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Supplemental Information

Structural Insights into the Niemann-Pick C1 (NPC1)-Mediated Cholesterol Transfer and Ebola Infection

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Supplemental Experimental Procedures

Expression and purification of the human NPC1 (hNPC1) and NPC2 (hNPC2)

The complementary DNAs of full-length hNPC1 and hNPC2 were each subcloned into the pCAG vector. A C-terminal His_{10} tag was fused for hNPC1 and a C-terminal FLAG plus His_{10} tag (for ITC) or FLAG tag (for transfer assay) was used for hNPC2. HEK 293F cells (Invitrogen) were cultured in SMM 293T-I medium (Sino Biological Inc.) at 37 °C under 5% CO_2 in a Multitron-Pro shaker (Infors, 130 rpm). When the cell density reached 2.0×10^6 cells per ml, the cells were transiently transfected with the expression plasmids and polyethylenimines (PEIs) (Polysciences). Approximately 1 mg plasmids were pre-mixed with 3 mg PEIs in 50 ml fresh medium for 15-30 min before transfection. For transfection, 50 ml mixture was added to one liter cell culture and incubated for 30 min. Transfected cells were cultured for 48 h before harvesting.

For purification of hNPC1, the 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) digitonin (Sigma). After centrifugation at 25,000 g for 45 min, the supernatant was collected and incubated with nickel affinity resin (Ni-NTA, Qiagen) at 4 °C for 30 min. The resin was rinsed with the wash buffer containing 25 mM Tris pH 8.0, 150 mM NaCl, 30 mM imidazole, 0.1% digitonin (w/v), and protease inhibitors. The protein was eluted from the affinity resin with the wash buffer plus 250 mM imidazole. The eluent was concentrated and applied to size exclusion chromatography (SEC, Superose 6, 10/300, GE Healthcare) in the buffer containing 25 mM Tris pH 8.0, 150 mM NaCl and 0.1% digitonin. The peak fractions were pooled and concentrated for EM analysis. The hNPC1

WT and variant proteins used for ITC or SPR were purified similarly, except that digitonin were replaced by 1% LMNG (w/v) or 0.02% C12E8 (w/v) in the extraction and purification procedure, respectively.

For purification of hNPC2, the secreted protein were collected and concentrated in the buffer containing 25 mM MES pH 6.0 and 150 mM NaCl. After centrifugation at 25,000 g for 10 min, the supernatant was collected and loaded to nickel affinity resin (Ni-NTA, Qiagen) at 4 °C. The resin was washed by the buffer containing 25 mM MES pH 6.0, 150 mM NaCl and 30 mM imidazole. The protein was eluted from the affinity resin with the wash buffer plus 250 mM imidazole. The eluent was concentrated and applied to SEC (Superose 6, 10/300, GE Healthcare) in the buffer containing 25 mM MES pH 6.0, 150 mM NaCl, 0.02% C12E8 (w/v) and 0.1 mM *tris*(2-carboxyethyl)phosphine (TCEP). The peak fractions were pooled and concentrated for ITC titration. The hNPC2 protein used for cholesterol transfer assay was purified similarly, except that the SEC buffer contained 25 mM MES pH 5.5, and 150 mM NaCl.

The protocol for expression, purification and processing of the glycoprotein of Ebola virus (EBOV-GPcl) was identical to that reported (Wang et al., 2016).

Isothermal titration calorimetry (ITC)

The binding affinities between hNPC2 and the indicated hNPC1 variants were measured using ITC. All the proteins were purified to homogeneity in the buffer containing 25 mM MES (pH 6.0) or 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.02% $C_{12}E_8$ (w/v) and 0.1 mM TCEP. For each injection, 2 μl hNPC2 with a concentration of approximately 300 μM was titrated into hNPC1 in the chamber. Various concentrations of the hNPC1

variants were tested and the optimal concentrations were used: WT, R518W, and ΔNTD at ~1 μM, L175A/L176A and Q88A/Q92A/R96A at ~5 μM, and Δlinker at ~10 μM. Multiple concentrations of NTD were tested, but none gave rise to a characteristic pattern that could be fitted. Shown in Figure S4D was the titration with 1 μM NTD. All experiments were performed with a VP-ITC microcalorimeter (MicroCal iTC200) at 18 °C. The data were fitted using the software ORIGIN 7.0 (Origin Lab).

[^{3}H]-cholesterol transfer assay

For preparation of the [^{3}H]-cholesterol bound hNPC2, 300 μl buffer A (25 mM MES, pH 5.5, and 150 mM NaCl) containing approximately 500 nM [^{3}H]-cholesterol (108.78 dpm/fmol; delivered in ethanol at final concentration of 3%) was mixed with approximately 80 μg FLAG-tagged hNPC2. After incubation for about 3 hr at room temperature, the mixture was diluted with 700 μl buffer A. After centrifugation at 15000 g for 20 min, the suspension was applied to FLAG M2 affinity column. After washing with 4 ml cold buffer A, the [^{3}H]-cholesterol-loaded protein was eluted with 1 ml cold buffer A plus 0.3 mg/ml FLAG peptide. The eluent containing [^{3}H]-cholesterol-bound hNPC2 was aliquoted and used for transfer assay immediately.

For transfer assay, His_{10}-tagged hNPC1 variants were prepared in 125 μl buffer containing buffer A plus 0.06% digitonin and mixed with 25 μl aforementioned hNPC2 eluent (with about 0.7 pmol [^{3}H]-cholesterol as measured by scintillation counting). After incubation at 4 °C for 30 min, each reaction was diluted with 875 μl cold buffer B (250 mM HEPES 7.5, 150 mM NaCl and 0.06% digitonin) before immediately loading to Ni-NTA column. After washing with 3 ml cold buffer C (25 mM HEPES 7.5, 150 mM

NaCl and 0.06% digitonin), the protein was eluted with 1 ml buffer C plus 250 mM imidazole. The amount of [^{3}H]-cholesterol in the eluent was quantified by scintillation counting. The transfer assay at pH 7.5 was carried out similarly expect that the pH of buffer A was adjusted to 7.5 with 100 mM HEPES. Each data point is the average of triple assays. Error bars represent SD.

Surface plasmon resonance (SPR)

The SPR experiments were performed using a Biacore® 3000 system at 25 °C with a flow rate of 30 μl/min in buffer A (50 mM MES, pH 6.0, 150 mM NaCl, 0.02% C12E8) or buffer B (10 mM Hepes, pH 7.5, 150 mM NaCl, 0.02% C12E8). The purified proteins for SPR analysis were changed into buffer A or buffer B through SEC. For SPR measurement at pH 6.0, GPcl was covalently coupled to a CM5 sensor chip with about 2000 response units, and a series of concentrations of the hNPC1 variants were flowed over the GPcl chip surface. For measurement at pH 7.5, NPC1 was covalently coupled to a CM5 sensor chip, and different concentrations of GPcl were flowed over the NPC1 chip surface. The binding kinetics was analyzed using the software BIAevaluation Version 4.1.

Cryo-EM data collection and processing of the complex between FL hNPC1 and EBOV-GPcl (the NPC1-GPcl complex)

The NPC1-GPcl complex (approximately 15 mg/ml) was reconstituted by incubating the purified NPC1 and GPcl at a mass ratio of approximately 0.6:1 at 4 °C for 5h. The procedure for cryo-EM sample preparation and data collection of the NPC1-GPcl

complex was nearly identical to that for NPC1. To be brief, a total of 1,379 micrographs of NPC1-GPcl complex were collected, from which 703,336 particles were automatically picked with RELION 1.4. After several rounds of 2D classification, a total of 391,702 particles were selected and 3D classified into 4 classes. One class that showed distinct features of the NPC1-GPcl complex was further 3D classified into 6 classes. Classes displaying similar features were combined, resulting in 3 classes based on the density of GPcl and hNPC1(NTD). The first and second classes have both NPC1 and GPcl, which were invisible at higher threshold in the third class. Among the first two classes, the NTD was invisible at a higher threshold in the second class. The first class was further subjected to 3D auto-refinement with RELION 1.4. The final particle number used was 50,223.

Model building and refinement

Please refer to Experimental Procedures for cryo-EM data acquisition and processing of hNPC1. The Class 1 map of hNPC1 at 4.4 Å was used for model building. The crystal structures of domain C (PDB: 5F1B, 5HNS) (Wang et al., 2016; Zhao et al., 2016) and NPC1(NTD) (PDB: 3GKI) (Kwon et al., 2009) were first docked into the corresponding map in Chimera (Pettersen et al., 2004). Thirteen transmembrane helices (TMs) were readily discernible in the transmembrane region, 12 of which can be fitted by a homologous crystal structure of AcrB (PDB: 1IWG) (Murakami et al., 2002). Since the 12 TMs exhibited a 2-fold pseudosymmetry, the AcrB crystal structure can be fitted into the transmembrane regions in two ways. The correct fitting was selected based on the connections between TMs and the extracellular domains. The remaining transmembrane

helix was TM1. Each TM was manually adjusted in COOT (Emsley et al., 2010) to better fit the EM map. After the assignment of NTD, domain C, and TMD, the remaining map in the extracellular region corresponded to domain I. The map of domain I resembles that of domain C, consistent with their 30% sequence similarity. The structure of domain I was manually built in COOT using domain C as a reference.

Since the overall resolution was insufficient for side chain assignment, the model was mostly built as poly Ala except for the directly docked NTD and domain C. Most of the predicted glycosylation sites can be observed in the EM maps of extracellular domains and 25 sugar molecules were built to 14 glycosylation sites. These glycosyl groups facilitated validation of model building. The structure was refined against the Class 1 map by PHENIX (Adams et al., 2010) in real space with secondary structure and geometry restraints.

For the NPC1-GPcl complex, the 4.4 Å cryo-EM structure of NPC1 and the crystal structure of an EBOV-GPcl trimer (PDB: 5F1B) were fitted into the EM map in Chimera. Model statistics can be found in Supplemental Tables 2 and 4.

Supplemental Table 1 | Statistics of the removed and remaining NPC1 particle numbers during random-phase 3D classifications. Related to Experimental Procedure.

NPC1 Classes	Datasets	Random-phase resolution, Å	# of iteration	Binning factor	# of remaining particles	# of removed particles
Class 1	subset I	40	33	2	103,751	4,533
		30	72	2	90,072	13,679
		25	89	2	81,548	8,524
		25	71	1	78,208	3,340
		20[a]	64	1	70,573	5,663
		16	50	1	66,245	4,328
		14	42	1	64,188	2,057
		12	33	1	60,977	3,211
		11	63	1	57,062	3,915
		10	29	1	52,976	4,086
Class 1	subset II	40	32	2	150,524	11,245
		30	31	2	144,778	5,746
		25	33	2	139,447	5,331
		20[b]	78	2	80,091	20,412
		25	63	1	69,108	10,983
		20	75	1	67,204	1,904
Class 1	Subsets I & II	25[c]	77	1	114,403	5,777
		20	98	1	109,947	4,456
		15	53	1	102,731	7,216
Class 2	subset I	40	33	2	119,504	7,435
		30	62	2	105,057	14,447
		25	91	2	93,620	11,437

[a,] The particles were re-3D classified before this cycle; [b,] A total of 38,944 particles were removed through 2D classification before this cycle; [c,] The dataset was obtained by combining the subsets I and II.

Supplemental Table 2 | Summary of data collection and model statistics of EM reconstructions of hNPC1. Related to Figure 1.

Data collection		
EM equipment	FEI Titan Krios	
Voltage (kV)	300	
Detector	K2 Summit (Gatan)	
Pixel size (Å)	1.307	
Electron dose ($e^-/Å^2$)	50	
Defocus range (μm)	1.5-3.0	
Reconstruction		
Software	RELION 1.4	
Maps	Class 1	Class 2
Number of used Particles	102,731	93,620
Symmetry	C1	
Final Resolution (Å)	4.4	6.7
Model building		
Software	COOT	
Refinement		
Software	Phenix	
Docking correlation coefficient		
Domain A	0.863	
Domain C	0.828	
AcrB_TMD	0.745	
NPC1_TMD	0.884	
Domain I	0.850	
Model composition		
Protein residues	1,139	
Sugar	25	
Validation		
R.m.s deviations		
Bonds length (Å)	0.005	
Bonds Angle (°)	0.802	
Ramachandran plot statistics (%)		
Preferred	90.6	
Allowed	7.5	
Outlier	1.9	

Supplemental Table 3 | Summary of Niemann-Pick disease type C-related sequence variations of hNPC1. Related to Figure 3.

NTD	Domain C	Domain I		TMD	
C63R	V378A	Q862L	NITDQF961-966S	M272R	Y825C
C74Y	L380F	S865L	N961S	W273S	S849I
Q92R	A388P	Y871C	N968S	R372W	I1094T
C113R	R389C	D874V	C976R	M631R	D1097N
T137M	P401T	P888S	R978C	G640R	N1137I
P166S	R404P	V889M	G986S	S652W	G1140V
C177G	R404Q	Y890C	G992A	G660S	M1142T
C177Y	R404W	Y899D	G992R	V664M	N1150K
N222S	P433L	G910S	G992W	S666N	N1156I
V231G	P434L	D917Y	M996R	C670W	N1156S
P237S	E451K	A926T	S1004L	G673V	V1165M
D242H	S473P	A927V	P1007A	L684F	F1167L
D242N	P474L	Q928P	G1012D	P691L	C1168Y
C247Y	C479Y	L929P	G1015V	L695V	A1174V
G248V	Y509S	R934Q	H1016R	D700N	R1186H
	H510P	S940L	V1023G	F703S	E1189G
	H512R	W942C	G1034R	L724P	T1205K
	R518Q	I943M	A1035V	V727F	T1205R
	R518W	D944N	T1036K	S734I	V1212L
	A521S	D945N	T1036M	E742K	L1213F
	F537L	D948H	A1054T	A745E	L1213V
	P543L	D948N	R1059Q	M754K	A1216V
	T574K	D948Y	I1061T	F763L	F1224L
	K576R	V950M	A1062V	A767V	G1236E
	A605V	S954L	T1066N	Q775P	G1240R
	E612D	C956Y	F1087L	R789C	S1249G
	R615C	R958L	Y1088C	R789G	
	R615L	R958Q	E1089K		
		V959E			

The mutations were extracted from a review (Scott and Ioannou, 2004) and the UNIPROT website: http://www.uniprot.org/uniprot/O15118

Supplemental Table 4 | Summary of data collection and model statistics for the cryo-EM reconstruction of the complex between hNPC1 and EBOV-GPcl. Related to Figure 6.

Data collection	
EM equipment	FEI Titan Krios
Voltage (kV)	300
Detector	K2 Summit (Gatan)
Pixel size (Å)	1.307
Electron dose ($e^-/Å^2$)	50
Defocus range (μm)	1.7-2.7
Reconstruction	
Software	RELION 1.4
Number of used Particles	50,223
Symmetry	C1
Final Resolution (Å)	6.6
Model Docking	
Software	Chimera
Docking correlation coefficient	
GP_trimer	0.864
hNPC1	0.844

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