Supporting Information for

Conformation switch of NPC1 epitomizes a luminal alternating access for nonpolar substrates

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Figures

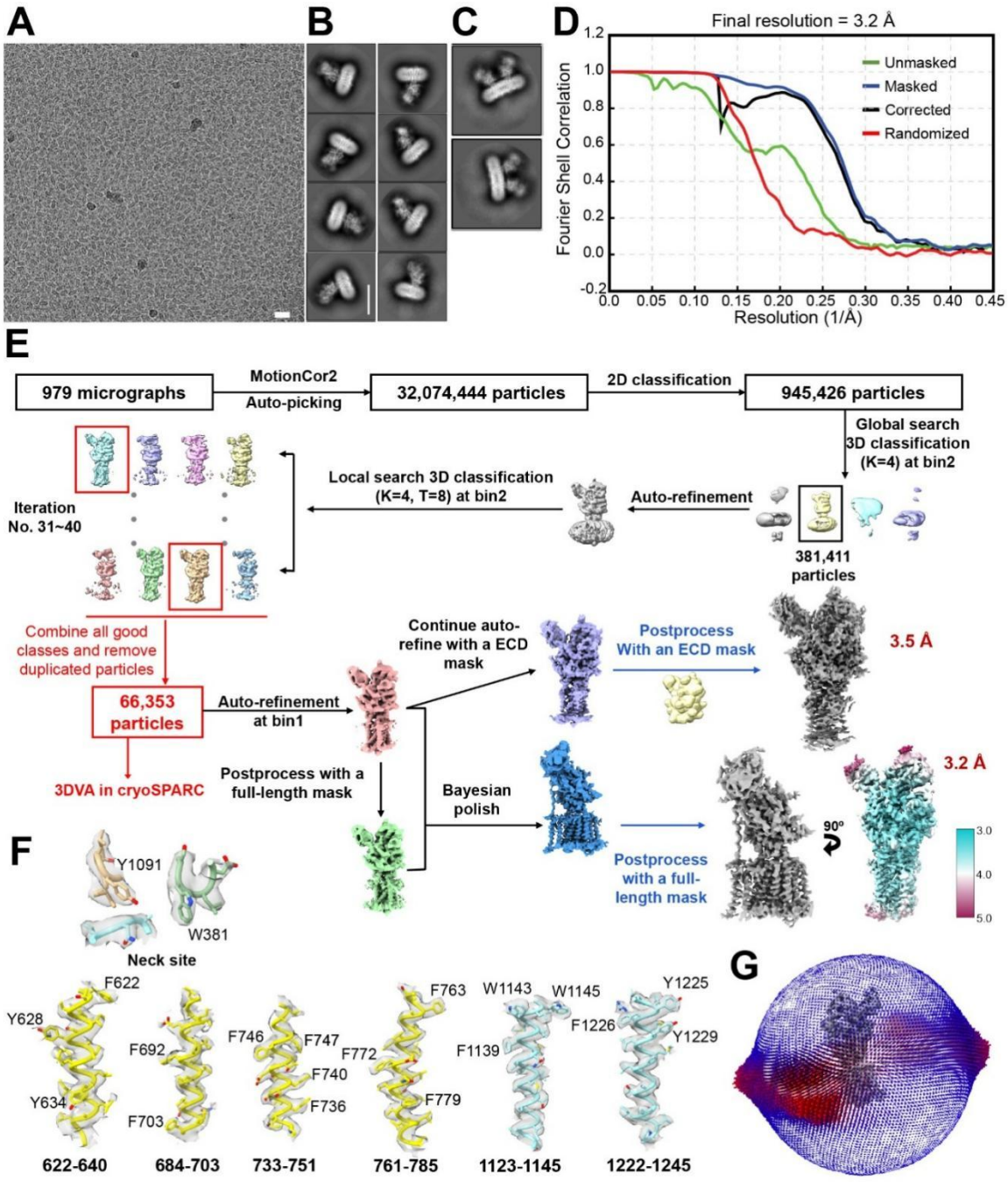


Fig. S1. Cryo-EM analysis of the bio-sterol bound NPC1-NPC2 complex. A. Representative raw micrograph; scale bar, 22 nm. **B.** Representative 2D averages for monomeric NPC1-NPC2 complex; scale bar, 11 nm. **C.** Representative 2D averages of the putative dimeric NPC1-NPC2 complex. **D.** FSC curves of the final map, determining the structure at 3.2 Å. **E.** Processing flowchart of the dataset, with most work done in RELION (1) and cryoSPARC (2). **F.** EM maps for representative elements in the bis-sterol bound NPC1-NPC2 complex. **G.** Angular distribution of particles constituting the final map.

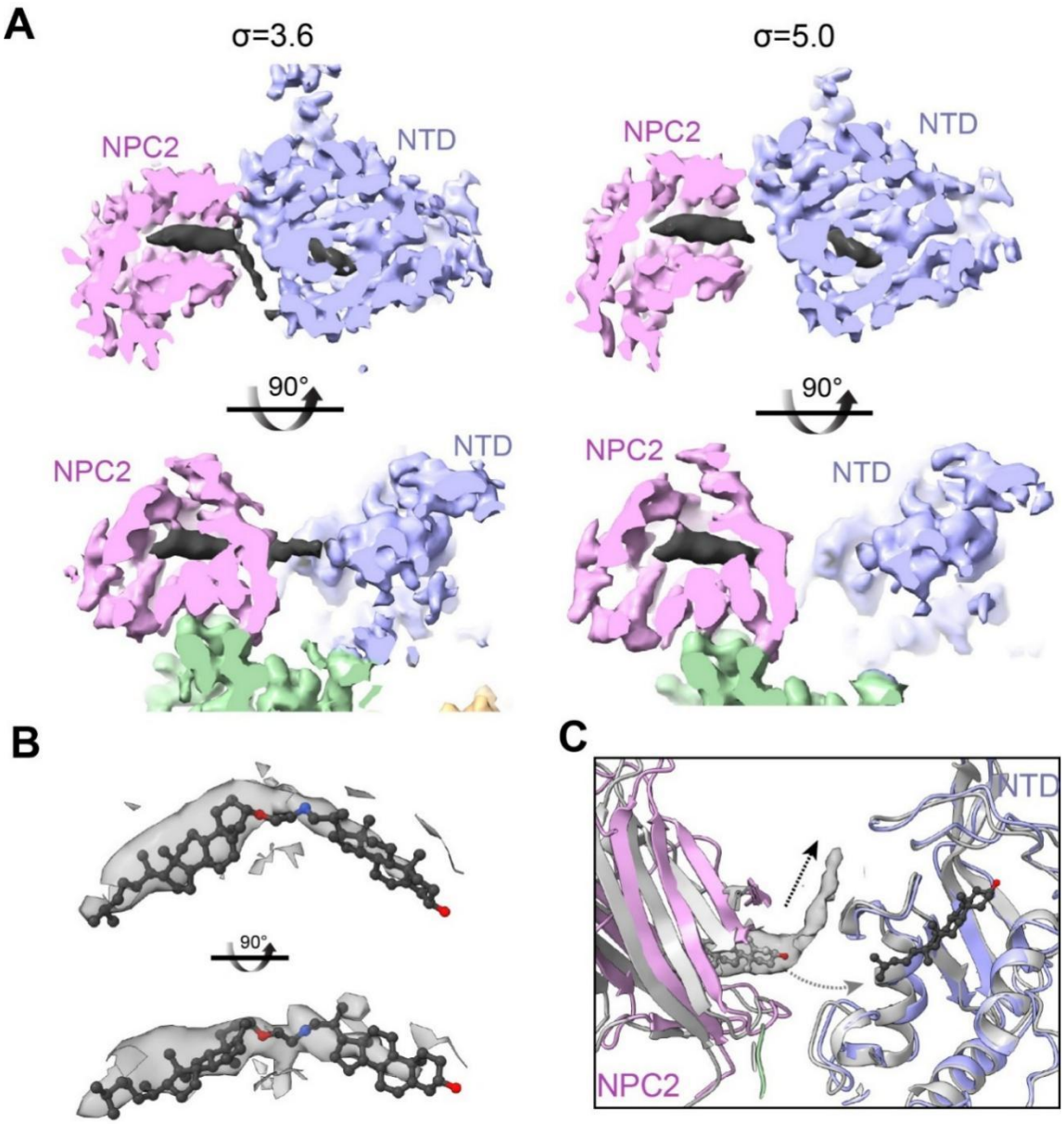


Fig. S2. A putative bis-sterol density in the ECD. A. Top and side views of NPC2-NTD density at σ = 3.6 and 5.0, with the former showing a swayed tail of the sterol density (black) and the latter cut off at the NPC1-engulfed steroid nucleus. **B.** Rough fitting of JM046 in the elongated sterol density at σ = 3.6. **C.** Molecular models showing the bis-sterol density relative to the NTD sterol-binding pocket. Black dotted arrow: JM046 orientation; grey dotted arrow: the path from NPC2 to the pocket entrance of NTD.

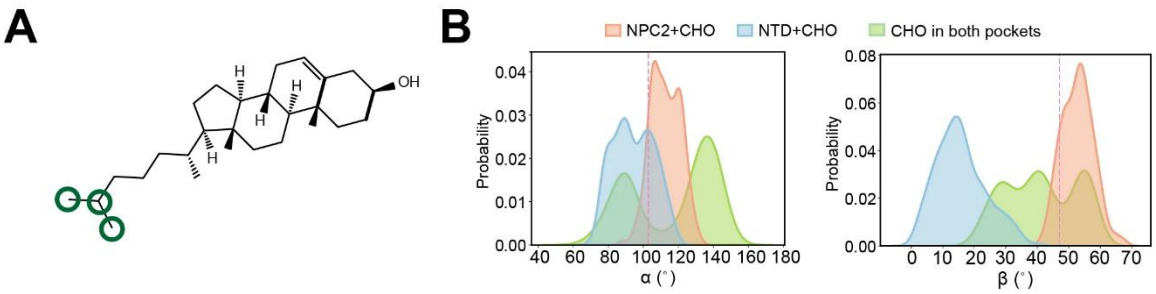


Fig. S3. MD simulation supplement. A. Definition of the "tail" of cholesterol circled in dark green: C25, C26 and C27**. B.** The distribution of α and β angles when both the NTD and NPC2 pockets (NPC2+CHO&NTD+CHO) are occupied by cholesterol molecules (green), the other two conditions are displayed for comparison.

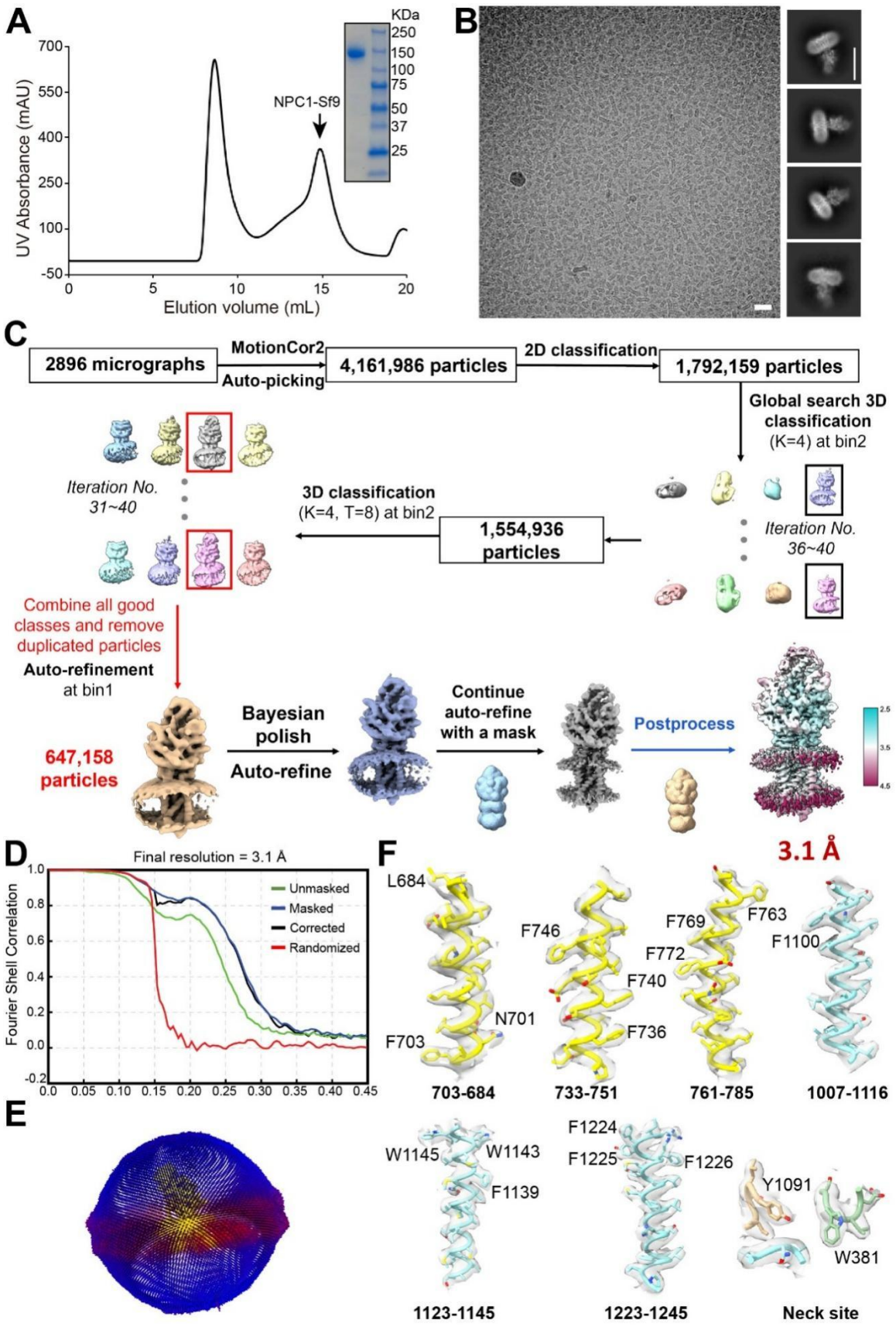

A
KDa
250
150
100
75
50
37
25
NPC1-Sf9
UV Absorbance (mAU)
700
550
400
250
100
-50
0
5
10
15
20
Elution volume (mL)
B
C
2896 micrographs
MotionCor2
Auto-picking
4,161,986 particles
2D classification
1,792,159 particles
Global search 3D classification
(K=4) at bin2
Iteration No.
31~40
Iteration No.
36~40
3D classification
(K=4, T=8) at bin2
1,554,936 particles
Combine all good classes and remove duplicated particles
Auto-refinement
at bin1
647,158 particles
Bayesian polish
Auto-refine
Continue auto-refine with a mask
Postprocess
2.5
3.5
4.5
D
Final resolution = 3.1 Å
Fourier Shell Correlation
Unmasked
Masked
Corrected
Randomized
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.0
0.05
0.10
0.15
0.20
0.25
0.30
0.35
0.40
0.45
E
F
3.1 Å
L684
F746
F769
F763
F772
F1100
F740
N701
F736
F703
703-684
733-751
761-785
1007-1116
W1145
W1143
F1139
F1224
F1225
F1226
Y1091
W381
1123-1145
1223-1245
Neck site

Fig. S4. Biochemical characterization and cryo-EM analysis of NPC1-5.5 (Sf9). A. Size exclusion chromatography profile and SDS-PAGE analysis of the peak fractions for NPC1 expressed and purified from Sf9 at pH 5.5. **B.** Representative raw micrograph (scale bar = 22 nm) and 2D images (scale bar, 11 nm) of the sample made from the indicated peak of **(A)**. **C.** Cryo-EM data processing flowchart of the Sf9 dataset using RELION. **D, E.** FSC curve (D) and particle angular distribution (E) of the map with a final resolution determined at 3.1 Å. **F.** EM maps for the representative element in the NPC1-5.5 (Sf9) structure.

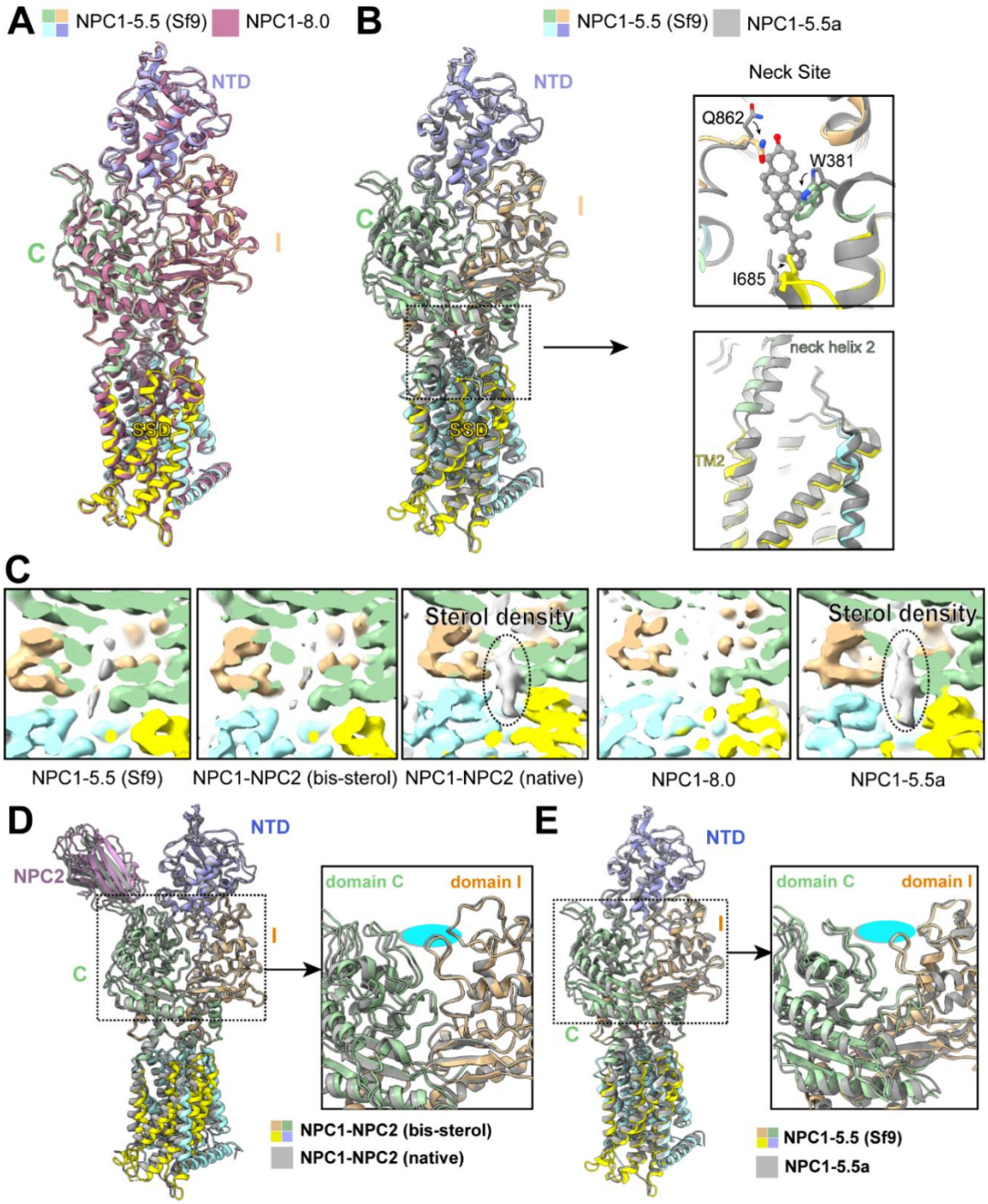
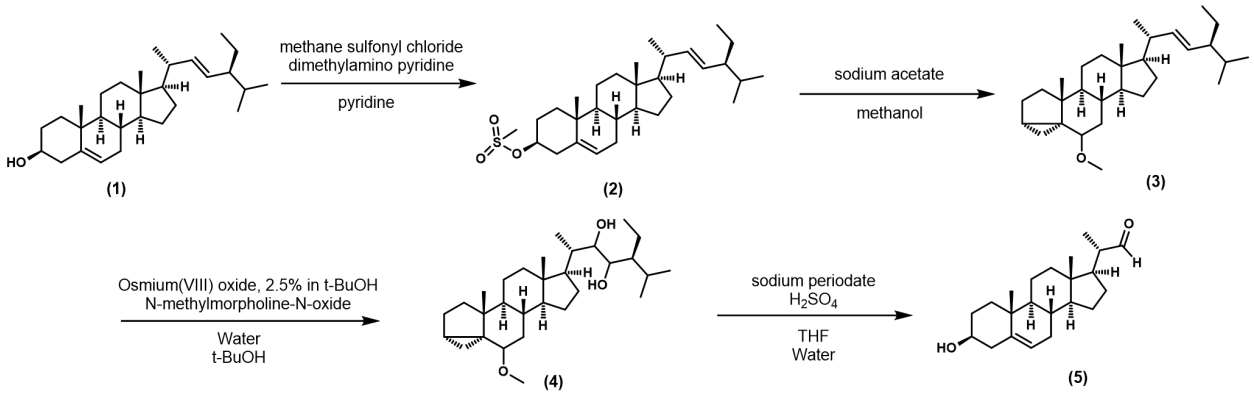


Fig. S5. Structural and map comparisons of NPC1-5.5 (Sf9) to other NPC1 structures at different pHs. A. Structural comparison of NPC1-5.5 (Sf9) to NPC1-8.0 (PDB code: 6W5S) shows very similar backbone arrangements. **B.** Structural comparison of NPC1-5.5 (Sf9) to our previous NPC1 structure at pH 5.5 expressed in HEK293F (3) (NPC1-5.5a, PDB code: 6W5T), shows deviation at both the neck cholesterol binding site (top right) and the Neck helix 2-TM2 (bottom right). **C.** Map comparison of the neck sites for the NPC1-5.5 (Sf9), bis-sterol-bound NPC1-NPC2 complex, the native NPC1-NPC2 complex (EMD-21549), NPC1-8.0 (EMD-21546) and NPC1-5.5a (EMD-21547). **D.** NPC1-NPC2 structural comparison between the native complex (grey) and bis-sterol bound complex (in colors), which are aligned by domain I. *Inset:* isolated domain C and I overlaid, with tunnel openings indicated in colored (bis-sterol bound) and grey

(Sf9) ellipses. **E.** NPC1 structural comparison between the HEK293F batch (NPC1-5.5a, grey) and the Sf9 batch (in colors), which are aligned by domain I. *Inset:* isolated domain C and I overlaid, with tunnel openings indicated in colored (Sf9) and grey (HEK293F) ellipses.

Supporting Information Text

Synthesis of Bis-Sterol probe JM046



Synthesis of (3S,8S,9S,10R,13R,14S,17R)-17-((2R,5S,E)-5-ethyl-6-methylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl methanesulfonate (2)

To a solution of methanesulfonylchloride (1.67 g, 14.54 mmol, 1.13 mL) and dimethyl aminopyridine (59.21 mg, 484.63 umol) in pyridine (15 mL) was added stigmasterol (2 g, 4.85 mmol) . The resultant mixture was allowed to stir for 12 h at room temperature, at which time the solution was poured into saturated aqueous sodium bicarbonate (500 mL) at which point a solid precipitated. The solid was collected by filtration and recrystallization from hot acetone provided the intermediate mesylate (**2**) which was used without further purification for the next step. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.40 (dd, *J* = 19.0, 5.0 Hz, 1H), 5.16 (dd, *J* = 15.2, 8.5 Hz, 1H), 5.03 (dd, *J* = 15.2, 8.6 Hz, 1H), 4.52 (tt, *J* = 11.0, 5.4 Hz, 1H), 3.00 (s, 2H), 2.56 – 2.43 (m, 2H), 2.27 (td, *J* = 14.5, 6.7 Hz, 1H), 2.10 – 1.67 (m, 7H), 1.48 (ddtd, *J* = 29.1, 14.6, 7.7, 3.2 Hz, 9H), 1.26 (s, 3H), 1.20 – 1.12 (m, 3H), 1.03 (t, *J* = 3.3 Hz, 7H), 0.87 – 0.82 (m, 4H), 0.80 (d, *J* = 6.9 Hz, 5H), 0.70 (s, 3H). ^{13}C NMR (100 MHz, CDCl3) δ 138.88, 138.38, 129.57, 123.98, 82.19, 56.93, 56.16, 51.41, 50.19, 42.41, 40.59, 39.76, 39.36, 38.96, 37.11, 36.59, 32.05, 32.04, 31.99, 29.18, 29.02, 25.55, 24.50, 21.38, 21.21, 19.35, 19.16, 12.37, 12.21.

Synthesis of (1aS,3aR,3bS,5aR,6R,8aS,8bS,10aS)-6-((2R,5S,E)-5-ethyl-6-methylhept-3-en-2-yl)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalene (3)

(3S,8S,9S,10R,13R,14S,17R)-17-((2R,5S,E)-5-ethyl-6-methylhept-3-en-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl methanesulfonate (1.26 g, 2.57 mmol) was dissolved in Methanol (15 mL). Anhydrous Sodium acetate, (1.16 g, 14.12 mmol, 757.09 uL) was added and the reaction was warmed to 50°C. and stirred overnight. The next morning the starting material was mostly consumed, and two close running, less polar, spots were observed. The reaction was concentrated to dryness, redissolved in water, and extracted twice with Ethyl acetate. The combined extracts were washed with brine and dried. Filtration followed by concentration on silica and purification by flash column chromatography (10% Ethyl acetate in Heptane) gave (1aS,3aR,3bS,5aR,6R,8aS,8bS,10aS)-6-((2R,5S,E)-5-ethyl-6-methylhept-3-en-2-yl)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalene (**3**) (911.9 mg, 2.14 mmol, 83.2% yield) as a mixture of methoxy diastereomers. ^{1}H NMR (400 MHz, CDCl3) δ 5.16 (dd, J = 15.2, 8.5 Hz, 1H), 5.02 (dd, J = 15.2, 8.6 Hz, 1H), 3.32 (s, 3H), 2.77 (t, J = 2.8 Hz, 1H), 2.07 – 2.02 (m, 1H), 1.97 (dt, J = 12.5, 3.3 Hz, 1H), 1.89 (dt, J = 13.5, 3.0 Hz, 1H), 1.73 (dtq,

J = 18.3, 8.8, 4.7 Hz, 4H), 1.54 (q, J = 4.7 Hz, 8H), 1.45 – 1.38 (m, 3H), 1.31 – 1.04 (m, 11H), 1.02 (d, J = 6.8 Hz, 6H), 0.87 – 0.83 (m, 6H), 0.80 (d, J = 6.3 Hz, 6H), 0.74 (s, 3H), 0.67 – 0.62 (m, 1H), 0.43 (dd, J = 8.0, 5.0 Hz, 1H). ^{13}C NMR (100 MHz, CDCl3) δ 138.33, 129.26, 82.44, 56.66, 56.52, 56.18, 51.24, 48.11, 43.41, 42.68, 40.45, 40.21, 35.32, 35.09, 33.37, 31.86, 30.49, 28.95, 25.36, 24.95, 24.25, 22.75, 21.49, 21.18, 21.02, 19.25, 18.98, 13.06, 12.42, 12.18.

Synthesis of (2S,5S)-5-ethyl-2-((1aS,3aR,3bS,5aS,6R,8aS,8bS,10aS)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalen-6-yl)-6-methylheptane-3,4-diol (4)

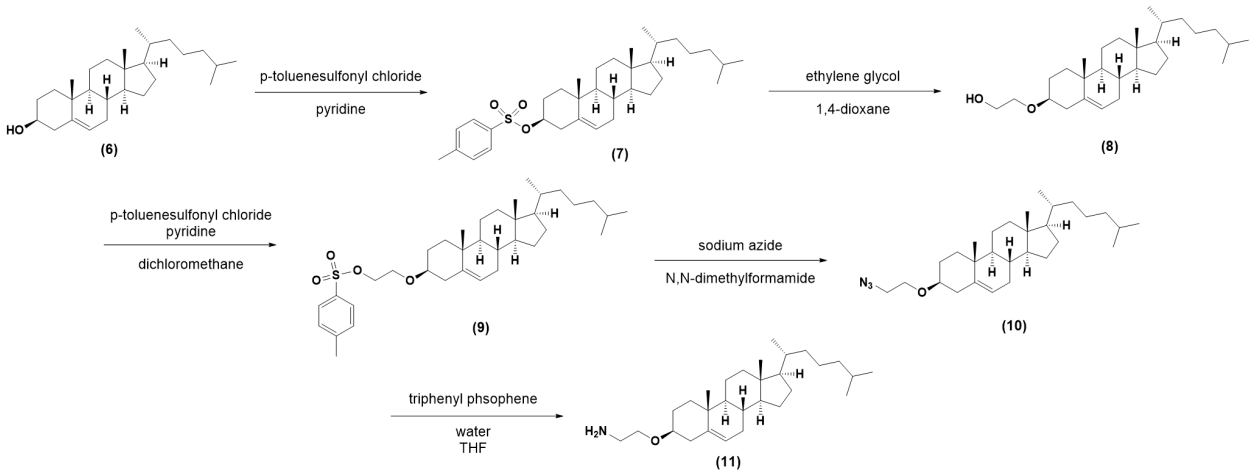
A 1 dram vial was charged with water (0.75 mL) and tert-butanol (5 mL) followed by ((1aS,3aR,3bS,5aR,6R,8aS,8bS,10aS)-6-((2R,5S,E)-5-ethyl-6-methylhept-3-en-2-yl)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalene (**3**) (548 mg, 1.28 mmol) . To this solution, osmium tetroxide (1.96 g, 192.63 µmol, 2.42 mL) was added as a 2.5% w/w solution in tBuOH. Lastly, N-methylmorpholine-N-oxide (225.66 mg, 1.93 mmol) was added, and the resultant was left to stir at room temperature. After ca. 96 hours, the starting material was ~90% consumed and a new polar spot (RF = 0.2 in 15% ethyl acetate in Heptane) was observed. The crude reaction mixture was diluted with saturated aq. sodium thiosulfate and stirred for 5 min. The resultant mixture was then extracted three times with DCM. The combined organic layers were then washed once with sat. sodium thiosulfate, followed by 1N HCl, and finally brine. The dichloromethane extract was then dried over anhydrous magnesium sulfate and concentrated. The resultant yellow oil was purified by FCC over silica (Heptane/Ethyl acetate, 0-40%), yielding (2S,5S)-5-ethyl-2-((1aS,3aR,3bS,5aS,6R,8aS,8bS,10aS)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalen-6-yl)-6-methylheptaneane-3,4-diol (4) (153 mg, 332.08 µmol, 25.9% yield) as an off-white foamy solid. ^{1}H NMR (400 MHz, $CDCl_3$-*d*) δ 3.61 (d, *J* = 6.2 Hz, 2H), 3.32 (d, *J* = 0.6 Hz, 3H), 2.76 (t, *J* = 2.5 Hz, 1H), 2.10 (td, *J* = 6.9, 2.3 Hz, 2H), 2.05 – 1.98 (m, 2H), 1.89 (dt, *J* = 13.4, 2.9 Hz, 2H), 1.75 (qd, *J* = 11.9, 4.5 Hz, 5H), 1.68 – 1.57 (m, 2H), 1.51 (dt, *J* = 13.6, 7.8 Hz, 3H), 1.45 – 1.30 (m, 5H), 1.31 – 1.05 (m, 11H), 1.02 (d, *J* = 5.2 Hz, 8H), 0.99 – 0.91 (m, 7H), 0.91 – 0.79 (m, 9H), 0.76 (d, *J* = 5.0 Hz, 4H), 0.64 (t, *J* = 4.4 Hz, 1H), 0.43 (dd, *J* = 8.0, 5.2 Hz, 1H).^{13}C NMR (100 MHz, $CDCl_3$) δ 82.54, 72.45, 70.75, 56.69, 56.37, 53.00, 49.88, 48.19, 43.54, 42.63, 40.45, 35.26, 33.53, 32.01, 30.68, 29.13, 28.28, 25.11, 24.58, 22.93, 21.61, 19.39, 18.73, 17.95, 14.21, 13.25, 12.29.

Synthesis of (S)-2-((3S,8S,9S,10R,13S,14S,17R)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)propanal (5)

A 2 dram vial was charged with THF (4ml) and water (0.4 ml) and (2S,5S)-5-ethyl-2-((1aS,3aR,3bS,5aS,6R,8aS,8bS,10aS)-10-methoxy-3a,5a-dimethylhexadecahydrocyclopenta[a]cyclopropa[2,3]cyclopenta[1,2-f]naphthalen-6-yl)-6-methylheptaneane-3,4-diol (140 mg, 303.87 µmol) was added. The resultant solution was stirred at RT and sodium periodate (194.98 mg, 911.60 µmol, 50.45 µL) was added in one portion. The reaction was stirred until complete conversion was observed by TLC (ca. 2h). After 2h, an insoluble precipitate had formed, and the mixture was partitioned between water and DCM. The aqueous layer extracted 3x with DCM and the combined organic extracts were dried over magnesium sulfate, filtered, and concentrated yielding the crude product as a brown oil.

The oil obtained above was then dissolved in THF (5 ml) and concentrated sulfuric acid was added (0.5 ml) and the mixture was heated to 50°C with stirring. After 10 minutes, the mixture was neutralized with sat. aq. sodium bicarbonate and extracted 3x with Ethyl acetate. The combined organic extracts were then washed with brine, dried over anhydrous magnesium sulfate, filtered and concentrated to yield a pale semi-solid, which purified by FCC (Heptane/Ethyl acetate, 10-55%) to yield (S)-2-((3S,8S,9S,10R,13S,14S,17R)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)propanal (25.1 mg, 75.94 µmol, 25.0% yield) as a white solid.^{1}H NMR (400 MHz, $CDCl_3$) δ 9.56 (dd, *J* = 11.2, 4.1 Hz, 1H), 5.35 (dd, *J* = 5.0, 1.8 Hz, 1H), 3.52 (tq, *J* = 11.2, 4.1 Hz, 1H), 2.40 – 2.19 (m,

3H), 2.03 – 1.94 (m, 2H), 1.91 – 1.81 (m, 3H), 1.72 – 1.61 (m, 3H), 1.60 – 1.43 (m, 9H), 1.13 & 1.04 (2d, *J* = 6.8 Hz, 3H), 1.01 (d, *J* = 8.0 Hz, 3H), 0.97 – 0.86 (m, 3H), 0.74 & 0.70 (2s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 205.86, 205.14, 141.05, 140.96, 121.64, 121.58, 71.92, 56.43, 56.21, 52.14, 51.27, 50.32, 49.63, 48.93, 43.14, 42.47, 42.31, 39.67, 38.96, 38.64, 37.45, 36.73, 32.09, 32.06, 32.01, 31.98, 27.21, 26.61, 24.82, 24.07, 21.20, 20.95, 19.53, 14.17, 13.72, 13.01, 12.40.



Synthesis of (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 4-methylbenzenesulfonate (7)

Cholesterol (3.61 g, 9.34 mmol) was dissolved in 30mL of dry pyridine at room temperature. To this solution, 4-methylbenzenesulfonyl chloride (3.56 g, 18.67 mmol) was added portion-wise. The reaction was stirred at room temperature for 48h then poured into 250mL of saturated sodium bicarbonate solution with stirring. Upon a addition, a precipitate was formed. The precipitate was filtered, and the resulting solid was washed twice with water (50mL) then twice with acetone (50mL). The material was then stirred with dichloromethane and filtered. The filtrate was dried over sodium sulfate and concentrated to give (3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 4-methylbenzenesulfonate (**7**) (3.21 g, 5.94 µmol, 63.6% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (d, *J* = 8.3 Hz, 2H), 7.32 (d, *J* = 8.4 Hz, 2H), 5.34 – 5.28 (m, 1H), 4.34 (td, *J* = 11.4, 5.6 Hz, 1H), 2.44 (s, 3H), 2.32 – 2.23 (m, 1H), 2.03 – 1.90 (m, 2H), 1.87 – 1.77 (m, 3H), 1.76 – 1.64 (m, 1H), 1.61 – 0.96 (m, 23H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.86 (dd, *J* = 6.6, 1.6 Hz, 6H), 0.66 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.49, 139.08, 135.03, 129.87, 127.80, 123.66, 82.54, 56.85, 56.33, 50.13, 42.49, 39.86, 39.69, 39.07, 37.09, 36.54, 36.35, 35.91, 32.03, 31.95, 28.83, 28.34, 28.16, 24.41, 23.99, 22.94, 22.70, 21.75, 21.17, 19.30, 18.87, 12.00.

Synthesis of 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-ol (8)

(3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl 4-methylbenzenesulfonate (2 g, 3.70 mmol) was dissolved in 15 mL of dry 1,4-dioxane. Ethylene glycol (5.74 g, 92.45 mmol, 5.16 mL) was added and the reaction was warmed to 85°C. After 8h of heating the reaction was cooled, diluted with Ethyl Acetate, and poured into saturated aqueous sodium bicarbonate solution. The organic layer was then extracted several times with sodium bicarbonate and then was dried over sodium sulfate. After filtration and concentration the crude residue was purified by FCC (0-40% Ethyl acetate in Heptane) to give 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-ol (**8**) (0.7722 g, 1.79 µmol, 48.5% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 – 5.32 (m, 1H), 3.75 – 3.67 (m, 2H), 3.59 (tt, *J* = 4.7, 2.7 Hz, 2H), 3.24 – 3.13 (m, 1H), 2.37 (ddd, *J*

= 13.1, 4.7, 2.1 Hz, 1H), 2.27 – 2.13 (m, 1H), 2.06 – 1.76 (m, 6H), 1.59 – 0.86 (m, 36H), 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.94, 121.90, 79.64, 69.11, 62.29, 56.98, 56.39, 50.42, 42.53, 39.99, 39.71, 39.32, 37.39, 37.06, 36.39, 35.95, 32.13, 32.10, 28.63, 28.39, 28.17, 24.46, 24.01, 22.95, 22.71, 21.26, 19.53, 18.90, 12.03.

Synthesis of 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl 4-methylbenzenesulfonate (9)

2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-ol (8) (632.5 mg, 1.47 mmol) was dissolved in 50 mL of dry Dichloromethane. To this solution, Pyridine (1.74 g, 22.03 mmol, 1.77 mL) was added followed by portion-wise addition of 4-methylbenzenesulfonyl chloride (335.96 mg, 1.76 mmol). The reaction was stirred at room temperature for 18 hours. After this time the reaction was poured into saturated aqueous sodium bicarbonate solution. The mixture was extracted three times with Dichloromethane and then the combined organic extracts were washed with 1N HCl (aq.) and then brine. The combined organic layers were then dried over sodium sulfate, decanted and concentrated onto silica to give the crude product. Purification by FCC (0-40% Ethyl acetate in Heptane) yielded 2-(((3S,8S,9S,10R,13R,^{14S},17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl 4-methylbenzenesulfonate (9) as a white solid (0.4576 g, 782.37 µmol, 53.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (d, *J* = 8.3 Hz, 2H), 7.33 (d, *J* = 8.5 Hz, 2H), 5.31 (d, *J* = 5.2 Hz, 1H), 4.19 – 4.12 (m, 2H), 3.69 – 3.62 (m, 2H), 3.10 (ddd, *J* = 15.5, 11.2, 4.3 Hz, 1H), 2.44 (s, 3H), 2.24 (ddd, *J* = 13.2, 4.7, 2.2 Hz, 1H), 2.15 – 2.05 (m, 1H), 2.05 – 1.93 (m, 2H), 1.82 (qd, *J* = 10.9, 3.3 Hz, 3H), 1.62 – 1.54 (m, 1H), 1.53 (d, *J* = 3.3 Hz, 2H), 1.49 – 0.99 (m, 16H), 0.97 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (dd, *J* = 6.6, 1.5 Hz, 6H), 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 144.81, 140.78, 133.52, 129.91, 128.17, 121.95, 79.80, 69.80, 65.56, 56.97, 56.38, 50.37, 42.52, 39.98, 39.70, 39.06, 37.29, 36.99, 36.38, 35.95, 32.11, 32.09, 28.38, 28.17, 24.46, 24.01, 22.95, 22.71, 21.77, 21.25, 19.49, 18.90, 12.03.

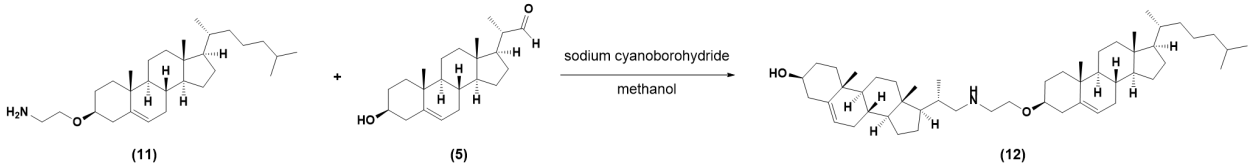
Synthesis of (3S,8S,9S,10R,13R,14S,17R)-3-(2-azidoethoxy)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene (10)

2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl 4-methylbenzenesulfonate (0.4576 g, 782.37 µmol) was dissolved in 15 mL of dry DMF. To this solution, sodium azide (254.31 mg, 3.91 mmol) was added. The solution was stirred at 40°C. After 18 hours, TLC showed complete consumption of the starting material. The reaction was poured into saturated sodium bicarbonate (aq.) and extracted 3 times with dichloromethane. The combined organic extracts were washed with water, brine and then dried over anhydrous sodium sulfate. The solution was decanted and filtered over a plug of silica (1:1 Heptane in Ethyl Acetate) to yield (3S,8S,9S,10R,13R,14S,17R)-3-(2-azidoethoxy)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene (10) (314 mg, 689.02 µmol, 88.1% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.39 – 5.31 (m, 1H), 3.71 – 3.61 (m, 2H), 3.40 – 3.33 (m, 2H), 3.21 (tt, *J* = 11.2, 4.4 Hz, 1H), 2.37 (ddd, *J* = 13.2, 4.7, 2.2 Hz, 1H), 2.28 – 2.18 (m, 1H), 2.06 – 1.76 (m, 5H), 1.62 – 1.43 (m, 8H), 1.42 – 1.22 (m, 4H), 1.21 – 1.03 (m, 7H), 1.01 (s, 4H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (dd, *J* = 6.6, 1.6 Hz, 6H), 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 140.91, 121.92, 79.89, 66.90, 56.99, 56.39, 51.22, 50.42, 42.53, 39.99, 39.70, 39.17, 37.37, 37.02, 36.39, 35.95, 32.13, 32.10, 28.50, 28.39, 28.17, 24.46, 24.01, 22.95, 22.71, 21.26, 19.53, 18.90, 12.03.

Synthesis of 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-amine (11)

(3S,8S,9S,10R,13R,14S,17R)-3-(2-azidoethoxy)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthrene (10) (0.2665 g, 584.79 µmol) was dissolved in THF (10 mL) / water (1 mL). Triphenylphosphine (184.06 mg, 701.75 µmol) was added, and the reaction was warmed to 65°C for 18h. Once complete the reaction was cooled and partitioned between ethyl acetate and water. The aqueous

layer was extracted twice with ethyl acetate and the combined organic extracts were combined and dried over sodium sulfate. The organic mixture was then filtered and concentrated onto silica. The product was purified by FCC (ethyl acetate/methanol /ammonium hydroxide (10 : 1: 0.1)0-100% gradient in ethyl acetate) to give the product 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-amine (203.3 mg, 473.10 µmol, 80.90% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38 – 5.32 (m, 1H), 3.52 (t, *J* = 5.2 Hz, 2H), 3.17 (tq, *J* = 9.1, 4.4 Hz, 1H), 2.87 (s, 2H), 2.37 (ddd, *J* = 13.2, 4.6, 2.1 Hz, 1H), 2.26 – 2.14 (m, 1H), 2.11 – 1.77 (m, 8H), 1.63 – 1.24 (m, 12H), 1.23 – 1.02 (m, 7H), 1.00 (s, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.87 (dd, *J* = 6.6, 1.6 Hz, 6H), 0.68 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 141.10, 121.76, 79.48, 69.83, 56.98, 56.39, 50.42, 42.52, 40.00, 39.70, 39.35, 37.43, 37.07, 36.39, 35.95, 32.13, 28.65, 28.39, 28.17, 24.46, 24.01, 22.95, 22.71, 21.26, 19.54, 18.89, 12.03.



(11) (5) (12)

Synthesis of Bis-Sterol Probe; (3S,8S,9S,10R,13S,14S,17R)-17-((S)-1-((2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl)amino)propan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol (12)

(S)-2-((3S,8S,9S,10R,13S,14S,17R)-3-hydroxy-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)propanal **(5)** (20 mg, 60.51 µmol) was combined with 2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethan-1-amine **(11)** (33.81 mg, 78.67 µmol) in methanol. The resultant solution was stirred at room temperature for 30 minutes, and then sodium cyanoborohydride (4.56 mg, 72.62 µmol) was added in one portion. The reaction was left to stir under ambient conditions overnight and monitored by TLC. After ca. 24h, the mixture was concentrated to dryness and dry loaded onto silica. The mixture was purified by FCC (DCM/MeOH, 0-10%) to yield (3S,8S,9S,10R,13S,14S,17R)-17-((S)-1-((2-(((3S,8S,9S,10R,13R,14S,17R)-10,13-dimethyl-17-((R)-6-methylheptanean-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-yl)oxy)ethyl)amino)propan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-3-ol **(12)** (18.8 mg, 25.26 µmol, 41.74% yield) as a white waxy solid.

^{1}H NMR (400 MHz, DMSO) δ 8.13 (bs, 1H), 5.34 (d, *J* = 2.3 Hz, 1H), 5.27 (d, *J* = 3.9 Hz, 1H), 4.55 (d, *J* = 4.3 Hz, 1H), 3.75 – 3.63 (m, 2H), 3.21 – 3.11 (m, 2H), 2.68 – 2.56 (m, 1H), 2.39 – 2.31 (m, 1H), 2.18 – 2.07 (m, 3H), 2.00 – 1.72 (m, 11H), 1.52 (dq, *J* = 19.2, 6.4 Hz, 8H), 1.43 – 1.21 (m, 13H), 1.18 – 1.06 (m, 9H), 1.04 – 0.89 (m, 19H), 0.85 (dd, *J* = 6.6, 1.7 Hz, 6H), 0.72 – 0.63 (m, 6H). ^{13}C NMR (151 MHz, DMSO) δ 141.29, 141.19, 140.20, 140.18, 121.49, 120.39, 120.33, 78.64, 78.56, 69.99, 69.97, 62.71, 62.26, 56.19, 56.18, 56.01, 55.99, 55.59, 53.65, 53.02, 52.47, 51.64, 49.61, 49.58, 49.53, 49.49, 46.91, 42.20, 42.11, 41.87, 41.69, 38.54, 38.49, 38.45, 36.97, 36.93, 36.61, 36.55, 36.28, 36.27, 36.06, 36.04, 35.66, 35.21, 33.43, 32.63, 31.45, 31.40, 31.37, 31.31, 31.25, 27.88, 27.79, 27.41, 27.22, 23.87, 23.83, 23.56, 23.19, 22.69, 22.42, 20.66, 20.62, 19.15, 19.11, 19.06, 18.57, 17.01, 16.69, 11.75, 11.70, 11.66.

Tables

Table S1.
Cryo-EM data collection, image processing, and reconstruction statistics

	NPC1-NPC2 (bis-sterol)	NPC1-5.5 (Sf9)
Data collection		
EM	Titan Krios (Thermo Fisher)	
Voltage (kV)	300	
Detector	K2 Summit (Gatan)	
Pixel size (Å/pixel)	1.114	
Electron dose (e^-/Å^2)	50	
Number of micrographs	979	2,896
Reconstruction		
Software	RELION 3.0	
Number of used Particles	66,353	260,257
Symmetry	C1	
Resolution	3.2 Å	3.1 Å
Map sharpening B-factor (Å^2)	-61	-75
Model building		
Software	Coot 0.9.8.5	
Refinement		
Software	Phenix 1.20.1	
Model composition		
Non-hydrogen atoms	10828	9708
Protein residues	1325	1193
Sugar moieties	21	19
Ligands	2	0
Validation		
MolProbity score	2.14	1.88
Clashscore	15.57	9.43
Rotamer outliers (%)	0.78	0.29
R.m.s deviations		
Bond length (Å)	0.003	0.003
Bond angle (°)	0.772	0.660
Ramachandran plot statistics (%)		
Preferred	93.01	94.44
Allowed	6.91	5.48
Outlier	0.08	0.08

Movie S1 (separate file):

Representative 500-ns MD simulation of NPC1-NPC2 complex with cholesterol bound in NPC2 only

Movie S2 (separate file):

Representative 500-ns MD simulation of NPC1-NPC2 complex with cholesterol bound in NPC1-NTD only

Movie S3 (separate file):

Representative 500-ns MD simulation of NPC1-NPC2 complex with cholesterol bound in both NPC2 and NPC1-NTD

Movie S4 (separate file):

3DVA volume series of NPC1-NPC2 (bis-sterol) complex dataset

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