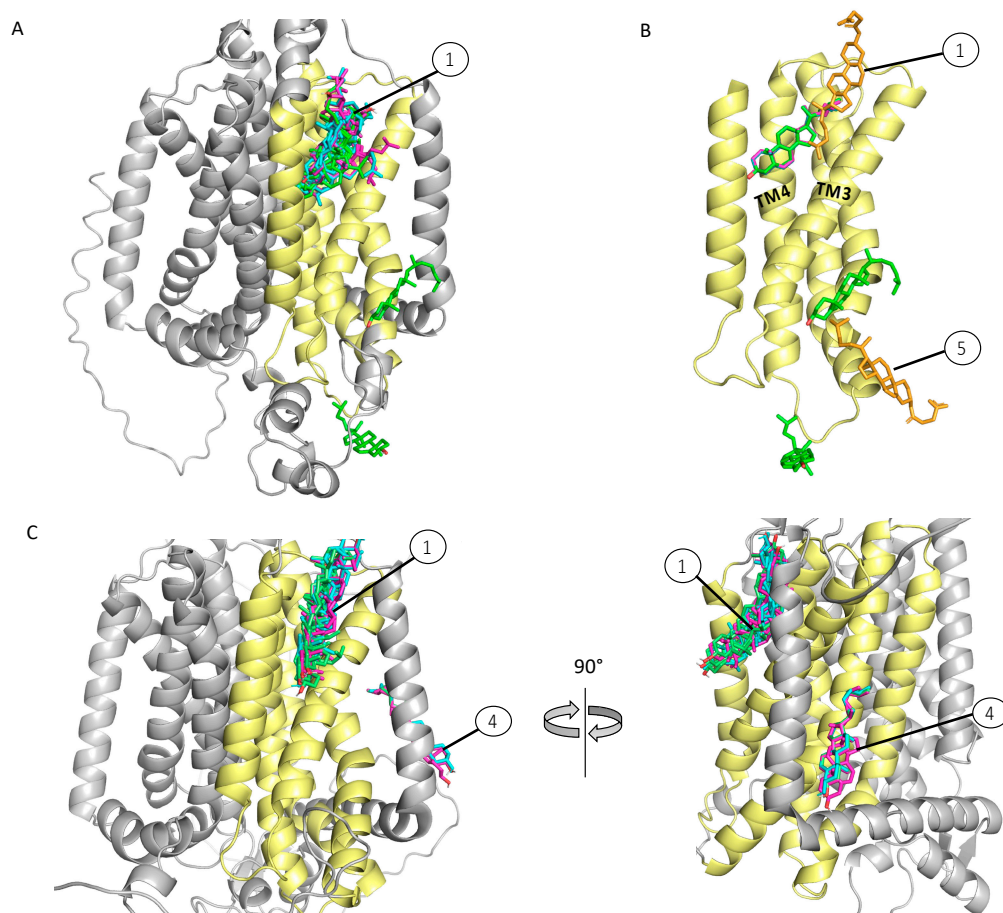


**S1 Optimization of PTCHD1 purification.** **A** Purification of PTCHD1 in either PBS or Tris-HCl. A clear band is visible in the elution fraction of the PBS purification, whereas no band is visible in the Tris-HCl elution fraction. **B** PTCHD1 purification following solubilization in 0.2 % GDN for either 2 h or overnight at + 4 °C. **C** Densitometry analysis shows a modest increase in o/n solubilization compared to 2 h solubilization (1.15 -fold increase), and a 1.9 -fold increase in eluted PTCHD1. **D** Clear differences in the intensity of PTCHD1 elution bands (~100 kDa) after altering the resin amount, detergent amount or detergent type. **E** Densitometry analysis shows a 1.5 -fold increase in eluted PTCHD1 when the resin amount was doubled, and 2.7 -fold increase when the amount of GDN used to solubilize PTCHD1 was increased to 0.8 %. Using 1 % DDM supplemented with 0.125 % CHS led to a decrease in eluted PTCHD1.



S2 Mass spectrometry sequence coverage of purified PTCHD1.

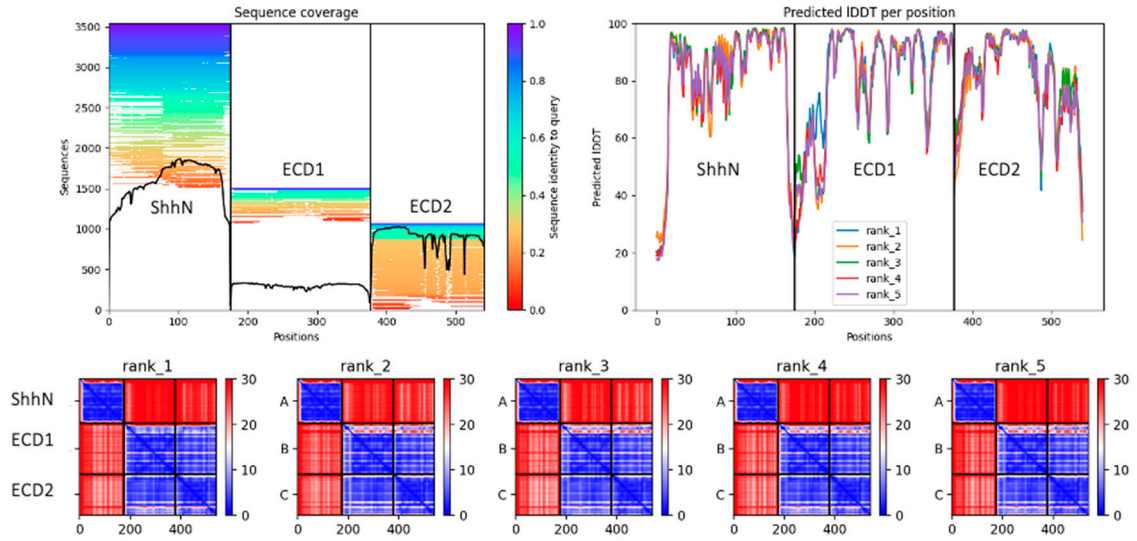


**S3 Docking of cholesterol to PTCHD1 and PTCHD1 indicates similar binding.** Docking was performed in triplicates, cholesterol from the first, second, and third runs are in cyan, lime, and pink, respectively. Sites are numbered as in Qi et al. (2018)[28] **A** All cholesterol docked to PTCHD1. 25/27 cholesterol were docked to a position corresponding to site 1. **B** The cholesterol with highest affinity to PTCHD1 (lime, top) overlaps with PTCH1 (6RMG) cholesterol site 1 (orange, top). Two outlying cholesterol were docked near PTCH1 cholesterol position 5 (orange, bottom) **C** Cholesterol dock to two identified sites on PTCH1 (AF-Q13635). Sites 1 and 4 are occupied by 23 and 4 cholesterol, respectively.

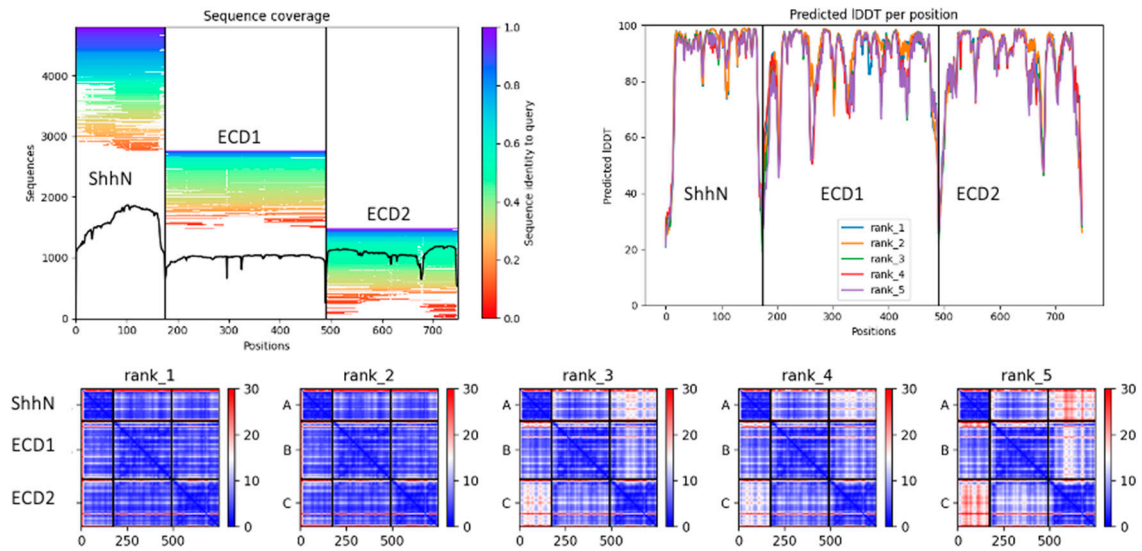
Supplementary table 1 Docking results

TARGET	RUN	MODE	AFFINITY (KCAL/MOL)	SITE	TARGET	RUN	MODE	AFFINITY (KCAL/MOL)	SITE
PTCHD1	1	1	-8,6	1	PTCH1	1	1	-8,4	4
PTCHD1	1	2	-8,6	1	PTCH1	1	2	-8,3	1
PTCHD1	1	3	-8,0	1	PTCH1	1	3	-8,1	1
PTCHD1	1	4	-7,7	1	PTCH1	1	4	-8,1	1
PTCHD1	1	5	-7,7	1	PTCH1	1	5	-8,0	1
PTCHD1	1	6	-7,7	1	PTCH1	1	6	-8,0	1
PTCHD1	1	7	-7,6	1	PTCH1	1	7	-7,6	1
PTCHD1	1	8	-7,5	1	PTCH1	1	8	-7,6	1
PTCHD1	1	9	-7,4	1	PTCH1	1	9	-7,6	1
PTCHD1	2	1	-8,6	1	PTCH1	2	1	-8,3	4
PTCHD1	2	2	-8,0	1	PTCH1	2	2	-8,2	1
PTCHD1	2	3	-7,6	1	PTCH1	2	3	-8,0	1
PTCHD1	2	4	-7,5	1	PTCH1	2	4	-7,9	1
PTCHD1	2	5	-7,5	1	PTCH1	2	5	-7,7	1
PTCHD1	2	6	-7,4	1	PTCH1	2	6	-7,7	1
PTCHD1	2	7	-7,4	1	PTCH1	2	7	-7,6	1
PTCHD1	2	8	-7,2	5	PTCH1	2	8	-7,5	1
PTCHD1	2	9	-7,2	5	PTCH1	2	9	-7,3	1
PTCHD1	3	1	-8,7	1	PTCH1	3	1	-8,1	4
PTCHD1	3	2	-8,0	1	PTCH1	3	2	-8,1	1
PTCHD1	3	3	-7,7	1	PTCH1	3	3	-7,8	1
PTCHD1	3	4	-7,7	1	PTCH1	3	4	-7,7	1
PTCHD1	3	5	-7,7	1	PTCH1	3	5	-7,6	1
PTCHD1	3	6	-7,5	1	PTCH1	3	6	-7,5	1
PTCHD1	3	7	-7,5	1	PTCH1	3	7	-7,4	1
PTCHD1	3	8	-7,5	1	PTCH1	3	8	-7,4	1
PTCHD1	3	9	-7,3	1	PTCH1	3	9	-7,3	4

**A**



**B**



**S4 AlphaFold2 prediction of ECD1:ECD2:ShhN complex formation.** (A) PTCHD1 ECDs do not form a complex with ShhN. The sequence coverage is acceptable throughout and the predicted local distance difference test (IDDT) score is above 50 excluding N-terminals of the ShhN and ECD1, and C-terminal of ECD2. The predicted aligned error is high in all ranks between both ECDs and ShhN. (B) PTCH1 ECDs form a complex with ShhN. The sequence coverage is acceptable throughout and the predicted IDDT is above 50 excluding N-terminals of the ShhN and ECD1, and C-terminal of ECD2. The predicted aligned error is low throughout in ranks 1 and 2, and in most positions of rank 4. In rank 5 the predicted aligned error is high between ShhN and ECD2.



Consensus	XXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXLHRXXXXX	58
Q13635 PTCH1_HUMAN	MASAGNAAEPPDRGGGGSGC	IGAPGRPAGGGRRRTGGLR	RAAAPDRDYLHRPSYCD	58
Q96NR3 PTCHD1_HUMAN	ML-----RQVLHRG----			9
Consensus	XX	XXXXXXXXXXXXXXXXXXLR	XXXXXXXXXXLGXXIXXXXXX	116
Q13635 PTCH1_HUMAN	AF	ALEQISKGKATGRKAPLWLR	AKFQRLLFKLGCTQKNCGK	116
Q96NR3 PTCHD1_HUMAN	--	-----LR	TC----FSRLGHFASHPVF	43
Consensus	XXXX	XXXXXXXXXXXXXXXXXXEX	XXXXXXXXXXXXXXXXXXJ	174
Q13635 PTCH1_HUMAN	KAAN	LETNVEELWVEVGGRVSR	ELNYTRQKIG-EEAMFNPQLMI	173
Q96NR3 PTCHD1_HUMAN	SRYQ	VESEVHLLAPQHS	LAKIERNLVNSLFFVNRSKHRLYS	101
Consensus	XXXXXL	XXXXXXXXXXLXXXXXXXXXXV	XXXXXXXXXXXXHJCXXXXXX	232
Q13635 PTCH1_HUMAN	ALLQHL	DSA-----I-----QASRVHV	YMYNRQWKLEHLQYKSGELI	222
Q96NR3 PTCHD1_HUMAN	QKANML	QDHHTDLILKHA	AVTKIQVPRPGFNITFAHILNND--	155
Consensus	XXXXXXXX	XXXXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXXXXXXLEELKX	290
Q13635 PTCH1_HUMAN	LYPELIIT	PLDCFWEQAKLQSGTAYLLG	KPPLRWTNFDPLEFLLEELKK	280
Q96NR3 PTCHD1_HUMAN			-----VLEELKNAR-----	164
Consensus	XXXXXXXXXX	XXXXXXXXXXXXXXXXXXXXXN	XXXXXXXXXXTXDXDXVX	348
Q13635 PTCH1_HUMAN	MLNKA	EVGHGYMDRPLN	PADPCPATAPNKN-----	331
Q96NR3 PTCHD1_HUMAN	-----	-----ATN	RTNFAITYPIITHLKDGRAVY	189
Consensus	XXXXXXXXXXXX	GGXXXXXXXSAXAJQXX	XXLXXXXXXXXXXXXXXXXXX	406
Q13635 PTCH1_HUMAN	RKYMHWQ	EELIVGGTVKNSTGKLVSAH	ADTMFOIMTPKQMYEHFKGYEYVS	389
Q96NR3 PTCHD1_HUMAN	-----HQL	GGVTVHSKDRVKS	AEATQLTYYQSINSLNDM-----	224
Consensus	XXXXXXXXXX	XXXXXXXXXXXXX	XXXXXXXXXXXXX	464
Q13635 PTCH1_HUMAN	DKAAAIL	EAQRTYVEV	VHQSAQNSTQKVLSTTTTDDILKSFSDVS	447
Q96NR3 PTCHD1_HUMAN	-----VAERWESSF	CDTVRLFKSNSKVMYPYT	SSSLREDFQKTRSRV	277
Consensus	LXXXXXALXXXXXDCX	KSXXXXXGLXGXXXXX	LXXXXX	522
Q13635 PTCH1_HUMAN	IMLAYACITMLRWDCS	KSOQAVGLAGVLLVALSVAA	GLCLCSHIGISFNAATTQVL	505
Q96NR3 PTCHD1_HUMAN	LVVTMAITCCSMQDCV	RSKPWLGLLGLVTISLATLT	AAGIINTGKYNSTF-LGV	334
Consensus	XXLGXGXXXXX	XXXXXX	XXXXXXXXXXXXX	580
Q13635 PTCH1_HUMAN	LALGVGVDDVFLAHAFS	ETGQNKRI	PFEDRTGECLKRTGASVALTSISNVTA	563
Q96NR3 PTCHD1_HUMAN	VMLGHGLYGTTEMLSSWR	KTR	EDQHV--KERTAAVYAD	390
Consensus	XXXXXXXXIXAKRFXXXXXXX	XFNXXXXVLXXXXX	LXXXXX	638
Q13635 PTCH1_HUMAN	--LIP	IPALRAESLQA	AVVVENFAMVLLIFPAISMDLY	619
Q96NR3 PTCHD1_HUMAN	SPFTNIEAARIECCNSCIAI	FENLYVL	SFYGSSLVFTGYIENNYQHSIFCRKV-P-K	446
Consensus	XX	XJQXXPXXXXX	XXXXXXXXXXXXXXXXXXXX	696
Q13635 PTCH1_HUMAN	SR	VIOVEPQMTDTHDNTRYSP	PPYSSHSFAHETQITMQST	677
Q96NR3 PTCHD1_HUMAN	PE	ALQEKPAWRF	-----VQLRTEYDPHTHYVT	461
Consensus	TXXX	XXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXX	754
Q13635 PTCH1_HUMAN	TAEP	RSEISVQPVTVTQDTLSCQS	PESTSSTRDLSQFSDSLH	734
Q96NR3 PTCHD1_HUMAN	T---	-----	ARFSEDTAEGEEANTYESHLVVC	485
Consensus	FXXXXX	XXXXJXXXXXXVXXXXLXX	JXXXXLXGXXXXVXXGDXLXB	812
Q13635 PTCH1_HUMAN	BAEKHY	APFLKPKAKVVVIFLFLGL	LGVSLYGTTRVRDGLDITDI	792
Q96NR3 PTCHD1_HUMAN	BLKRYV	CDWITNTYVKPFVLFYLIY	ISFALMGLYQVSEGS	543
Consensus	AQXYFSX	XXXXIXXXXXX	XXXXXXXXXXXXX	870
Q13635 PTCH1_HUMAN	AQFKYFSF	YNYMI--VTQKADY	PNIQHLLYDLHRSFSNVKYVMLEEN	846
Q96NR3 PTCHD1_HUMAN	AQQKYFSN	YSPVIGFYIYESIEYWN	-----TSVQEDVLE	589
Consensus	XXXBXLXXXX	XXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXLVXTGXXXXX	928
Q13635 PTCH1_HUMAN	YFRDWLQGLQ	DAFDSDWETGKIMPNNYKNG	SDDGVLAYKLLVQTSRDKP	904
Q96NR3 PTCHD1_HUMAN	SYLNYLRK--	-----	LNVTGLPKK--NFTDMLR	614
Consensus	XXXXX	XXXXXXXXXXXXX	XXXXXXXXXXXXX	986
Q13635 PTCH1_HUMAN	QRLVDADGIINP	SAFYIYLTAWVSN	DPVAYAAQANIRPHRPFWVHDKADYM	962
Q96NR3 PTCHD1_HUMAN	NSFLKAPQFSHF	QEDIF	S--KKYNDVDVVASRMFLVAKTMTNREELYDL	670
Consensus	JXXXXXXXXXXFX	XXXXXXXXXXXXXXXXXXXX	XXXXXXXXXXXXX	1044
Q13635 PTCH1_HUMAN	IPAAEPIEYAE	PFYLNGLRDTSDFVEAIEKVRT	ICSNYTSLSGLSSYPNGYPFL	1020
Q96NR3 PTCHD1_HUMAN	LSVTSKVKFIV	---	-----NPSFVYMDRVA	703
Consensus	XXXXXXXXXX	XXXXXXXXXX	XXXXXXXXXX	1102
Q13635 PTCH1_HUMAN	YIGLRHLLISVVL	ACTFLVCAVFLNPWTAGII	VMVLALMTVE--LFCMGLI	1076
Q96NR3 PTCHD1_HUMAN	CI--SAFLLEFSA--	---	FLV-ADSLINVTITL--TVVSV	747
Consensus	XLXXXXXX	XXXXXXXXXX	XXXXALEXXXXXX	1160

**S5 Structure based sequence alignment PTCH1 and PTCHD1.** ECD1 and ECD2 loops participating in ShhN binding are highlighted in turquoise and purple, respectively. Residues that participate in hydrogen bonds with ShhN in PTCH1 (6RMG) are indicated with dots. Conserved proline, identified as a key residue for PTCH1 and NPC1 function, is highlighted in yellow.