 6 9 10 12 13 15 17 19 20 22 23
C
Error-prone PCR library from Nb 17, 20, 23
100 nM Ptch1 NNQ
Selected 1
5 nM Ptch1 NNQ
Selected 2
0.8 nM Ptch1 NNQ
Selected 3
FACS
0.5 nM Ptch1 NNQ
1 μM Nb 17, 20, 23
170 min chase
Validation
D
170 min chase
selected
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selected
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Ptch1-NNQ binding
HA-FITC
Nb expression
E
One-pot mutagenesis
FACS
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Validation
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24 hr chase
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Ptch1 binding
HA-FITC
Nb expression
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CDR2
T77N
CDR3
Y102I
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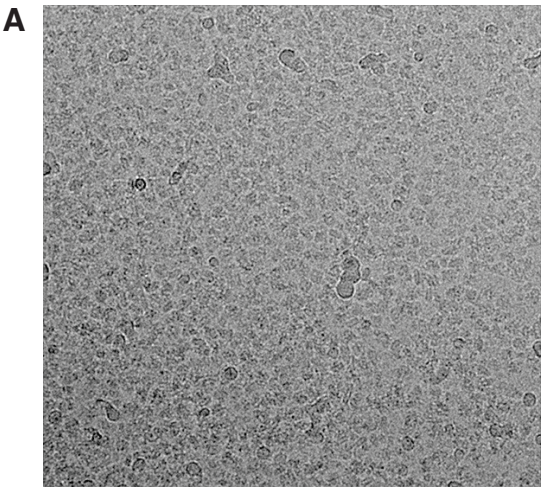
H
Nb23
T23
TI23
Ptch1-NNQ-1D4-Alexa 647
Ptch1-WT-PrC-Alexa 488
Ctrl beads
Nanobody expressing cells

Fig. S2. Cryo-EM data validation. (A) Protein particles are clearly visible in raw cryo-EM micrographs. (B) The parameters for contrast transfer function (CTF) are well fitted for this dataset. (C) 2D classification revealed clear views of PTCH1-TI23 complex. (D) Cryo-EM data processing was summarized in the flow chart here. All steps were carried out in cryoSPARC, except for the last local refinement step, which was performed with cisTEM. (E) The orientation of the particles is summarized in the spherical histogram here. Most particles are oriented along the equator of the protein. (F) The FSC curves of the final refinement were plotted here. The resolution of the final map is estimated to be 3.4 Å according to the 0.143 gold standard FSC. (G) Local resolution of the final reconstruction was estimated in cryoSPARC and shown in the 3D models here. Most regions were well resolved except for part of the nanobody.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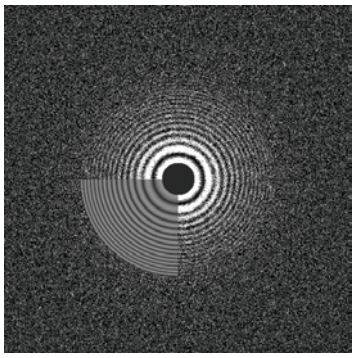
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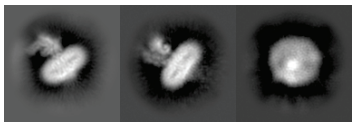
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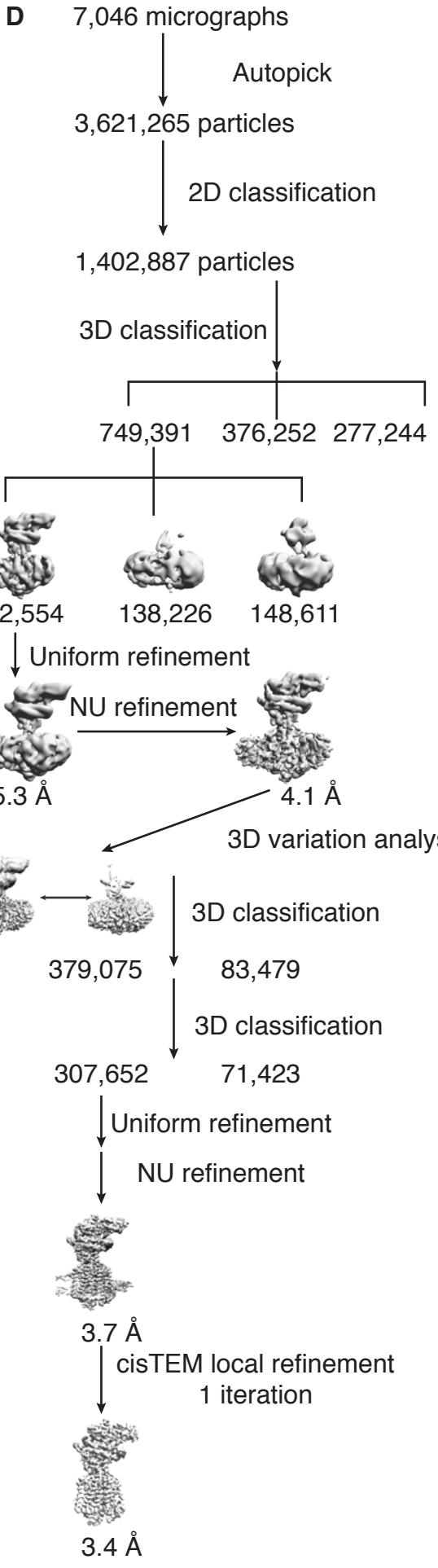
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138,226
148,611
Uniform refinement
NU refinement
5.3 Å
4.1 Å
3D variation analysis
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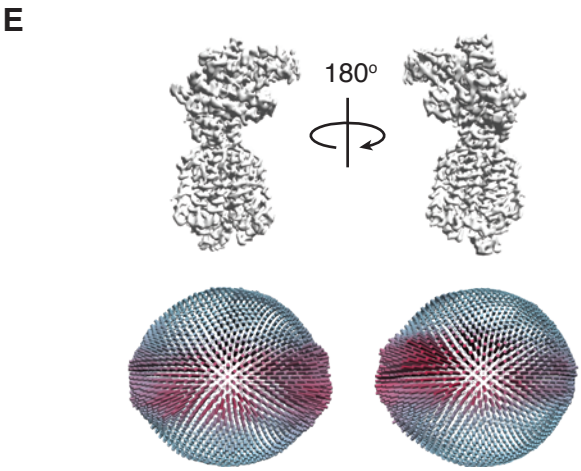
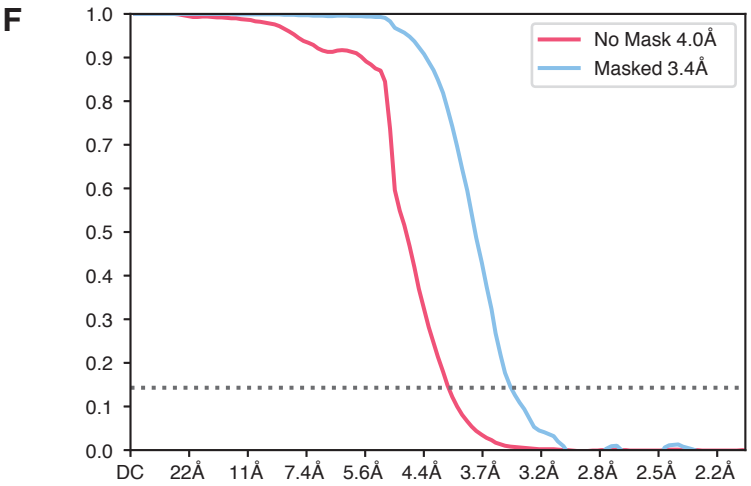
NU refinement
3.7 Å
cisTEM local refinement
1 iteration
3.4 Å


E
180°


F
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Masked 3.4Å
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0.5
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0.2
0.1
0.0
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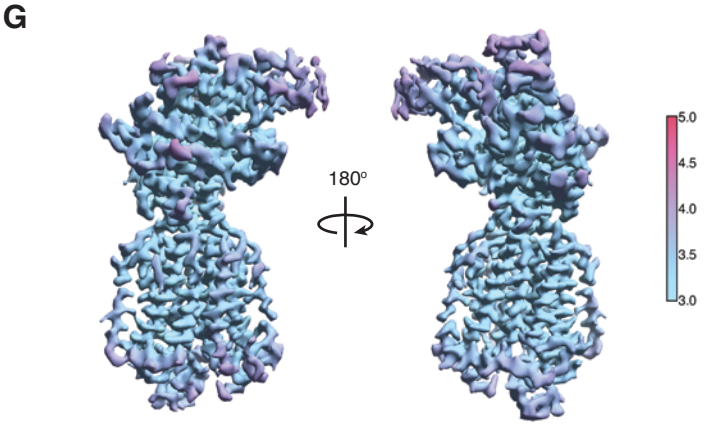
G
180°
5.0
4.5
4.0
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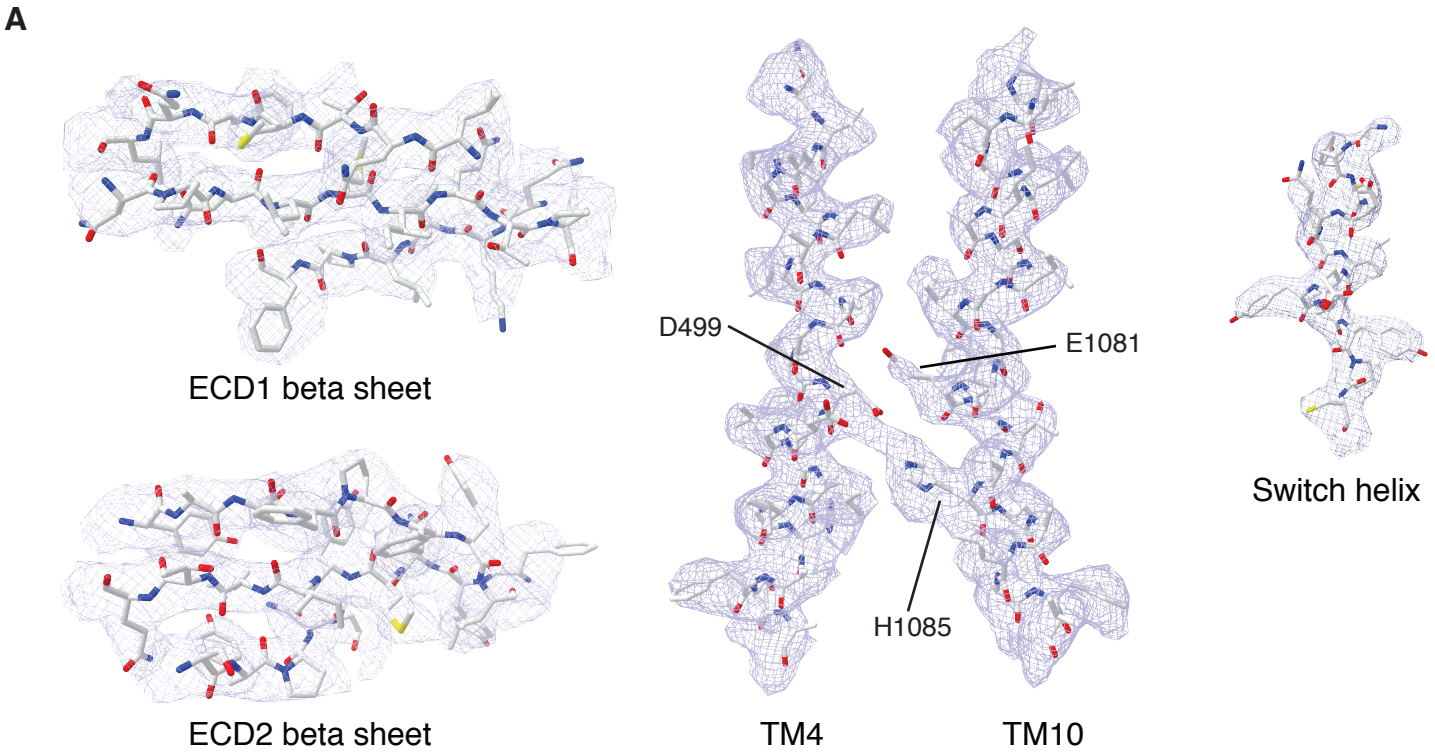
3.0

Figure S3. Features of the protein model. (A) The protein model fits the cryo-EM well. The high quality map enables confident modeling of not only alpha helical structures but also beta strands in the extracellular domain. Presence of clear side chain densities in the key transmembrane helices 4 and 10 enables modeling of the interaction of the key charged triad. (B) A large density present in the extracellular domain fits well with GDN and is thus likely to be a bound GDN molecule. (C) The model fits well with the cryo-EM map, as indicated by the model-map FSC curves. (D) The interactions between TM4 and TM10 are distinct between the TI23 bound murine PTCH1 structure (left) and the SHH-bound human PTCH1 structure (right; H1099, E1095, and D513 correspond to murine residues H1085, E1081, and D499).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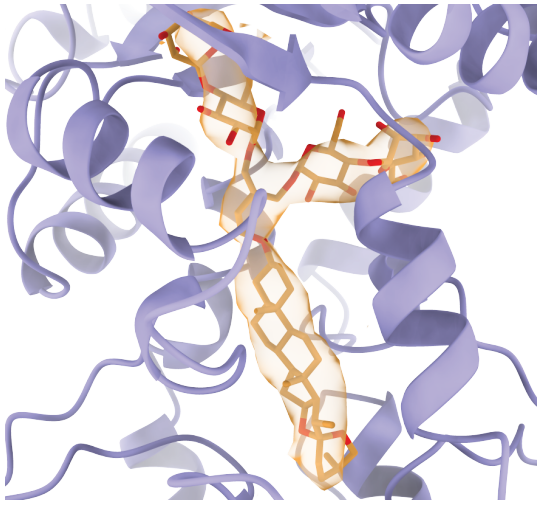
ECD2 beta sheet
D499
E1081
H1085
TM4
TM10
Switch helix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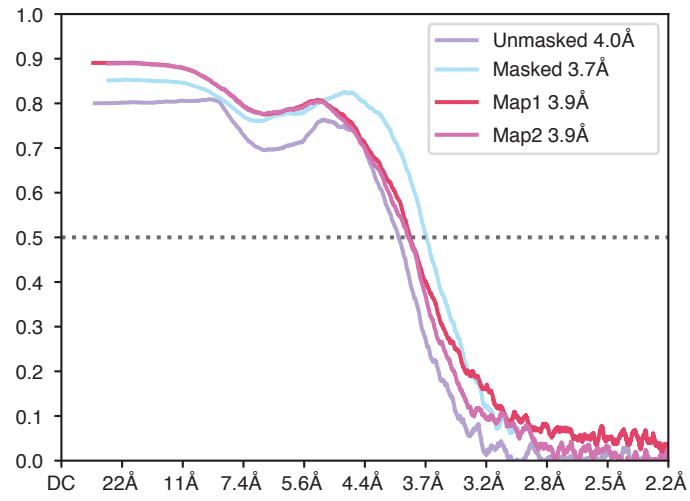

C
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DC
22Å
11Å
7.4Å
5.6Å
4.4Å
3.7Å
3.2Å
2.8Å
2.5Å
2.2Å
Unmasked 4.0Å
Masked 3.7Å
Map1 3.9Å
Map2 3.9Å

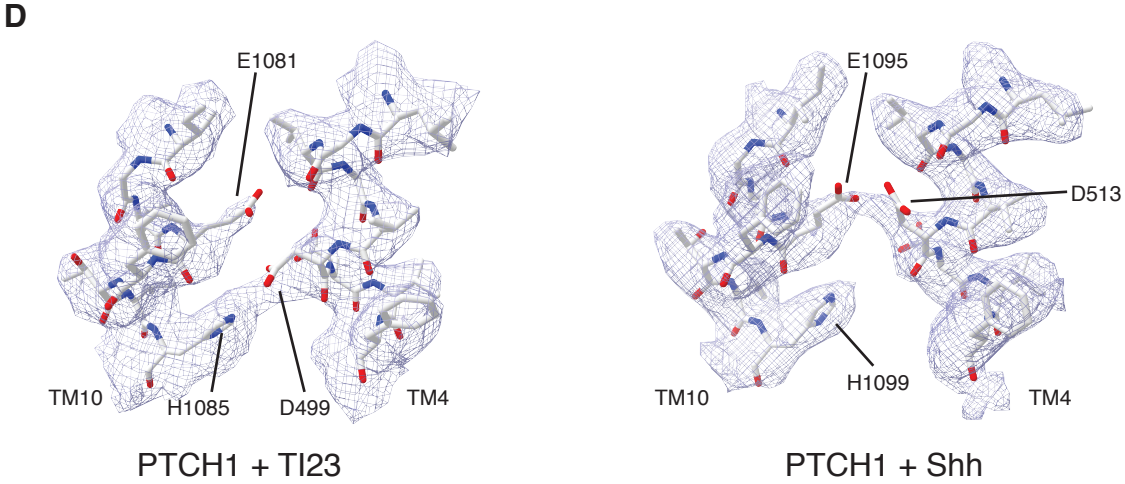
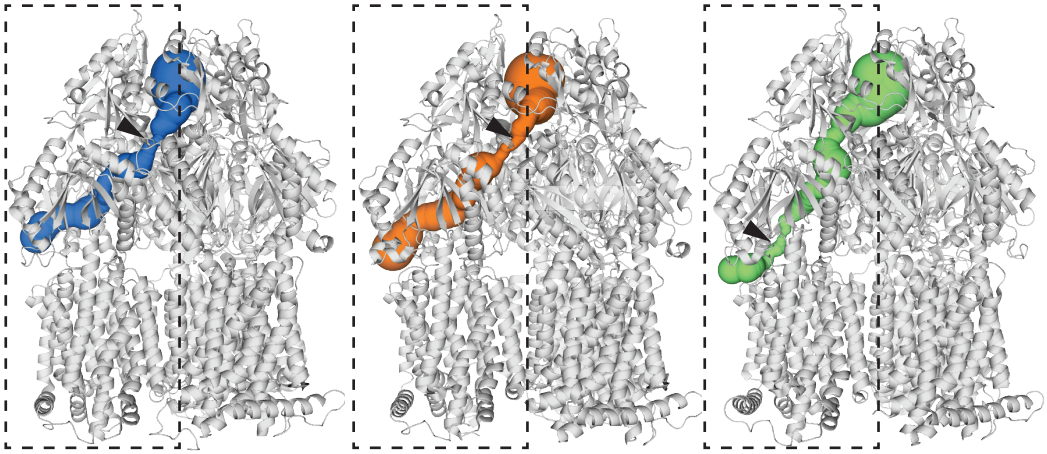

D
E1081
TM10
H1085
D499
TM4
PTCH1 + TI23
E1095
D513
TM10
H1099
TM4
PTCH1 + Shh

Figure S4. Comparison of AcrB and PTCH1 conformational changes (A) Two distinct sites (marked by triangles, one lower site close to the membrane plane and one upper site close to the upper exit of the extracellular domain) alternatively open and close in three distinct conformations of AcrB (PDB ID: 2gif, L state shown in chain A, T state shown in chain B, O state shown in chain C). (B) A single site distal to the membrane alters conformation in known PTCH1 structures. PTCH1:TI23 and PTCH1 alone (6mg8) are shown here as examples.

A

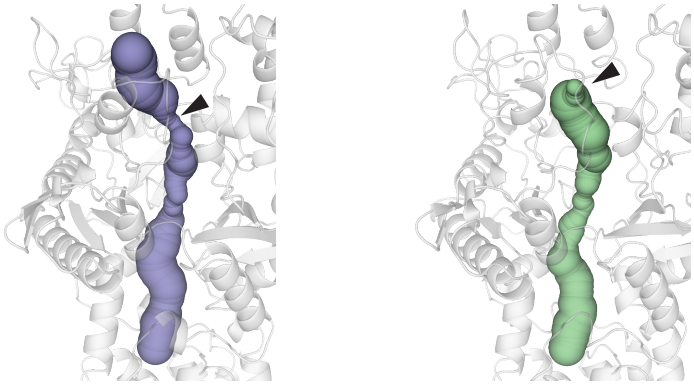


AcrB L state
2gifA

AcrB T state
2gifB

AcrB O state
2gifC

B



PTCH1::TI23

PTCH1

Table S1. Summary of cryo-EM data collection and model refinement

Data collection/processing	
Voltage (kV)	300
Magnification	22,500
Defocus range (μm)	-1.0 – -3.0
Pixel size (Å)	1.059
Total electron dose ($e^-/Å^2$)	38
Exposure time (s)	12
Number of images	7046
Number of frames/image	60
Initial particle number	3,621,265 (autopick)
	1,402,887 (2D select)
Final particle number	307,652
Resolution (unmasked, Å)	4.0
Resolution (masked, Å)	3.4
Refinement	
Composition	
Number of atoms	8796
Number of residues	1120 (protein)
Ligands	NAG: 7
	Q7G: 4
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.698
Ramachandran	
Favored (%)	97.13
Allowed (%)	2.87
Outlier (%)	0.00
Clash score	8.32
Rotamer outliers (%)	0.00
Cβ outliers (%)	0.00
Peptide plane (%)	
Cis proline/general	2.1/0.0
Twisted proline/general	0.0/0.0
Molprobity score	1.61
EMRinger score	2.44

SI References

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