Supporting Information

Weinglass *et al.* 10.1073/pnas.0800936105

SI Text

Materials. Restriction enzymes and Pfusion polymerase were from New England Biolabs. pCDNA5-FRT and pCR3.1, and STBL4 competent cells and T4 DNA ligase were purchased from Invitrogen. Synthetic oligonucleotides were synthesized by IDT. dNTPs were purchased from Roche Diagnostics and Chromaspin columns from Clontech. Dye terminator sequence reactions were performed with the ABI Big Dye 3.1 sequencing kit and analyzed with an ABI3100 genetic analyzer, both from Applied Biosystems. Fugene6 transfection reagent was from Roche. MDCKII cells were from Piet Borst (Netherland Cancer Institute, Amsterdam) (1) and TsA201 cells were a gift of Robert DuBridge (Pdl Biopharm, Freeman, CA). Procedures for handling TsA-201 cells and their transfection with Fugene6 have been described (2). MDCKII cells were maintained in DMEM + Glutamax (Sigma). All media were supplemented with 10% FBS, penicillin and streptomycin and cells were grown at 37°C in 5% CO_2. All other reagents were obtained from commercial sources and were of the highest purity commercially available. EZE, ezetimibe glucuronide (EZE-gluc), EZE-gluc-enantiomer (ent-1) (3), the propargyl sulfonamide, 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]prop-1-yn-1-yl}phenyl)-4-oxoazetidin-2-yl]phenyl methyl-β-D-glucopyranosiduronate (PS), the alkyl sulfonamide, 4-[(2S,3R)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-1-(4-{3-[(methylsulfonyl)amino]propyl}phenyl)-4-oxoazetidin-2-yl]phenyl β-D-glucopyranosiduronic acid (AS), and [^{3}H]AS were prepared as described (2).

Expression of Dog NPC1L1 and dog NPC1L1-K_v1.1 in MDCKII-Flp Cells. The generation of MDCKII-Flp and dog NPC1L1/MDCKII-Flp cells has been described (2). A dog NPC1L1-K_v1.1/MDCKII II–Flp stable cell line was generated by transfecting MDCKII-Flp cells with pCDNA5/FRT-dog NPC1L1-K_v1.1 using lipofectamine, followed by selection on 200 μg/ml hygromycin B. Clones were isolated with cloning rings and selected for levels of [^{3}H]AS binding in the absence, or presence, of 10 mM sodium butyrate, to identify cells expressing high amounts of dog NPC1L1-K_v1.1.

Cell-Based [^{3}H]AS Binding. Dog NPC1L1/MDCKII-Flp or dog NPC1L1-K_v1.1/MDCKII-Flp cells were seeded at a density of 25,000 cells per well in 96-well tissue culture treated plates, and cells were allowed to attach and polarize for ≈48 h at 37°C. 4 mM sodium butyrate was added and cells were incubated for an additional 24 h at 37°C. TsA201 cells were seeded at a density of 10,000 cells per well in 96-well polyD-lysine coated plates and cells were allowed to attach for ≈18 h at 37°C. TsA201 cells were subsequently transfected with NPC1L1 cDNA variants according to the manufacturer's instructions (Roche) and incubated for 3 days at 37°C. For all binding studies, [^{3}H]AS was added to the well, and cells were incubated under normal growth conditions for determined periods of time. Duplicate samples were averaged for each experimental point. For saturation binding experiments, cells were incubated with increasing concentrations of [^{3}H]AS for 4 h. Nonspecific binding was defined in the presence of 100 μM EZE-gluc. At the end of the incubation period, cells were washed twice with 200 μl of DMEM to separate bound from free ligand, and radioactivity associated with cells was determined using a β-counter. Data from saturation experiments were analyzed as described (2).

Cell-Based [^{3}H]Cholesterol Flux. Flux assays were performed essentially as described in refs. 2 and 4. Briefly, cell growth medium was completely aspirated and replaced with 200 μl of 5% LPDS containing the appropriate concentration of compound and incubated at 37°C for 3 h in a 5% CO_2 incubator. Media were subsequently aspirated, and cells were incubated in 200 μl of 4.5% methyl-β-cyclodextrin (βmCD), dissolved in 5% LPDS and filtered through a 0.22 μM filter, at 37°C for 45 min in a 5% CO_2 incubator. Media were removed, and cells were washed twice with 125 μl of 5% LPDS followed by addition of [^{3}H]cholesterol [51Ci/mmol, (Perkin–Elmer)] complexed to BSA in 5% LPDS (4). After a 45-min incubation, cells were washed twice with DMEM, and 1% SDS was added. Solubilized cell content was transferred to vials for radioactive analyses.

Membrane Preparation. Dog NPC1L1/MDCKII-Flp and dog NPC1L1-K_v1.1/MDCKII-Flp membranes were prepared from frozen cell pellets by isotonic lysis and separation on a sucrose step gradient. Pellets were pottered in 14 ml of homogenization buffer (Tris-buffered saline containing protease inhibitors and 1 μg/ml DNaseI (Roche Molecular Biochemicals) and centrifuged at low speed (5 min at 3,000 × *g*). Pellets were homogenized two more times with the same procedure and combined supernatants underlain with 8 ml of 1.3 M sucrose/10 mM Tris-HCl pH 7 in thin-walled ultracentrifugation tubes. After centrifugation (30 min. at 30,000 rpm, Sorvall Surespin 630), interfaces were collected, diluted with 40 ml 20 mM Tris-HCl pH 7.4 (membrane dilution buffer, MDP), pelleted by ultracentrifugation and stored as shock frozen aliquots after resuspension in MDP. Protein content was determined by a Bradford assay (Bio-Rad) according to the manufacturer's instructions.

Immunoprecipitation. Dog NPC1L1/MDCKII-Flp and dog NPC1L1-K_v1.1/MDCKII-Flp membranes at 0.2 mg/ml in 20 mM Tris-Cl, 100 mM NaCl pH 7.4 were treated with 0.03% sodium taurocholate/0.05% digitonin and 20 nM [^{3}H]AS overnight in the absence or presence of 100 μM EZE-gluc. Free [^{3}H]AS was removed by centrifugation at 80,000 rpm for 25 min in a TLA 100.4 rotor. Pellet was resuspended at a final concentration of ~0.2 mg/ml membranes using a homogenizer. Resuspended pellet (M) was evaluated for [^{3}H]AS-binding activity by filtering with 120 mM NaCl, 0.1% sodium taurocholate, 20 mM Mes pH 6.7 (3) and solubilized with 1% digitonin/0.03% sodium taurocholate for 30 min at 4°C with rotation. After solubilization, material was spun at 80,000 rpm for 20 min in a TLA 100.4 rotor. The supernatant (S) was evaluated for [^{3}H]AS-binding activity by filtration (20 mM Tris, 10 mM $MgCl_2$, 10% PEG).

Protein A Sepharose beads coated with anti-K_v1.1 antibody were prepared by washing twice with TBS wash buffer (150 mM NaCl, 20 mM Tris-Cl pH 7.4, 0.1% digitonin) and mixing with anti-K_v1.1 antibody for 30 min followed by three washes with TBS wash buffer to remove unbound antibody. Solubilized material (S) was incubated with anti-K_v1.1 beads for 4 h at 4°C. Sepharose beads were spun down and supernatant was assessed for [^{3}H]AS binding activity (**U**). The beads pellet (P) was washed 3 times with TBS buffer and associated [^{3}H]AS radioactivity determined by placing the tube in a β-counter. Bound proteins were then eluted from the beads with 1x Laemmli buffer. For monitoring affinity purification efficiency and specificity, equivalent sample aliquots were resolved on analytical 7.5% SDS/PAGE gels and electroblotted on 0.45 μm PVDF membranes (Millipore) using the MiniProteanII wet blot system (Bio-Rad)

and Bjerrum transfer buffer. Membranes were blocked with 3% BSA (Grade V, Roth) in PBS + 0.05% Tween-20, incubated first with anti-K_v1.1 antibody (1:50,000), washed (3 × 10 min) and incubated with HRP-coupled anti-rabbit IgG (Santa Cruz Biotechnology). After washing (5 × 30 min), blots were developed with ECL+ and exposed to Hyperfilm according to the manufacturers instructions (Amersham Biosciences). The major portion (90%) of eluted protein was run into SDS/PAGE gels and, following silver-staining, lanes were excised (each as 2 samples) and subjected to *in-gel* tryptic digestion as described (5).

Mass spectrometric analysis was carried out as detailed in ref. 5. Peptides from digested samples were vacuum-dried and resolubilized in 0.5% trifluoroacetic acid. Using an UltiMate 3000 HPLC system (Dionex) peptide samples were concentrated on a C18 PepMap100 precolumn (5 μm; Dionex) and loaded onto ReproSil-Pur 120 ODS-3 (C18; 3 μm; Dr. A. Maisch (HPLC). Ammerbuch-Entringen) manually packed into a PicoTip Emitter (75 μm; tip: 8 ± 1 μm; New Objective). Peptides were eluted with an aqueous-organic gradient (solvent A: 0.5% acetic acid; solvent B: 80% acetonitrile / 0.5% acetic acid; gradient: 60 min from 3% B to 30% B, 15 min to 100% B; flow rate: 300 nl/min) and sprayed into a LTQ-FT mass spectrometer (Thermo Electron) via a nanoelectrospray source (Proxeon). Scan cycles consisted of one FTMS full scan and up to five ITMS dependent MS/MS scans of the five most intense ions with charge state 2+, 3+ or 4+. Dynamic exclusion was set enabled (duration 30 sec, mass width 20 ppm) as well as monoisotopic precursor selection. Extracted MS/MS spectra were searched against the NCBInr database (mammalia) using the Mascot search engine (Matrix Science) accepting common variable modifications and one missed trypsin cleavage (peptide tolerance was ± 10 ppm and MS/MS tolerance was ± 1.0 Da; score threshold was 20).

Proteins with at least two specific peptides with score >20 were considered unambiguously identified. For evaluation, exogeneous contaminations (trypsin, keratins, immunoglobulins, and albumin) were removed from the datasets. As a semiquantitative measure, the relative peptide query score (rPQScore, 34) was used. In the histogram of Fig. 1I, the logarithmic ratios of peptide queries (PQ) obtained for any identified protein in NPC1L1-K_v1.1- versus NPC1L1-cell-derived material were binned in 0.2 intervals. Proteins absent in all four control measurements evaluated were assigned a threshold maximum value of 0.25 queries. A specificity threshold of around 4 is suggested by the distribution as published previously (5).

Generation of Dog/Mouse NPC1L1 Chimeras and Point Mutations. Dog/mouse serial chimeras were constructed by aligning dog (GenBank NP_001091019) and mouse (GenBank AAI31789) NPC1L1 cDNA sequences to identify those regions of high homology (Table S2). Two-step PCR mutagenesis of the genes encoding dog and mouse NPC1L1 (in plasmids pCDNA5/FRT and pCR3.1, respectively) generated products containing dog/mouse chimeras 1–6 [1 ($D_{1–264}/M_{265–1333}$), 2 ($D_{1–629}/M_{630–1333}$), 3 ($D_{1–806}/M_{807–1333}$), 4 ($D_{1–873}/M_{874–1333}$), 5 ($D_{1–1102}/M_{1104–1333}$) and 6 ($D_{1–1268}/M_{1270–1333}$)]. For chimeras 7 ($M_{1–386}/D_{387–629}/M_{630–1333}$) and 8 ($D_{1–386}/M_{387–629}/D_{630–1325}$), three first-round PCR products were generated using mouse and dog NPC1L1 cDNA and subsequently these were spliced together. For chimeras 9 ($M_{1–386}/D_{387–434}/M_{435–1333}$), 10 ($M_{1–386}/D_{387–509}/M_{510–1333}$), and 11 ($M_{1–386}/D_{387–571}/M_{572–1333}$), chimera 7 plasmid DNA linearized with NdeI was used as the template to generate a PCR product encoding the N-terminal region of NPC1L1 and mouse NPC1L1 cDNA was used to amplify the C-terminal region of NPC1L1. These PCR products were subcloned back into pCR3.1 as XhoI/HindIII fragments. All constructs were confirmed by DNA sequencing.

Point mutations in dog or mouse NPC1L1 were introduced by *in vitro* mutagenesis using Quikchange XL (Stratagene) following the manufacturer's protocol. Dog NPC1L1 cDNA was modified to encode the mutants Phe-532→Tyr, Met-543→Ile and Phe-532→Tyr/ Met-543→Ile. Mouse NPC1L1 cDNA was modified to encode the mutants Tyr-532→Phe, Ile-543→Met and Tyr-532→Phe/Ile-543→Met. All plasmids encoding point mutations of dog or mouse NPC1L1 were grown in STBL4 cells and the entire coding sequence was verified by DNA sequencing.

1. Louvard D (1980) Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc Natl Acad Sci USA* 77:4132–4136.
2. Weinglass AB, *et al.* (2008) MDCKII cells: A pharmacologically validated system for NPC1L1-mediated cholesterol uptake. *Mol Pharmacol*, 73:1072—1084.
3. Garcia-Calvo M, *et al.* (2005) The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). *Proc Natl Acad Sci USA* 102:8132–8137.
4. Yu L, *et al.* (2006) Cholesterol-regulated translocation of NPC1L1 to the cell surface facilitates free cholesterol uptake. *J Biol Chem* 281:6616—6624.
5. Berkefeld H, *et al.* (2006) BKCa-Cav channel complexes mediate rapid and localized Ca2+-activated K+ signaling. *Science* 314:615–620.

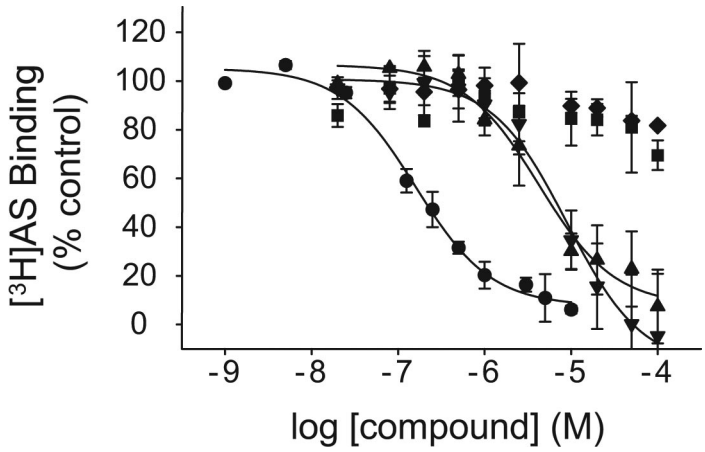

Fig. S1. Pharmacology of [^{3}H]AS binding to mouse NPC1L1. Cells were incubated with 150 nM [^{3}H]AS in the presence or absence of increasing concentrations of PS (●), EZE-gluc (▲), EZE (▼), EZE-enantiomer (■) or EZE-gluc enantiomer (♦) for 4 h at 37°C. Inhibition of binding was assessed relative to an untreated control. Specific binding was fit to a single-site inhibition model, yielding K_i values of (●) 51 nM (PS), (▲) 1.23 μM (EZE-gluc), (▼) 2.49 μM (EZE), (■) N.D. (EZE-enantiomer) and (♦) N.D. EZE-gluc enantiomer.

Table S1. MASCOT results of the NPC1L1-specific proteins identified (for search parameters, see *Experimental Procedures*)

gi|148223061 **Mass:** 143802 **Score:** 346 **Queries matched:** 12
NPC1 (Niemann-Pick disease, type C1, gene)-like 1 [Canis lupus familiaris]

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
712	488.2420	974.4694	974.4669	2.66	0	(31)	0.47	1	R.SLEDEINR.T
713	488.2421	974.4696	974.4669	2.86	0	48	0.01	1	R.SLEDEINR.T
1173	374.5406	1120.6000	1120.5989	0.95	0	24	1.4	1	R.SFAVSTRPTR.L
1175	561.3076	1120.6006	1120.5989	1.55	0	(23)	1.7	1	R.SFAVSTRPTR.L
1319	591.8419	1181.6692	1181.6656	3.11	0	(86)	4e-007	1	R.VGLDQELALPK.D
1320	591.8424	1181.6702	1181.6656	3.96	0	87	3.8e-007	1	R.VGLDQELALPK.D
1352	599.2744	1196.5342	1196.5309	2.79	0	81	8.3e-006	1	R.NSEDYTEALR.V
1353	599.2745	1196.5344	1196.5309	2.95	0	(58)	0.0014	1	R.NSEDYTEALR.V
1358	599.7654	1197.5162	1197.5149	1.11	0	(75)	2.7e-005	1	R.NSEDYTEALR.V+Deamidated (NQ)
1590	654.7930	1307.5714	1307.5672	3.23	0	42	0.048	1	R.LDVCCCVSAPK.L+3 Carbamidomethyl (C)
1635	675.8923	1349.7700	1349.7667	2.50	0	65	3.9e-005	1	K.LPAPGQSEGLLLR.V
1636	675.8926	1349.7706	1349.7667	2.94	0	(59)	0.00012	1	K.LPAPGQSEGLLLR.V

gi|73963782 **Mass:** 44446 **Score:** 76 **Queries matched:** 2
PREDICTED: similar to Thioredoxin domain containing protein 1 precursor (Transmembrane Trx-related protein)

Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Peptide
804	509.7644	1017.5142	1017.5131	1.14	0	24	2.9	1	R.IITDENWK.E
1475	629.3276	1256.6406	1256.6361	3.62	0	52	0.0033	1	K.VDVTEQPGLSGR.F

Each listed query corresponds to a peptide fragment spectrum (MS/MS) that has been matched to the assigned peptide with high probability (score >20).

Table S2. Dog (genbank NP_001091019), mouse (Genback AAI31789), human (Genbank AAS5693), and rat (Genbank EDM00336) NC1L1 sequences are aligned

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dog    1    MADTGLRGWLLWALLLHVAQSELYTPIHQPGYCAFYDECGKNPELSGGLAPLSNVSCLSN   60
mouse  1    MA-AAWQGWLLWALLLNSAQGELYTPTHKAGFCTFYEECGKNPELSGGLTSLSNISCLSN   59
human  1    MAEAGLRGWLLWALLLRLAQSEPYTTIHQPGYCAFYDECGKNPELSGSLMTLSNVSCLSN   60
rat    1    MA-AAWLGWLLWALLLSAAQGELYTPKHEAGVCTFYEECGKNPELSGGLTSLSNVSCLSN   59

dog    61   TPAPRVTGEHLTLLQRICPRLYTG-TTTYACCSPKQLLSLETSLAVTKALLTRCPTCSDN  119
mouse  60   TPARHVTGDHLALLQRVCPRLYNGPNDTYACCSTKQLVSLDSSLSITKALLTRCPACSEN  119
human  61   TPARKITGDHLILLQKICPRLYTGP-NTQACCSAKQLVSLEASLSITKALLTRCPACSDN  119
rat    60   TPARHVTGEHLALLQRICPRLYNGPNTTFACCSTKQLLSLESSMSITKALLTRCPACSDN  119

dog    120  FVNLHCQNTCSPNQSLFINVTRVAGGGGGRPQAVVAYEAFYQDTFAQQTYDSCSRVRIPA  179
mouse  120  FVSIHCHNTCSPDQSLFINVTRVVQRDPGQLPAVVAYEAFYQRSFAEKAYESCSRVRIPA  179
human  120  FVNLHCHNTCSPNQSLFINVTRVAQLGAGQLPAVVAYEAFYQHSFAEQSYDSCSRVRVPA  179
rat    120  FVSLHCHNTCSPDQSLFINVTRVVERGAGEPPAVVAYEAFYQRSFAEKAYESCSQVRIPA  179

dog    180  AATLAVGTMCGVYGSTLCNAQRWLNFQGDTSNGLAPLDITFHLMEPGQALGSGMQALTGE  239
mouse  180  AASLAVGSMCGVYGSALCNAQRWLNFQGDTGNGLAPLDITFHLLEPGQALADGMKPLDGK  239
human  180  AATLAVGTMCGVYGSALCNAQRWLNFQGDTGNGLAPLDITFHLLEPGQAVGSGIQPLNEG  239
rat    180  AASLAVGSMCGVYGSALCNAQRWLNFQGDTGNGLAPLDITFHLLEPGQALPDGIQPLNGK  239

                                    F1                          TMD1
dog    240  IRPCNESQGNGTVACSCQDCAASCPTIPQPQALDSTFYLGGLEGGLALVIILCSAFALLT  299
mouse  240  ITPCNESQGEDSAACSCQDCAASCPVIPPPPALRPSFYMGRMPGWLALIIIFTAVFVLLS  299
human  240  VARCNESQGDDVATCSCQDCAASCPAIARPQALDSTFYLGQMPGSLVLIIILCSVFAVVT  299
rat    240  IAPCNESQGDDSAVCSCQDCAASCPVIPPPEALRPSFYMGRMPGWLALIIIFTAVFVLLS  299

dog    300  TFLVGTRLASSCGKDKTPDPKAGMSLSDKLSLSTNVILSQCFQNWGTWVASWPLTILLVS  359
mouse  300  VVLVYLRVASNRNKNKTAGSQEAPNLPRKRRFSPHTVLGRFFESWGTRVASWPLTVLALS  359
human  300  ILLVGFRVAPARDKSKMVDPKKGTSLSDKLSFSTHTLLGQFFQGWGTWVASWPLTILVLS  359
rat    300  AVLVRLRVVSNRNKNKAEGPQEAPKLPHKHKLSPHTILGRFFQNWGTRVASWPLTVLALS  359

             TMD2                    F7,8,9,10,11
dog    360  IAVVLALSGGLAFVELTTDPVELWSAPSSQARSEKAFHDQHFGPFLRTNQVILTAPNRPS  419
mouse  360  FIVVIALSVGLTFIELTTDPVELWSAPKSQARKEKAFHDEHFGPFFRTNQIFVTAKNRSS  419
human  360  VIPVVALAAGLVFTELTTDPVELWSAPNSQARSEKAFHDQHFGPFFRTNQVILTAPNRSS  419
rat    360  FIVVIALAAGLTFIELTTDPVELWSAPKSQARKEKSFHDEHFGPFFRTNQIFVTARNRSS  419

                        F9
dog    420  YHYDSLLLGPKNFSGVLASDLLLELLELQETLRHLQVWSPEEQRHISLQDICFAPLNPHN  479
mouse  420  YKYDSLLLGPKNFSGILSLDLLQELLELQERLRHLQVWSHEAQRNISLQDICYAPLNPHN  479
human  420  YRYDSLLLGPKNFSGILDLDLLLELLELQERLRHLQVWSPEAQRNISLQDICYAPLNPDN  479
rat    420  YKYDSLLLGSKNFSGILSLDFLLELLELQERLRHLQVWSPEAERNISLQDICYAPLNPYN  479
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Table S2. ***Continued***

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                                                F10
dog    480  ASLSDCCINSLLQYFQSNRTHLLLTANQTLTGQTSQVDWRDHFLYCANAPLTFKDGTALA  539
mouse  480  TSLTDCCVNSLLQYFQNNHTLLLLTANQTLNGQTSLVDWKDHFLYCANAPLTYKDGTALA  539
human  480  TSLYDCCINSLLQYFQNNRTLLLLTANQTLMGQTSQVDWKDHFLYCANAPLTFKDGTALA  539
rat    480  TSLSDCCVNSLLQYFQNNRTLLMLTANQTLNGQTSLVDWKDHFLYCANAPLTFKDGTSLA  539

                                                    F11
dog    540  LSCMADYGGPVFPFLAVGGYKGKDYSEAEALIMTFSLNNYAPGDPRLAQAKLWEAAFLEE  599
mouse  540  LSCIADYGAPVFPFLAVGGYQGTDYSEAEALITTFSINNYPADDPRMAHAKLWEEAFLKE  599
human  540  LSCMADYGAPVFPFLAIGGYKGKDYSEAEALIMTFSLNNYPAGDPRLAQAKLWEEAFLEE  599
rat    540  LSCMADYGAPVFPFLAVGGYQGTDYSEAEALIITFSLNNYPADDPRMAQAKLWEEAFLKE  599

                                              F2,7,8              TMD3
dog    600  MKAFQRRTAGTFQVTFMAERSLEDEINRTTAEDLPIFGVSYIIIFLYISLALGSYSSWRR  659
mouse  600  MQSFQRSTADKFQIAFSAERSLEDEINRTTIQDLPVFAISYLIVFLYISLALGSYSRWSR  659
human  600  MRAFQRRMAGMFQVTFMAERSLEDEINRTTAEDLPIFATSYIVIFLYISLALGSYSSWSR  659
rat    600  MESFQRNTSDKFQVAFSAERSLEDEINRTTIQDLPVFAVSYIIVFLYISLALGSYSRCSR  659

                              TMD4                                  TMD5
dog    660  VPVDSKVTLGLGGVAVVLGAVTAAMGFFSYLGVPSSLVILQVVPFLVLAVGADNIFIFVL  719
mouse  660  VAVDSKATLGLGGVAVVLGAVVAAMGFYSYLGVPSSLVIIQVVPFLVLAVGADNIFIFVL  719
human  660  VMVDSKATLGLGGVAVVLGAVMAAMGFFSYLGIRSSLVILQVVPFLVLSVGADNIFIFVL  719
rat    660  VAVESKATLGLGGVIVVLGAVLAAMGFYSYLGVPSSLVIIQVVPFLVLAVGADNIFIFVL  719

                                                    TMD6
dog    720  EYQRLPRRPGEPREAHIGRALGSVAPSMLLCSLSEAICFFLGALTPMPAVKTFALISGFA  779
mouse  720  EYQRLPRMPGEQREAHIGRTLGSVAPSMLLCSLSEAICFFLGALTSMPAVRTFALTSGLA  779
human  720  EYQRLPRRPGEPREVHIGRALGRVAPSMLLCSLSEAICFFLGALTPMPAVRTFALTSGLA  779
rat    720  EYQRLPRMPGEQREAHIGRTLGSVAPSMLLCSLSEAICFFLGALTPMPAVRTFALTSGLA  779

                     TMD7                  F3
dog    780  IVLDFLLQVSAFVALLSLDSRRQEASRLDVCCCVSAPKLPAPGQSEGLLLRVFRKFYVPV  839
mouse  780  IIFDFLLQMTAFVALLSLDSKRQEASRPDVVCCFSSRNLPPPKQKEGLLLCFFRKIYTPF  839
human  780  VILDFLLQMSAFVALLSLDSKRQEASRLDVCCCVKPQELPPPGQGEGLLLGFFQKAYAPF  839
rat    780  IILDFLLQMTAFVALLSLDSKRQEASRPDVLCCFSTRKLPPPKEKEGLLLRFFRKIYAPF  839

                                    TMD8            F4
dog    840  LLHRVTRAVVLLLFTGLFGVGLYFMCHIRVGLDQELALPKDSYLLDYFFFLNRYFEVGAP  899
mouse  840  LLHRFIRPVVLLLFLVLFGANLYLMCNISVGLDQDLALPKDSYLIDYFLFLNRYLEVGPP  899
human  840  LLHWITRGVVLLLFLALFGVSLYSMCHISVGLDQELALPKDSYLLDYFLFLNRYFEVGAP  899
rat    840  LLHRFIRPVVMLLFLTLFGANLYLMCNINVGLDQELALPKDSYLIDYFLFLNRYLEVGPP  899

dog    900  VYFVTTGGYNFSSEAGMNAVCSSAGCDSYSLTQKIQYATEFPEESYLAIPASSWVDDFID  959
mouse  900  VYFDTTSGYNFSTEAGMNAICSSAGCESFSLTQKIQYASEFPNQSYVAIAASSWVDDFID  959
human  900  VYFVTTLGYNFSSEAGMNAICSSAGCNNFSFTQKIQYATEFPEQSYLAIPASSWVDDFID  959
rat    900  VYFVTTSGFNFSSEAGMNATCSSAGCKSFSLTQKIQYASEFPDQSYVAIAASSWVDDFID  959

dog    960  WLTPSS-CCRLYAFGANKDKFCPSTVNSLACLKNCVNFTLGPVRPSVDQFHKYLPWFLSD 1018
mouse  960  WLTPSSSCCRIYTRGPHKDEFCPSTDTSFNCLKNCMNRTLGPVRPTTEQFHKYLPWFLND 1019
human  960  WLTPSS-CCRLYISGPNKDKFCPSTVNSLNCLKNCMSITMGSVRPSVEQFHKYLPWFLND 1018
rat    960  WLTPSSSCCRLYIRGPHKDEFCPSTDTSFNCLKNCMNRTLGPVRPTAEQFHKYLPWFLND 1019

dog   1019  PPNIKCPKGGLAAYNTSVHLGSDGQVLASRFMAYHKPLRNSEDYTEALRVSRALAANITA 1078
mouse 1020  TPNIRCPKGGLAAYRTSVNLSSDGQIIASQFMAYHKPLRNSQDFTEALRASRLLAANITA 1079
human 1019  RPNIKCPKGGLAAYSTSVNLTSDGQVLASRFMAYHKPLKNSQDYTEALRAARELAANITA 1078
rat   1020  PPNIRCPKGGLAAYRTSVNLSSDGQVIASQFMAYHKPLRNSQDFTEALRASRLLAANITA 1079

                                        F5                  TMD9
dog   1079  QLRQVPGTDPAFEVFPYTITNVFYEQYLSVVPEGLFMLAICLLPTFVVCCLLLGMDLRSG 1138
mouse 1080  ELRKVPGTDPNFEVFPYTISNVFYQQYLTVLPEGIFTLALCFVPTFVVCYLLLGLDIRSG 1139
human 1079  DLRKVPGTDPAFEVFPYTITNVFYEQYLTILPEGLFMLSLCLVPTFAVSCLLLGLDLRSG 1138
rat   1080  DLRKVPGTDPNFEVFPYTISNVFYQQYLTVLPEGIFTLALCFVPTFVVCYLLLGLDMCSG 1139
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Table S2. ***Continued***

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                          TMD10                          TMD11
dog    1139 LLNLFSIVMILVDTVGFMALWGISYNAVSLINLVTAVGISVEFVSHITRSFAVSTRPTRL 1198
mouse  1140 ILNLLSIIMILVDTIGLMAVWGISYNAVSLINLVTAVGMSVEFVSHITRSFAVSTKPTRL 1199
human  1139 LLNLLSIVMILVDTVGFMALWGISYNAVSLINLVSAVGMSVEFVSHITRSFAISTKPTWL 1198
rat    1140 ILNLLSIIMILVDTIGLMAVWGISYNAVSLINLVTAVGMSVEFVSHITRSFAVSTKPTRL 1199

                              TMD12                                  TMD13
dog    1199 ERAKEATISMGSAVFAGVAMTNLPGILVLGLAKAQLIQIFFFRLNLLITVLGLLHGLVFL 1258
mouse  1200 ERAKDATIFMGSAVFAGVAMTNFPGILILGFAQAQLIQIFFFRLNLLITLLGLLHGLVFL 1259
human  1199 ERAKEATISMGSAVFAGVAMTNLPGILVLGLAKAQLIQIFFFRLNLLITLLGLLHGLVFL 1258
rat    1200 ERAKDATVFMGSAVFAGVAMTNFPGILILGFAQAQLIQIFFFRLNLLITLLGLLHGLVFL 1259

                      F6
dog    1259 PVVLSYLGPDINAALVLDQKKTEEAIGA------PAHLVPTSTASSTYVNYGFQHP-ANG 1311
mouse  1260 PVVLSYLGPDVNQALVLEEKLATEA-AMVSEPSCPQYPFPADANTSDYVNYGFNPEFIPE 1318
human  1259 PVILSYVGPDVNPALALEQKRAEEAVAAVMVASCPNHPSRVSTADNIYVNHSFEGS-IKG 1317
rat    1260 PVVLSYLGPDVNQALVQEEKLASEA-AVAPEPSCPQYPSPADADAN--VNYGFAPELAHG 1316

dog    1312 VVGDSSLPRSGPD-L  1325
mouse  1319 INAASSSLPKSDQKF  1333
human  1318 AGAISNFLPNNGRQF  1332
rat    1317 ANAARSSLPKSDQKF  1331
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TMD prediction was made with HMMTOP and TMHMM servers available through http://expasy.org/tools/#ptm and manually refined. Transmembranes are denoted with dark green text. The point at which the amino acid sequence between dog and mouse NPC1L1 diverges in chimera's is highlighted in red text as the fusion point (F1-F11). Molecular determinants of high affinity in dog NPC1L1 (Tyr532 and Met543) are highlighted in purple. Hot-spot of cholesterol hypo-absorption polymorphisms is highlighted in blue. Tryptic peptides identified by LC/MS/MS are highlighted in pink.