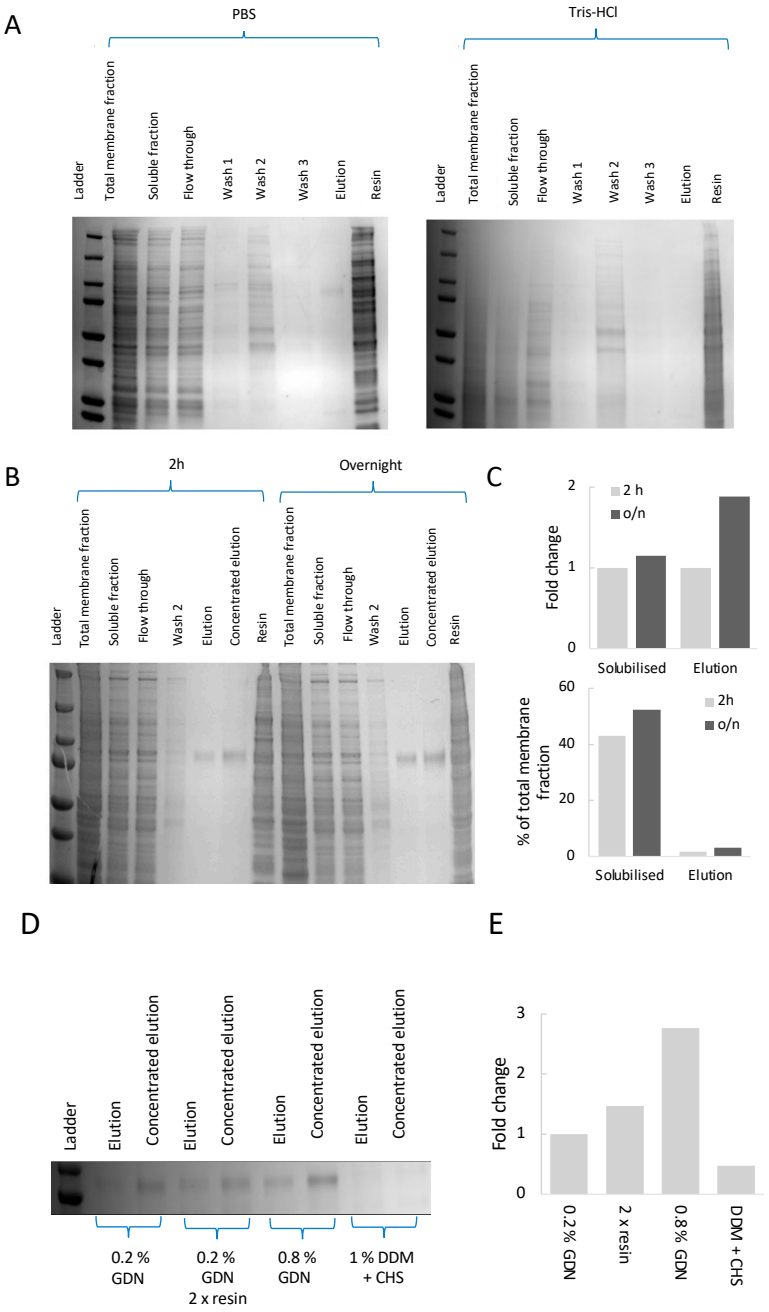
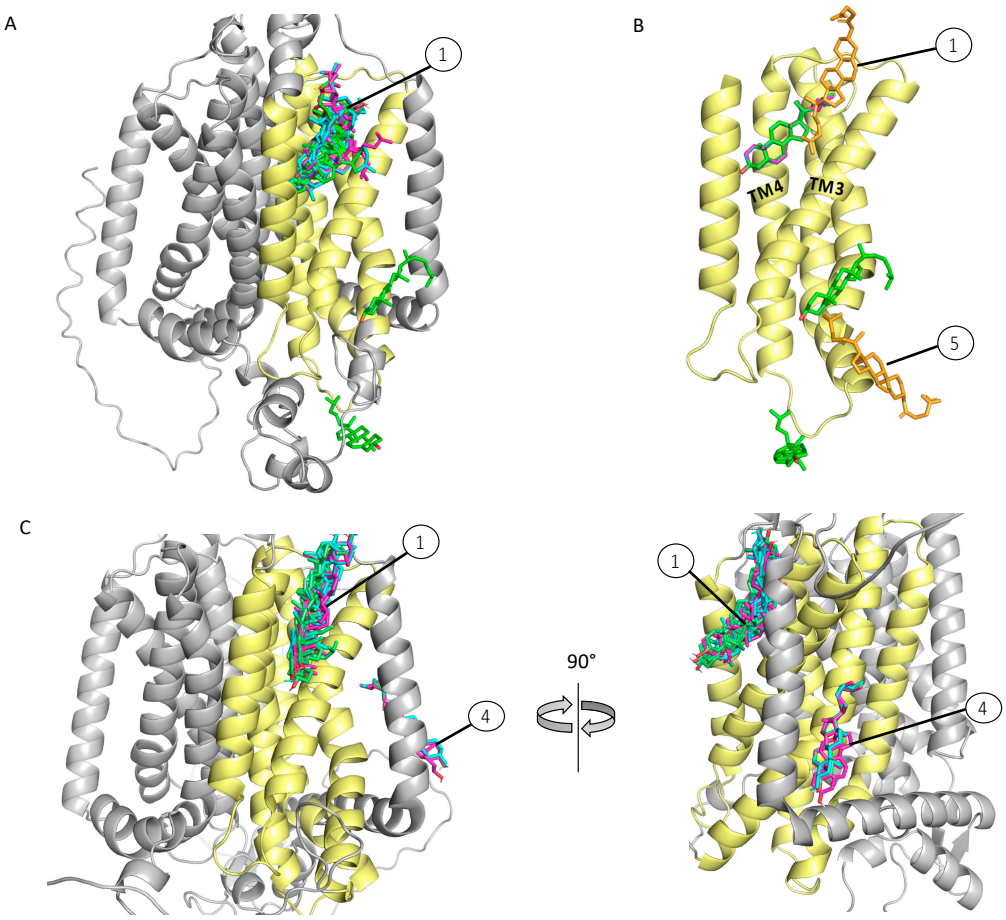


S1 Optimization of PTCHD1 purification. A Purification of PTCHD1 in either PBS or Tris-HCl. A clear band is visible in the elution fraction of the PBS purification, whereas no band is visible in the Tris-HCl elution fraction. **B** PTCHD1 purification following solubilization in 0.2 % GDN for either 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.

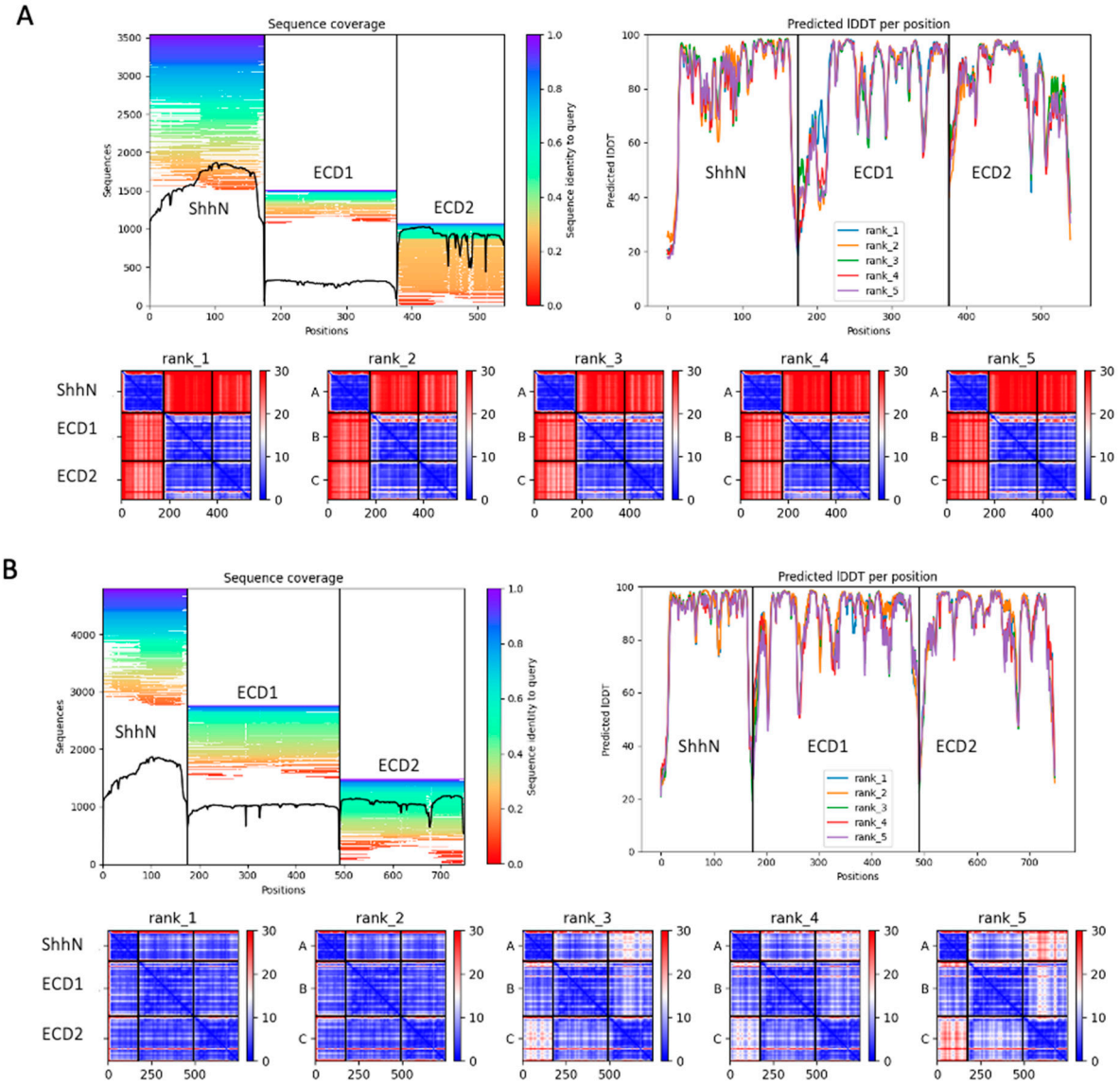
S2 Mass spectrometry sequence coverage of purified PTCHD1.



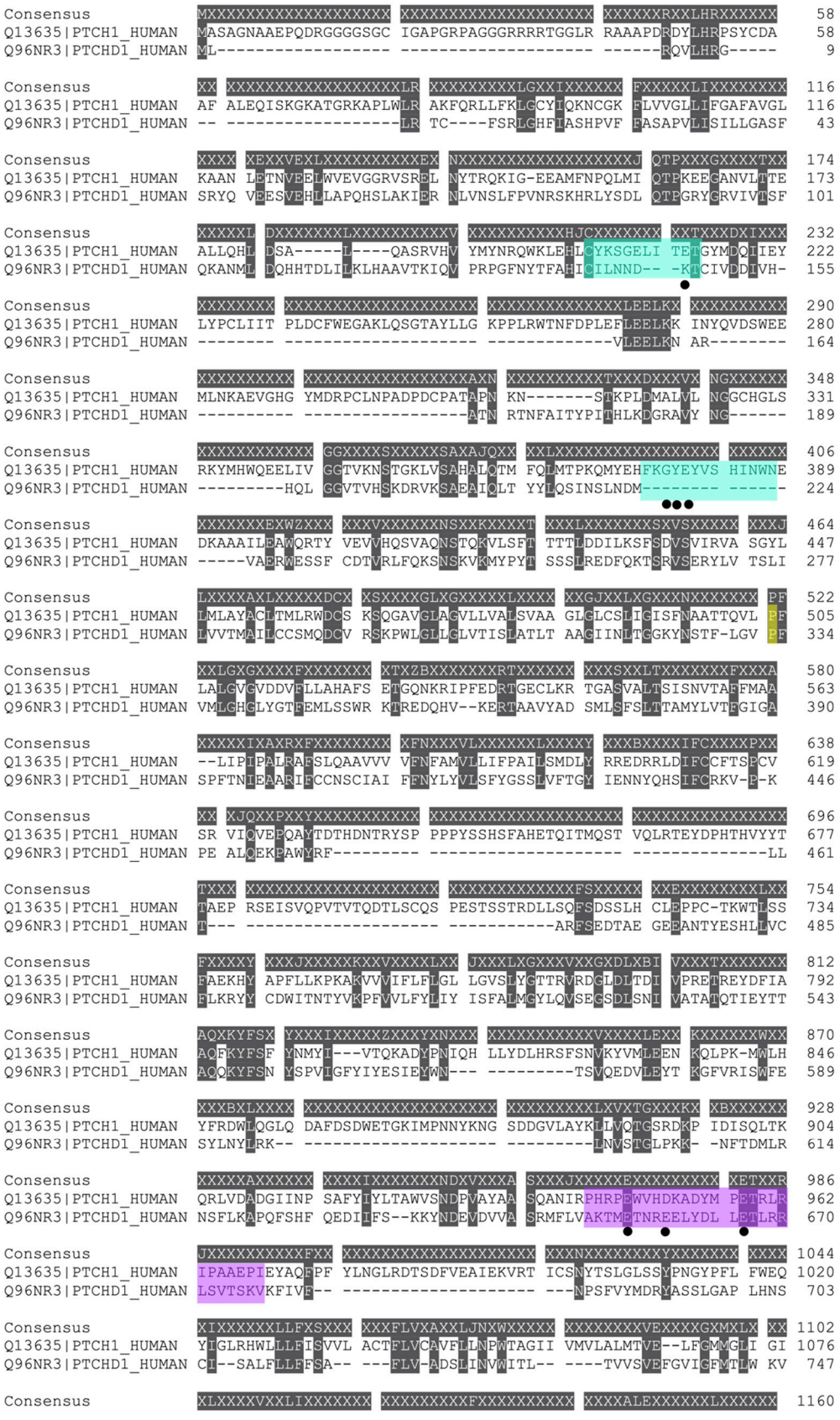
S3 Docking of cholesterol to PTCHD1 and PTCHD1 indicates similar binding. Docking was performed in triplicates, cholesterols from the first, second, and third runs are in cyan, lime, and pink, respectively. Sites are numbered as in Qi et al. (2018)[28] **A** All cholesterols docked to PTCHD1. 25/27 cholesterols 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 cholesterols were docked near PTCH1 cholesterol position 5 (orange, bottom) **C** Cholesterols dock to two identified sites on PTCH1 (AF-Q13635). Sites 1 and 4 are occupied by 23 and 4 cholesterols, 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



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 (lDDT) 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 lDDT 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.



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.