

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
24 February 2005 (24.02.2005)

PCT

(10) International Publication Number
WO 2005/015988 A1

(51) International Patent Classification⁷: **A01K 67/00**,
67/033

(21) International Application Number:
PCT/US2003/040113

(22) International Filing Date:
16 December 2003 (16.12.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10/621,758 17 July 2003 (17.07.2003) US
10/646,301 22 August 2003 (22.08.2003) US
10/663,208 16 September 2003 (16.09.2003) US

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR,
CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE,
HR, HU, ID, IL, IN, IS, JP, KG, KR, KZ, LC, LK, LR, LT,
LU, LV, MA, MD, MG, MK, MN, MX, MZ, NI, NO, NZ,
PG, PH, PL, PT, RO, RU, SC, SE, SG, SK, SL, SY, TJ, TM,
TN, TR, TT, TZ, UA, UZ, VC, VN, YU, ZA, ZM.

(84) Designated States (*regional*): ARIPO patent (BW, GH,
GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE,
SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA,
GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii)) for all designations
- as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii)) for all designations
- as to the applicant's entitlement to claim the priority of the
earlier application (Rule 4.17(iii)) for all designations

Published:

- with international search report

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: NPC1L1 (NPC3) AND METHODS OF USE THEREOF

(57) Abstract: The present invention provides human, rat and mouse NPC1L1 polypeptides and polynucleotides encoding the polypeptides. Also provided are methods for detecting agonists and antagonists of NPC1L1. Inhibitors of NPC1L1 can be used for inhibiting intestinal cholesterol absorption in a subject.



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NPC1L1 (NPC3) AND METHODS OF USE THEREOF

This application is a continuation-in-part of U.S. Patent Application No. 10/663,208; filed September 16, 2003 which is a continuation-in-part of U.S. Patent Application No. 10/646,301; filed August 22, 2003 which is a continuation-in-part of U.S. Patent Application No. 10/621,758; filed July 17, 2003 which claims the benefit of U.S. Provisional Patent Application No. 60/397,442; filed July 19, 2002 each of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention includes NPC1L1 polypeptides and polynucleotides which encode the polypeptides along with methods of use thereof.

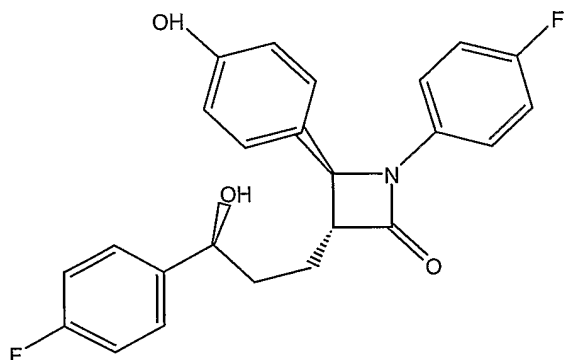
BACKGROUND OF THE INVENTION

A factor leading to development of vascular disease, a leading cause of death in industrialized nations, is elevated serum cholesterol. It is estimated that 19% of Americans between the ages of 20 and 74 years of age have high serum cholesterol. The most prevalent form of vascular disease is arteriosclerosis, a condition associated with the thickening and hardening of the arterial wall. Arteriosclerosis of the large vessels is referred to as atherosclerosis. Atherosclerosis is the predominant underlying factor in vascular disorders such as coronary artery disease, aortic aneurysm, arterial disease of the lower extremities and cerebrovascular disease.

Cholesteryl esters are a major component of atherosclerotic lesions and the major storage form of cholesterol in arterial wall cells. Formation of cholesteryl esters is also a step in the intestinal absorption of dietary cholesterol. Thus, inhibition of cholesteryl ester formation and reduction of serum cholesterol can inhibit the progression of atherosclerotic lesion formation, decrease the accumulation of cholesteryl esters in the arterial wall, and block the intestinal absorption of dietary cholesterol.

The regulation of whole-body cholesterol homeostasis in mammals and animals involves the regulation of intestinal cholesterol absorption, cellular cholesterol trafficking, dietary cholesterol and modulation of cholesterol biosynthesis, bile acid biosynthesis, steroid biosynthesis and the catabolism of the cholesterol-containing plasma lipoproteins. Regulation of intestinal cholesterol absorption has proven to be an effective

means by which to regulate serum cholesterol levels. For example, a cholesterol absorption inhibitor, ezetimibe (



), has been shown to be effective in this regard. A pharmaceutical composition

5 containing ezetimibe is commercially available from Merck/Schering-Plough Pharmaceuticals, Inc. under the tradename Zetia®. Identification of a gene target through which ezetimibe acts is important to understanding the process of cholesterol absorption and to the development of other, novel absorption inhibitors. The present invention addresses this need by providing a rat and a mouse homologue of human NPC1L1 (also
10 known as NPC3; Genbank Accession No. AF192522; Davies, *et al.*, (2000) Genomics 65(2):137-45 and Ioannou, (2000) Mol. Genet. Metab. 71(1-2):175-81), an ezetimibe target.

NPC1L1 is an N-glycosylated protein comprising a YQRL (SEQ ID NO: 38) motif (*i.e.*, a *trans*-golgi network to plasma membrane transport signal; see Bos, *et al.*,
15 (1993) EMBO J. 12:2219-2228; Humphrey, *et al.*, (1993) J. Cell. Biol. 120:1123-1135; Ponnambalam, *et al.*, (1994) J. Cell. Biol. 125:253-268 and Rothman, *et al.*, (1996) Science 272:227-234) which exhibits limited tissue distribution and gastrointestinal abundance. Also, the human *NPC1L1* promoter includes a Sterol Regulated Element Binding Protein 1 (SREBP1) binding consensus sequence (Athaniar, *et al.*, (1998) Proc.
20 Natl. Acad. Sci. USA 95:4935-4940; Ericsson, *et al.*, (1996) Proc. Natl. Acad. Sci. USA 93:945-950; Metherall, *et al.*, (1989) J. Biol. Chem. 264:15634-15641; Smith, *et al.*, (1990) J. Biol. Chem. 265:2306-2310; Bennett, *et al.*, (1999) J. Biol. Chem. 274:13025-13032 and Brown, *et al.*, (1997) Cell 89:331-340). NPC1L1 has 42% amino acid sequence homology to human NPC1 (Genbank Accession No. AF002020), a receptor
25 responsible for Niemann-Pick C1 disease (Carstea, *et al.*, (1997) Science 277:228-231). Niemann-Pick C1 disease is a rare genetic disorder in humans which results in accumulation of low density lipoprotein (LDL)-derived unesterified cholesterol in lysosomes (Pentchev, *et al.*, (1994) Biochim. Biophys. Acta. 1225: 235-243 and Vanier, *et al.*, (1991) Biochim. Biophys. Acta. 1096:328-337). In addition, cholesterol

accumulates in the *trans*-golgi network of *npc1⁻* cells, and relocation of cholesterol, to and from the plasma membrane, is delayed. NPC1 and NPC1L1 each possess 13 transmembrane spanning segments as well as a sterol-sensing domain (SSD). Several other proteins, including HMG-CoA Reductase (HMG-R), Patched (PTC) and Sterol
5 Regulatory Element Binding Protein Cleavage-Activation Protein (SCAP), include an SSD which is involved in sensing cholesterol levels possibly by a mechanism which involves direct cholesterol binding (Gil, *et al.*, (1985) Cell 41:249-258; Kumagai, *et al.*, (1995) J. Biol. Chem. 270:19107-19113 and Hua, *et al.*, (1996) Cell 87:415-426).

SUMMARY OF THE INVENTION

10 The present invention includes an isolated polypeptide comprising 42 or more contiguous amino acids from an amino acid sequence selected from SEQ ID NOs: 2 and 12, preferably comprising the amino acid sequence selected from SEQ ID NOs: 2 and 12. The present invention also comprises an isolated polypeptide comprising the amino acid
15 sequence of SEQ ID NO: 4. The invention also includes an isolated polynucleotide encoding a polypeptide of SEQ ID NO: 2, 4 or 12, preferably comprising a nucleotide sequence selected from SEQ ID NOs: 1, 3, 5-10, 11 and 13. A recombinant vector comprising a polynucleotide of the invention is also provided along with a host cell comprising the vector.

20 The present invention also provides an isolated antibody which specifically binds to or was raised against NPC1L1 (*e.g.*, rat NPC1L1, mouse NPC1L1 or human NPC1L1) or any antigenic fragment thereof, preferably rat NPC1L1, more preferably a polypeptide comprising an amino acid sequence selected from SEQ ID NO: 39-42. Preferably, the antibody is an isolated polyclonal or monoclonal antibody. In one embodiment, the
25 antibody is obtained from a rabbit.

The present invention also includes a method for making an NPC1L1 polypeptide of the invention comprising culturing a host cell of the invention under conditions in which the nucleic acid in the cell which encodes the NPC1L1 polypeptide is expressed. Preferably, the method includes the step of isolating the polypeptide from the culture.

30 The present invention includes methods for identifying an agonist or antagonist of NPC1L1 comprising (a) contacting a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of a
35 detectably labeled (*e.g.*, with ³H, ¹⁴C or ¹²⁵I) substituted azetidinone (*e.g.*, ezetimibe), with

a sample to be tested for the presence of an NPC1L1 agonist or antagonist; and (b) measuring the amount of detectably labeled substituted azetidinone (*e.g.*, ezetimibe) specifically bound to the polypeptide; wherein an NPC1L1 agonist or antagonist in the sample is identified by measuring substantially reduced binding of the detectably labeled substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide, compared to what would be measured in the absence of such an agonist or antagonist.

Another method for identifying an agonist or antagonist of NPC1L1 is also provided. The method comprises (a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer (*e.g.*, yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene), to which a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface are attached; (b) adding, to the suspension, a radiolabeled (*e.g.*, with ^3H , ^{14}C or ^{125}I) substituted azetidinone (*e.g.*, ezetimibe) and a sample to be tested for the presence of an antagonist or agonist, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon the binding of the substituted azetidinone (*e.g.*, ezetimibe) to the polypeptide to produce light energy, whereas radiolabeled substituted azetidinone (*e.g.*, ezetimibe) that does not bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and (c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 agonist or antagonist in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such an agonist or antagonist.

Also provided is a method for identifying an agonist or antagonist of NPC1L1 comprising (a) contacting a host cell (*e.g.*, chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell or a Caco2 cell) expressing an polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 or SEQ ID NO: 12 or a functional fragment thereof on a cell surface with detectably labeled (*e.g.*, with ^3H , ^{14}C or ^{125}I) sterol (*e.g.*, cholesterol) or 5α -stanol and with a sample to be tested for the presence of an antagonist or agonist; and (b) measuring the amount of detectably labeled sterol (*e.g.*, cholesterol) or 5α -stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol (*e.g.*, cholesterol) or 5α -stanol within the host cell, compared to what would be measured in the absence of such an antagonist and wherein an NPC1L1 agonist in the sample is identified by measuring

substantially increased detectably labeled sterol (*e.g.*, cholesterol) or 5 α -stanol within the host cell, compared to what would be measured in the absence of such an agonist.

The present invention includes methods for inhibiting NPC1L1-mediated intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol uptake, in a subject, by administering a substance identified by the screening methods described herein to the subject. Such substances include compounds such as small molecule antagonists of NPC1L1 other than ezetimibe. Also contemplated are methods for antagonizing NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5 α -stanol absorption by administering anti-NPC1L1 antibodies. NPC1L1-mediated absorption of sterol (*e.g.*, cholesterol) or 5 α -stanol can also be antagonized by any method which reduces expression of NPC1L1 in an organism. For example, NPC1L1 expression can be reduced by introduction of anti-sense *NPC1L1* mRNA into a cell of an organism or by genetic mutation of the *NPC1L1* gene in an organism (*e.g.*, by complete knockout, disruption, truncation or by introduction of one or more point mutations).

Also included in the present invention is a mutant transgenic mammal (*e.g.*, mouse, rat, dog, rabbit, pig, guinea pig, cat, horse), preferably a mouse comprising a homozygous or heterozygous mutation (*e.g.*, disruption, truncation, one or more point mutations, knock out) of endogenous, chromosomal *NPC1L1* wherein, preferably, the mouse does not produce any functional NPC1L1 protein. Preferably, the mutant mouse, lacking functional NPC1L1, exhibits a reduced level of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption and/or a reduced level of serum sterol (*e.g.*, cholesterol) or 5 α -stanol and/or a reduced level of liver sterol (*e.g.*, cholesterol) or 5 α -stanol as compared to that of a non-mutant mouse comprising functional NPC1L1. Preferably, in the mutant mouse chromosome, the region of *NPC1L1* (SEQ ID NO: 45) deleted is from nucleotide 790 to nucleotide 998. In one embodiment, *NPC1L1* (SEQ ID NO: 11) is deleted from nucleotide 767 to nucleotide 975. Any offspring or progeny of a parent *NPC1L1* mutant mouse (*i.e.*, *npc1l1*) of the invention which has inherited an *npc1l1* mutant allele is also part of the present invention.

The scope of the present invention also includes a method for screening a sample for an intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption antagonist comprising (a) feeding a sterol (*e.g.*, cholesterol) or 5 α -stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ¹⁴C-cholesterol or ³H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional *NPC1L1*; (b) administering the sample to the first mouse comprising a

functional *NPC1L1* but not to the second mouse; (c) measuring the amount of sterol (e.g., cholesterol) or 5 α -stanol absorption in the intestine of said first, second and third mouse (e.g., by measuring serum cholesterol); and (d) comparing the levels of intestinal sterol (e.g., cholesterol) or 5 α -stanol absorption in each mouse; wherein the sample is
5 determined to contain the intestinal sterol (e.g., cholesterol) or 5 α -stanol absorption antagonist when the level of intestinal sterol (e.g., cholesterol) or 5 α -stanol absorption in the first mouse and third mouse are less than the amount of intestinal sterol (e.g., cholesterol) or 5 α -stanol absorption in the second mouse.

The present invention also encompasses a kit comprising (a) a substituted
10 azetidinone (e.g., ezetimibe) in a pharmaceutical dosage form (e.g., a pill or tablet comprising 10mg substituted azetidinone (e.g., ezetimibe)); and (b) information, for example in the form of an insert, indicating that NPC1L1 is a target of ezetimibe. The kit may also include simvastatin in a pharmaceutical dosage form (e.g., a pill or tablet comprising 5 mg, 10 mg, 20 mg, 40 mg or 80mg simvastatin). The simvastatin in
15 pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form can be associated in a single pill or tablet or in separate pills or tablets.

The present invention also provides any isolated mammalian cell (e.g., isolated mouse cell, isolated rat cell or isolated human cell) which lacks a gene which encodes or can produce a functional NPC1L1 polypeptide. The isolated cell of can be isolated from
20 a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein. Further, the mutation can be in a gene, which when un-mutated, encodes an amino acid sequence of SEQ ID NO: 12 (e.g., comprising a nucleotide sequence of SEQ ID NO: 11). The cell can be isolated or derived from duodenum, gall bladder, liver, small intestine or stomach
25 tissue. The cell can be an enterocyte.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes an NPC1L1 polypeptide from rat, human and from mouse along with polynucleotides encoding the respective polypeptides. Preferably, the
30 rat NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 2, the human NPC1L1 comprises the amino acid sequence set forth in SEQ ID NO: 4 and the mouse NPC1L1 polypeptide comprises the amino acid sequence set forth in SEQ ID NO.12. The rat *NPC1L1* polynucleotide of SEQ ID NO:1 or 10 encodes the rat NPC1L1 polypeptide. The human *NPC1L1* polynucleotide of SEQ ID NO: 3 encodes the human

NPC1L1 polypeptide. The mouse *NPC1L1* polynucleotide of SEQ ID NO:11 or 13 encodes the mouse NPC1L1 polypeptide.

The present invention includes any isolated polynucleotide or isolated polypeptide comprising a nucleotide or amino acid sequence referred to, below, in Table 1.

5

Table 1. Polynucleotides and Polypeptides of the Invention.

Polynucleotide or Polypeptide	Sequence Identifier
Rat <i>NPC1L1</i> polynucleotide	SEQ ID NO: 1
Rat NPC1L1 polypeptide	SEQ ID NO: 2
Human <i>NPC1L1</i> polynucleotide	SEQ ID NO: 3
Human NPC1L1 polypeptide	SEQ ID NO: 4
Rat <i>NPC1L1</i> expressed sequence tag 603662080F1 (partial sequence)	SEQ ID NO: 5
Rat <i>NPC1L1</i> expressed sequence tag 603665037F1 (partial sequence)	SEQ ID NO: 6
Rat <i>NPC1L1</i> expressed sequence tag 604034587F1 (partial sequence)	SEQ ID NO: 7
EST 603662080F1 with downstream sequences added	SEQ ID NO: 8
EST 603662080F1 with upstream and downstream sequences added	SEQ ID NO: 9
Back-translated polynucleotide sequence of rat NPC1L1	SEQ ID NO: 10
Mouse <i>NPC1L1</i> polynucleotide	SEQ ID NO: 11
Mouse NPC1L1 polypeptide	SEQ ID NO: 12
Back-translated polynucleotide sequence of mouse NPC1L1	SEQ ID NO: 13
Back-translated polynucleotide sequence of human NPC1L1	SEQ ID NO: 51

A human NPC1L1 is also disclosed under Genbank Accession Number AF192522. As discussed below, the nucleotide sequence of the rat *NPC1L1* set forth in SEQ ID NO: 1 was obtained from an expressed sequence tag (EST) from a rat jejunum enterocyte cDNA library. SEQ ID NOs: 5-7 include partial nucleotide sequences of three independent cDNA clones. The downstream sequence of the SEQ ID NO: 5 EST

10

(603662080F1) were determined; the sequencing data from these experiments are set forth in SEQ ID NO: 8. The upstream sequences were also determined; these data are set forth in SEQ ID NO: 9.

5 SEQ ID NOS: 43 and 44 are the nucleotide and amino acid sequence, respectively, of human NPC1L1 which is disclosed under Genbank Accession No.: AF192522 (see Davies, *et al.*, (2000) Genomics 65(2):137-45).

SEQ ID NO: 45 is the nucleotide sequence of a mouse *NPC1L1* which is disclosed under Genbank Accession No. AK078947.

10 NPC1L1 mediates intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption. Inhibition of NPC1L1 in a patient is a useful method for reducing intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption and serum sterol (*e.g.*, cholesterol) or 5 α -stanol in the patient. Reducing the level of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption and serum sterol (*e.g.*, cholesterol) or 5 α -stanol in a patient is a useful way in which to treat or prevent the occurrence of atherosclerosis, particularly diet-induced
15 atherosclerosis.

As used herein, the term "sterol" includes, but is not limited to, cholesterol and phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol)).

20 As used herein, the term "5 α -stanol" includes, but is not limited to, cholestanol, 5 α -campestanol and 5 α -sitostanol.

Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of
25 the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook, *et al.*, 1989"); DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. (1985)); Transcription And Translation (B.D. Hames & S.J. Higgins, eds. (1984)); Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984); F.M. Ausubel, *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

The back-translated sequences of SEQ ID NO: 10 and of SEQ ID NO: 13 uses the single-letter code shown in Table 1 of Annex C, Appendix 2 of the PCT Administrative Instruction in the Manual of Patent Examination Procedure.

A "polynucleotide", "nucleic acid" or "nucleic acid molecule" may refer to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester analogs thereof, such as phosphorothioates and thioesters, in single stranded form, double-stranded form or otherwise.

A "polynucleotide sequence", "nucleic acid sequence" or "nucleotide sequence" is a series of nucleotide bases (also called "nucleotides") in a nucleic acid, such as DNA or RNA, and means any chain of two or more nucleotides.

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in production of the product.

The term "gene" means a DNA sequence that codes for or corresponds to a particular sequence of ribonucleotides or amino acids which comprise all or part of one or more RNA molecules, proteins or enzymes, and may or may not include regulatory DNA sequences, such as promoter sequences, which determine, for example, the conditions under which the gene is expressed. Genes may be transcribed from DNA to RNA which may or may not be translated into an amino acid sequence.

The present invention includes nucleic acid fragments of any of SEQ ID NOs: 1, 5-11 or 13. A nucleic acid "fragment" includes at least about 30 (*e.g.*, 31, 32, 33, 34), preferably at least about 35 (*e.g.*, 25, 26, 27, 28, 29, 30, 31, 32, 33 or 34), more preferably at least about 45 (*e.g.*, 35, 36, 37, 38, 39, 40, 41, 42, 43 or 44), and most preferably at least about 126 or more contiguous nucleotides (*e.g.*, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 150, 160, 170, 180, 190, 200, 300, 400, 500, 1000 or 1200) from any of SEQ ID NOs: 1, 5-11 or 13.

The present invention also includes nucleic acid fragments consisting of at least about 7 (*e.g.*, 9, 12, 17, 19), preferably at least about 20 (*e.g.*, 30, 40, 50, 60), more preferably about 70 (*e.g.*, 80, 90, 95), yet more preferably at least about 100 (*e.g.*, 105, 110, 114) and even more preferably at least about 115 (*e.g.*, 117, 119, 120, 122, 124, 125, 126) contiguous nucleotides from any of SEQ ID NOs: 1, 5-11 or 13.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, or 90), that may be

hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest. Oligonucleotides can be labeled, *e.g.*, by incorporation of ³²P-nucleotides, ³H-nucleotides, ¹⁴C-nucleotides, ³⁵S-nucleotides or nucleotides to which a label, such as biotin, has been covalently
5 conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the gene, or to detect the presence of nucleic acids. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer.

10 A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" may refer to a series of two or more amino acids in a protein, peptide or polypeptide.

"Protein", "peptide" or "polypeptide" includes a contiguous string of two or more amino acids. Preferred peptides of the invention include those set forth in any of SEQ ID
15 NOs: 2 or 12 as well as variants and fragments thereof. Such fragments preferably comprise at least about 10 (*e.g.*, 11, 12, 13, 14, 15, 16, 17, 18 or 19), more preferably at least about 20 (*e.g.*, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40), and yet more preferably at least about 42 (*e.g.*, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 110, 120 or 130) or more contiguous amino acid residues from any of SEQ ID NOs: 2 or 12.

20 The present invention also includes polypeptides, preferably antigenic polypeptides, consisting of at least about 7 (*e.g.*, 9, 10, 13, 15, 17, 19), preferably at least about 20 (*e.g.*, 22, 24, 26, 28), yet more preferably at least about 30 (*e.g.*, 32, 34, 36, 38) and even more preferably at least about 40 (*e.g.*, 41, 42) contiguous amino acids from any of SEQ ID NOs: 2 or 12.

25 The polypeptides of the invention can be produced by proteolytic cleavage of an intact peptide, by chemical synthesis or by the application of recombinant DNA technology and are not limited to polypeptides delineated by proteolytic cleavage sites. The polypeptides, either alone or cross-linked or conjugated to a carrier molecule to render them more immunogenic, are useful as antigens to elicit the production of
30 antibodies and fragments thereof. The antibodies can be used, *e.g.*, in immunoassays for immunoaffinity purification or for inhibition of NPC1L1, *etc.*

The terms "isolated polynucleotide" or "isolated polypeptide" include a polynucleotide (*e.g.*, RNA or DNA molecule, or a mixed polymer) or a polypeptide, respectively, which are partially or fully separated from other components that are
35 normally found in cells or in recombinant DNA expression systems. These components

include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

5 "Amplification" of DNA as used herein may denote the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239:487.

10 The term "host cell" includes any cell of any organism that is selected, modified, transfected, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression or replication, by the cell, of a gene, a DNA or RNA sequence or a protein. Preferred host cells include chinese hamster ovary (CHO) cells, murine macrophage J774 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

15 The nucleotide sequence of a nucleic acid may be determined by any method known in the art (*e.g.*, chemical sequencing or enzymatic sequencing). "Chemical sequencing" of DNA includes methods such as that of Maxam and Gilbert (1977) (Proc. Natl. Acad. Sci. USA 74:560), in which DNA is randomly cleaved using individual base-specific reactions. "Enzymatic sequencing" of DNA includes methods such as that of
20 Sanger (Sanger, *et al.*, (1977) Proc. Natl. Acad. Sci. USA 74:5463).

The nucleic acids herein may be flanked by natural regulatory (expression control) sequences, or may be associated with heterologous sequences, including promoters, internal ribosome entry sites (IRES) and other ribosome binding site sequences, enhancers, response elements, suppressors, signal sequences, polyadenylation
25 sequences, introns, 5'- and 3'- non-coding regions, and the like.

In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence. A promoter sequence is, in general, bounded at its 3' terminus by the transcription initiation
30 site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at any level. Within the promoter sequence may be found a transcription initiation site (conveniently defined, for example, by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. The promoter may be operably
35 associated with other expression control sequences, including enhancer and repressor

sequences or with a nucleic acid of the invention. Promoters which may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist, *et al.*, (1981) *Nature* 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, (1980) *Cell* 22:787-797), the herpes thymidine kinase promoter (Wagner, *et al.*, (1981) *Proc. Natl. Acad. Sci. USA* 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster, *et al.*, (1982) *Nature* 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Komaroff, *et al.*, (1978) *Proc. Natl. Acad. Sci. USA* 75:3727-3731), or the *tac* promoter (DeBoer, *et al.*, (1983) *Proc. Natl. Acad. Sci. USA* 80:21-25); see also "Useful proteins from recombinant bacteria" in *Scientific American* (1980) 242:74-94; and promoter elements from yeast or other fungi such as the *Gal 4* promoter, the *ADC* (alcohol dehydrogenase) promoter, *PGK* (phosphoglycerol kinase) promoter or the alkaline phosphatase promoter.

A coding sequence is "under the control of", "functionally associated with" or "operably associated with" transcriptional and translational control sequences in a cell when the sequences direct RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene, RNA or DNA sequence to become manifest; for example, producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene. A DNA sequence is expressed in or by a cell to form an "expression product" such as an RNA (*e.g.*, mRNA) or a protein. The expression product itself may also be said to be "expressed" by the cell.

The term "transformation" means the introduction of a nucleic acid into a cell. The introduced gene or sequence may be called a "clone". A host cell that receives the introduced DNA or RNA has been "transformed" and is a "transformant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or from cells of a different genus or species.

The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence.

Vectors that can be used in this invention include plasmids, viruses, bacteriophage, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of the host. Plasmids are the most

commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, e.g., Pourwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Butterworth, Boston, MA.

The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

Expression of nucleic acids encoding the NPC1L1 polypeptides of this invention can be carried out by conventional methods in either prokaryotic or eukaryotic cells. Although *E. coli* host cells are employed most frequently in prokaryotic systems, many other bacteria, such as various strains of *Pseudomonas* and *Bacillus*, are known in the art and can be used as well. Suitable host cells for expressing nucleic acids encoding the NPC1L1 polypeptides include prokaryotes and higher eukaryotes. Prokaryotes include both gram-negative and gram-positive organisms, e.g., *E. coli* and *B. subtilis*. Higher eukaryotes include established tissue culture cell lines from animal cells, both of non-mammalian origin, e.g., insect cells, and birds, and of mammalian origin, e.g., human, primates, and rodents.

Prokaryotic host-vector systems include a wide variety of vectors for many different species. A representative vector for amplifying DNA is pBR322 or many of its derivatives (e.g., pUC18 or 19). Vectors that can be used to express the NPC1L1 polypeptides include, but are not limited to, those containing the *lac* promoter (pUC-series); *trp* promoter (pBR322-*trp*); *Ipp* promoter (the pIN-series); lambda-pP or pR promoters (pOTS); or hybrid promoters such as *ptac* (pDR540). See Brosius *et al.*, "Expression Vectors Employing Lambda-, *trp*-, *lac*-, and *Ipp*-derived Promoters", in Rodriguez and Denhardt (eds.) Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Butterworth, Boston, pp. 205-236. Many polypeptides can be expressed, at high levels, in an *E. coli*/T7 expression system as disclosed in U.S. Patent Nos. 4,952,496, 5,693,489 and 5,869,320 and in Davanloo, P., *et al.*, (1984) Proc. Natl. Acad. Sci. USA 81: 2035-2039; Studier, F. W., *et al.*, (1986) J. Mol. Biol. 189: 113-130; Rosenberg, A. H., *et al.*, (1987) Gene 56: 125-135; and Dunn, J. J., *et al.*, (1988) Gene 68: 259.

Higher eukaryotic tissue culture cells may also be used for the recombinant production of the NPC1L1 polypeptides of the invention. Although any higher eukaryotic tissue culture cell line might be used, including insect baculovirus expression systems, mammalian cells are preferred. Transformation or transfection and propagation of such cells have become a routine procedure. Examples of useful cell lines include HeLa cells, chinese hamster ovary (CHO) cell lines, J774 cells, Caco2 cells, baby rat kidney (BRK) cell lines, insect cell lines, bird cell lines, and monkey (COS) cell lines. Expression vectors for such cell lines usually include an origin of replication, a promoter, a translation initiation site, RNA splice sites (if genomic DNA is used), a polyadenylation site, and a transcription termination site. These vectors also, usually, contain a selection gene or amplification gene. Suitable expression vectors may be plasmids, viruses, or retroviruses carrying promoters derived, *e.g.*, from such sources as adenovirus, SV40, parvoviruses, vaccinia virus, or cytomegalovirus. Examples of expression vectors include pCR[®]3.1, pCDNA1, pCD (Okayama, *et al.*, (1985) Mol. Cell Biol. 5:1136), pMC1neo Poly-A (Thomas, *et al.*, (1987) Cell 51:503), pREP8, pSVSPORT and derivatives thereof, and baculovirus vectors such as pAC373 or pAC610. One embodiment of the invention includes membrane bound NPC1L1. In this embodiment, NPC1L1 can be expressed in the cell membrane of a eukaryotic cell and the membrane bound protein can be isolated from the cell by conventional methods which are known in the art.

The present invention also includes fusions which include the NPC1L1 polypeptides and *NPC1L1* polynucleotides of the present invention and a second polypeptide or polynucleotide moiety, which may be referred to as a "tag". The fusions of the present invention may comprise any of the polynucleotides or polypeptides set forth in Table 1 or any subsequence or fragment thereof (discussed above). The fused polypeptides of the invention may be conveniently constructed, for example, by insertion of a polynucleotide of the invention or fragment thereof into an expression vector. The fusions of the invention may include tags which facilitate purification or detection. Such tags include glutathione-S-transferase (GST), hexahistidine (His6) tags, maltose binding protein (MBP) tags, haemagglutinin (HA) tags, cellulose binding protein (CBP) tags and myc tags. Detectable tags such as ³²P, ³⁵S, ³H, ^{99m}Tc, ¹²³I, ¹¹¹In, ⁶⁸Ga, ¹⁸F, ¹²⁵I, ¹³¹I, ^{113m}In, ⁷⁶Br, ⁶⁷Ga, ^{99m}Tc, ¹²³I, ¹¹¹In and ⁶⁸Ga may also be used to label the polypeptides and polynucleotides of the invention. Methods for constructing and using such fusions are very conventional and well known in the art.

Modifications (*e.g.*, post-translational modifications) that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned

gene in a host, for instance, the nature and extent of the modifications, in large part, will be determined by the host cell's post-translational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*.

5 Accordingly, when glycosylation is desired, a polypeptide can be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out post-translational glycosylations which are similar to those of mammalian cells. For this reason, insect cell expression systems have been developed to express, efficiently, mammalian proteins having native patterns of glycosylation. An insect cell which may be
10 used in this invention is any cell derived from an organism of the class *Insecta*. Preferably, the insect is *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusia ni* (High 5). Examples of insect expression systems that can be used with the present invention, for example to produce NPC1L1 polypeptide, include Bac-To-Bac (Invitrogen Corporation, Carlsbad, CA) or Gateway (Invitrogen Corporation, Carlsbad, CA). If desired,
15 deglycosylation enzymes can be used to remove carbohydrates attached during production in eukaryotic expression systems.

Other modifications may also include addition of aliphatic esters or amides to the polypeptide carboxyl terminus. The present invention also includes analogs of the NPC1L1 polypeptides which contain modifications, such as incorporation of unnatural
20 amino acid residues, or phosphorylated amino acid residues such as phosphotyrosine, phosphoserine or phosphothreonine residues. Other potential modifications include sulfonation, biotinylation, or the addition of other moieties. For example, the NPC1L1 polypeptides of the invention may be appended with a polymer which increases the half-life of the peptide in the body of a subject. Preferred polymers include polyethylene
25 glycol (PEG) (e.g., PEG with a molecular weight of 2 kDa, 5 kDa, 10 kDa, 12 kDa, 20 kDa, 30 kDa and 40 kDa), dextran and monomethoxypolyethylene glycol (mPEG).

The peptides of the invention may also be cyclized. Specifically, the amino- and carboxy-terminal residues of an NPC1L1 polypeptide or two internal residues of an NPC1L1 polypeptide of the invention can be fused to create a cyclized peptide. Methods
30 for cyclizing peptides are conventional and very well known in the art; for example see Gurrath, *et al.*, (1992) Eur. J. Biochem. 210:911-921.

The present invention contemplates any superficial or slight modification to the amino acid or nucleotide sequences which correspond to the polypeptides of the invention. In particular, the present invention contemplates sequence conservative
35 variants of the nucleic acids which encode the polypeptides of the invention. "Sequence-

conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon results in no alteration in the amino acid encoded at that position. Function-conservative variants of the polypeptides of the invention are also contemplated by the present invention. "Function-conservative variants" are those in which one or more amino acid residues in a protein or enzyme have been changed without altering the overall conformation and function of the polypeptide, including, but, by no means, limited to, replacement of an amino acid with one having similar properties. Amino acids with similar properties are well known in the art. For example, polar/hydrophilic amino acids which may be interchangeable include asparagine, glutamine, serine, cysteine, threonine, lysine, arginine, histidine, aspartic acid and glutamic acid; nonpolar/hydrophobic amino acids which may be interchangeable include glycine, alanine, valine, leucine, isoleucine, proline, tyrosine, phenylalanine, tryptophan and methionine; acidic amino acids which may be interchangeable include aspartic acid and glutamic acid and basic amino acids which may be interchangeable include histidine, lysine and arginine.

The present invention includes polynucleotides encoding rat, human or mouse NPC1L1 and fragments thereof as well as nucleic acids which hybridize to the polynucleotides. Preferably, the nucleic acids hybridize under low stringency conditions, more preferably under moderate stringency conditions and most preferably under high stringency conditions. A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook, *et al.*, *supra*). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Typical low stringency hybridization conditions are 55°C, 5X SSC, 0.1% SDS, 0.25% milk, and no formamide at 42°C; or 30% formamide, 5X SSC, 0.5% SDS at 42°C. Typical, moderate stringency hybridization conditions are similar to the low stringency conditions except the hybridization is carried out in 40% formamide, with 5X or 6X SSC at 42°C. High stringency hybridization conditions are similar to low stringency conditions except the hybridization conditions are carried out in 50% formamide, 5X or 6X SSC and, optionally, at a higher temperature (*e.g.*, higher than 42°C: 57 °C, 59 °C, 60 °C, 62 °C, 63 °C, 65°C or 68 °C). In general, SSC is 0.15M NaCl and 0.015M Na-citrate. Hybridization requires that the two nucleic acids contain complementary sequences, although, depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing

nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the higher the stringency under which the nucleic acids may hybridize. For hybrids of greater than 100 nucleotides in length, equations for calculating the melting temperature have been derived (see Sambrook, *et al.*, *supra*, 9.50-9.51). For hybridization with shorter nucleic acids, *i.e.*, oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook, *et al.*, *supra*).

Also included in the present invention are polynucleotides comprising nucleotide sequences and polypeptides comprising amino acid sequences which are at least about 70% identical, preferably at least about 80% identical, more preferably at least about 90% identical and most preferably at least about 95% identical (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 1 or 5-10) and amino acid sequences (*e.g.*, SEQ ID NO: 2), reference human *NPC1L1* nucleotide (*e.g.*, SEQ ID NO: 3) and amino acid sequences (*e.g.*, SEQ ID NO: 4) or the reference mouse *NPC1L1* nucleotide (*e.g.*, any of SEQ ID NOs: 11 or 13) and amino acids sequences (*e.g.*, SEQ ID NO: 12), when the comparison is performed by a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences. Polypeptides comprising amino acid sequences which are at least about 70% similar, preferably at least about 80% similar, more preferably at least about 90% similar and most preferably at least about 95% similar (*e.g.*, 95%, 96%, 97%, 98%, 99%, 100%) to the reference rat NPC1L1 amino acid sequence of SEQ ID NO: 2, reference human NPC1L1 amino acid sequence of SEQ ID NO: 4 or the reference mouse NPC1L1 amino acid sequence of SEQ ID NO: 12, when the comparison is performed with a BLAST algorithm wherein the parameters of the algorithm are selected to give the largest match between the respective sequences over the entire length of the respective reference sequences, are also included in the present invention.

Sequence identity refers to exact matches between the nucleotides or amino acids of two sequences which are being compared. Sequence similarity refers to both exact matches between the amino acids of two polypeptides which are being compared in addition to matches between nonidentical, biochemically related amino acids. Biochemically related amino acids which share similar properties and may be interchangeable are discussed above.

The following references regarding the BLAST algorithm are herein incorporated by reference: **BLAST ALGORITHMS:** Altschul, S.F., *et al.*, (1990) J. Mol. Biol. 215:403-410; Gish, W., *et al.*, (1993) Nature Genet. 3:266-272; Madden, T.L., *et al.*, (1996) Meth. Enzymol. 266:131-141; Altschul, S.F., *et al.*, (1997) Nucleic Acids Res. 25:3389-3402; Zhang, J., *et al.*, (1997) Genome Res. 7:649-656; Wootton, J.C., *et al.*, (1993) Comput. Chem. 17:149-163; Hancock, J.M., *et al.*, (1994) Comput. Appl. Biosci. 10:67-70; **ALIGNMENT SCORING SYSTEMS:** Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., (1991) J. Mol. Biol. 219:555-565; States, D.J., *et al.*, (1991) Methods 3:66-70; Henikoff, S., *et al.*, (1992) Proc. Natl. Acad. Sci. USA 89:10915-10919; Altschul, S.F., *et al.*, (1993) J. Mol. Evol. 36:290-300; **ALIGNMENT STATISTICS:** Karlin, S., *et al.*, (1990) Proc. Natl. Acad. Sci. USA 87:2264-2268; Karlin, S., *et al.*, (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877; Dembo, A., *et al.*, (1994) Ann. Prob. 22:2022-2039; and Altschul, S.F. "Evaluating the statistical significance of multiple distinct local alignments." in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

Protein Purification

The proteins, polypeptides and antigenic fragments of this invention can be purified by standard methods, including, but not limited to, salt or alcohol precipitation, affinity chromatography (*e.g.*, used in conjunction with a purification tagged NPC1L1 polypeptide as discussed above), preparative disc-gel electrophoresis, isoelectric focusing, high pressure liquid chromatography (HPLC), reversed-phase HPLC, gel filtration, cation and anion exchange and partition chromatography, and countercurrent distribution. Such purification methods are well known in the art and are disclosed, *e.g.*, in "Guide to Protein Purification", Methods in Enzymology, Vol. 182, M. Deutscher, Ed., 1990, Academic Press, New York, NY.

Purification steps can be followed by performance of assays for receptor binding activity as described below. Particularly where an NPC1L1 polypeptide is being isolated from a cellular or tissue source, it is preferable to include one or more inhibitors of

proteolytic enzymes in the assay system, such as phenylmethanesulfonyl fluoride (PMSF), Pefabloc SC, pepstatin, leupeptin, chymostatin and EDTA.

Antibody Molecules

5 Antigenic (including immunogenic) fragments of the NPC1L1 polypeptides of the invention are within the scope of the present invention (*e.g.*, 42 or more contiguous amino acids from SEQ ID NO: 2, 4 or 12). The antigenic peptides may be useful, *inter alia*, for preparing isolated antibody molecules which recognize NPC1L1. Isolated anti-NPC1L1 antibody molecules are useful NPC1L1 antagonists.

10 An antigen is any molecule that can bind specifically to an antibody. Some antigens cannot, by themselves, elicit antibody production. Those that can induce antibody production are immunogens.

 Preferably, isolated anti-NPC1L1 antibodies recognize an antigenic peptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42 (*e.g.*, an antigen
15 derived from rat NPC1L1). More preferably, the antibody is A0715, A0716, A0717, A0718, A0867, A0868, A1801 or A1802.

 The term "antibody molecule" includes, but is not limited to, antibodies and fragments (preferably antigen-binding fragments) thereof. The term includes monoclonal antibodies, polyclonal antibodies, bispecific antibodies, Fab antibody fragments, F(ab)₂
20 antibody fragments, Fv antibody fragments (*e.g.*, V_H or V_L), single chain Fv antibody fragments and dsFv antibody fragments. Furthermore, the antibody molecules of the invention may be fully human antibodies, mouse antibodies, rat antibodies, rabbit antibodies, goat antibodies, chicken antibodies, humanized antibodies or chimeric antibodies.

25 Although it is not always necessary, when NPC1L1 polypeptides are used as antigens to elicit antibody production in an immunologically competent host, smaller antigenic fragments are, preferably, first rendered more immunogenic by cross-linking or concatenation, or by coupling to an immunogenic carrier molecule (*i.e.*, a macromolecule having the property of independently eliciting an immunological response in a host
30 animal, such as diphtheria toxin or tetanus). Cross-linking or conjugation to a carrier molecule may be required because small polypeptide fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, *i.e.*, they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders them more immunogenic through
35 what is commonly known as the "carrier effect".

Carrier molecules include, *e.g.*, proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides *etc.* Protein carrier molecules are especially preferred, including, but not limited to, keyhole limpet hemocyanin and mammalian serum proteins such as human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in the art. Preferably, the protein carrier will be foreign to the host animal in which antibodies against the fragments are to be elicited.

Covalent coupling to the carrier molecule can be achieved using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the fragments of the invention can be coupled, *e.g.*, using water-soluble carbodiimides such as dicyclohexylcarbodiimide or glutaraldehyde.

Coupling agents, such as these, can also be used to cross-link the fragments to themselves without the use of a separate carrier molecule. Such cross-linking into aggregates can also increase immunogenicity. Immunogenicity can also be increased by the use of known adjuvants, alone or in combination with coupling or aggregation.

Adjuvants for the vaccination of animals include, but are not limited to, Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); Freund's complete or incomplete adjuvant; mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The polypeptides could also be administered following incorporation into liposomes or other microcarriers.

Information concerning adjuvants and various aspects of immunoassays are disclosed, *e.g.*, in the series by P. Tijssen, Practice and Theory of Enzyme Immunoassays, 3rd Edition, 1987, Elsevier, New York. Other useful references covering methods for preparing polyclonal antisera include Microbiology, 1969, Hoeber Medical Division, Harper and Row; Landsteiner, Specificity of Serological Reactions, 1962, Dover Publications, New York, and Williams, *et al.*, Methods in Immunology and Immunochemistry, Vol. 1, 1967, Academic Press, New York.

The anti-NPC1L1 antibody molecules of the invention preferably recognize human, mouse or rat NPC1L1; however, the present invention includes antibody

molecules which recognize NPC1L1 from any species, preferably mammals (*e.g.*, cat, sheep or horse). The present invention also includes complexes comprising an NPC1L1 polypeptide of the invention and an anti-NPC1L1 antibody molecule. Such complexes can be made by simply contacting the antibody molecule with its cognate polypeptide.

5 Various methods may be used to make the antibody molecules of the invention. Human antibodies can be made, for example, by methods which are similar to those disclosed in U.S. Patent Nos. 5,625,126; 5,877,397; 6,255,458; 6,023,010 and 5,874,299.

Hybridoma cells which produce the monoclonal anti-NPC1L1 antibodies may be produced by methods which are commonly known in the art. These methods include, but are not limited to, the hybridoma technique originally developed by Kohler, *et al.*, (1975) (Nature 256:495-497), as well as the trioma technique (Hering, *et al.*, (1988) Biomed. Biochim. Acta. 47:211-216 and Hagiwara, *et al.*, (1993) Hum. Antibod. Hybridomas 4:15), the human B-cell hybridoma technique (Kozbor, *et al.*, (1983) Immunology Today 4:72 and Cote, *et al.*, (1983) Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030), and the EBV-
10 hybridoma technique (Cole, *et al.*, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1985). ELISA may be used to determine if hybridoma cells are expressing anti-NPC1L1 antibodies.

The anti-NPC1L1 antibody molecules of the present invention may also be produced recombinantly (*e.g.*, in an *E.coli*/T7 expression system as discussed above). In
20 this embodiment, nucleic acids encoding the antibody molecules of the invention (*e.g.*, V_H or V_L) may be inserted into a pet-based plasmid and expressed in the *E.coli*/T7 system. There are several methods by which to produce recombinant antibodies which are known in the art. An example of a method for recombinant production of antibodies is disclosed in U.S. Patent No. 4,816,567. See also Skerra, A., *et al.*, (1988) Science 240:1038-1041; Better, M., *et al.*, (1988) Science 240:1041-1043 and Bird, R.E., *et al.*, (1988) Science 242:423-426.

The term "monoclonal antibody," includes an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible, naturally occurring mutations
30 that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Monoclonal antibodies are advantageous in that they may be synthesized by a hybridoma culture, essentially uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to
35 be construed as requiring production of the antibody by any particular method. The

monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method as described by Kohler, *et al.*, (1975) Nature 256:495.

The term "polyclonal antibody" includes an antibody which was produced among or in the presence of one or more other, non-identical antibodies. In general, polyclonal
5 antibodies are produced from a B-lymphocyte in the presence of several other B-lymphocytes which produced non-identical antibodies. Typically, polyclonal antibodies are obtained directly from an immunized animal (*e.g.*, a rabbit).

A "bispecific antibody" comprises two different antigen binding regions which bind to distinct antigens. Bispecific antibodies, as well as methods of making and using
10 the antibodies, are conventional and very well known in the art.

Anti-idiotypic antibodies or anti-idiotypes are antibodies directed against the antigen-combining region or variable region (called the idiotype) of another antibody molecule. As disclosed by Jerne (Jerne, N. K., (1974) Ann. Immunol. (Paris) 125c:373 and Jerne, N. K., *et al.*, (1982) EMBO 1:234), immunization with an antibody molecule
15 expressing a paratope (antigen-combining site) for a given antigen (*e.g.*, NPC1L1) will produce a group of anti-antibodies, some of which share, with the antigen, a complementary structure to the paratope. Immunization with a subpopulation of the anti-idiotypic antibodies will, in turn, produce a subpopulation of antibodies or immune cell subsets that are reactive to the initial antigen.

20 The term "fully human antibody" refers to an antibody which comprises human immunoglobulin sequences only. Similarly, "mouse antibody" refers to an antibody which comprises mouse immunoglobulin sequences only.

"Human/mouse chimeric antibody" refers to an antibody which comprises a mouse variable region (V_H and V_L) fused to a human constant region.

25 "Humanized" anti-NPC1L1 antibodies are also within the scope of the present invention. Humanized forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region of the
30 recipient are replaced by residues from a complementary determining region of a nonhuman species (donor antibody), such as mouse, rat or rabbit, having a desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are also replaced by corresponding non-human residues.

"Single-chain Fv" or "sFv" antibody fragments include the V_H and/or V_L domains
35 of an antibody, wherein these domains are present in a single polypeptide chain.

Generally, the sFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. Techniques described for the production of single chain antibodies (U.S. Patent Nos. 5,476,786; 5,132,405 and 4,946,778) can be adapted to produce anti-NPC1L1 specific,
 5 single chain antibodies. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenberg and Moore eds. Springer-Verlag, N.Y., pp. 269-315 (1994).

“Disulfide stabilized Fv fragments” and “dsFv” include molecules having a variable heavy chain (V_H) and/or a variable light chain (V_L) which are linked by a
 10 disulfide bridge.

Antibody fragments within the scope of the present invention also include F(ab)₂ fragments which may be produced by enzymatic cleavage of an IgG by, for example, pepsin. Fab fragments may be produced by, for example, reduction of F(ab)₂ with dithiothreitol or mercaptoethylamine.

15 An F_V fragment is a V_L or V_H region.

Depending on the amino acid sequences of the constant domain of their heavy chains, immunoglobulins can be assigned to different classes. There are at least five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.*, IgG-1, IgG-2, IgG-3 and IgG-4; IgA-1
 20 and IgA-2.

The anti-NPC1L1 antibody molecules of the invention may also be conjugated to a chemical moiety. The chemical moiety may be, *inter alia*, a polymer, a radionuclide or a cytotoxic factor. Preferably, the chemical moiety is a polymer which increases the half-life of the antibody molecule in the body of a subject. Suitable polymers include, but are
 25 by no means limited to, polyethylene glycol (PEG) (*e.g.*, PEG with a molecular weight of 2kDa, 5 kDa, 10 kDa, 12kDa, 20 kDa, 30kDa or 40kDa), dextran and monomethoxypolyethylene glycol (mPEG). Methods for producing PEGylated anti-IL8 antibodies which are described in U.S. Patent No. 6,133,426 can be applied to the production of PEGylated anti-NPC1L1 antibodies of the invention. Lee, *et al.*, (1999)
 30 (Bioconj. Chem. 10:973-981) discloses PEG conjugated single-chain antibodies. Wen, *et al.*, (2001) (Bioconj. Chem. 12:545-553) discloses conjugating antibodies with PEG which is attached to a radiometal chelator (diethylenetriaminopentaacetic acid (DTPA)).

The antibody molecules of the invention may also be conjugated with labels such as ⁹⁹Tc, ⁹⁰Y, ¹¹¹In, ³²P, ¹⁴C, ¹²⁵I, ³H, ¹³¹I, ¹¹C, ¹⁵O, ¹³N, ¹⁸F, ³⁵S, ⁵¹Cr, ⁵⁷To, ²²⁶Ra, ⁶⁰Co,
 35 ⁵⁹Fe, ⁵⁷Se, ¹⁵²Eu, ⁶⁷CU, ²¹⁷Ci, ²¹¹At, ²¹²Pb, ⁴⁷Sc, ¹⁰⁹Pd, ²³⁴Th, ⁴⁰K, ¹⁵⁷Gd, ⁵⁵Mn, ⁵²Tr or ⁵⁶Fe.

The antibody molecules of the invention may also be conjugated with fluorescent or chemiluminescent labels, including fluorophores such as rare earth chelates, fluorescein and its derivatives, rhodamine and its derivatives, isothiocyanate, phycoerythrin, phycocyanin, allophycocyanin, o-phthalaldehyde, fluorescamine, ¹⁵²Eu, dansyl, umbelliferone, luciferin, luminal label, isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, an aequorin label, 2,3-dihydrophthalazinediones, biotin/avidin, spin labels and stable free radicals.

The antibody molecules may also be conjugated to a cytotoxic factor such as diphtheria toxin, *Pseudomonas aeruginosa* exotoxin A chain, ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, *Aleurites fordii* proteins and compounds (e.g., fatty acids), dianthin proteins, *Phytolacca americana* proteins PAPI, PAPII, and PAP-S, *momordica charantia* inhibitor, curcin, crotin, *saponaria officinalis* inhibitor, mitogellin, restrictocin, phenomycin, and enomycin.

Any method known in the art for conjugating the antibody molecules of the invention to the various moieties may be employed, including those methods described by Hunter, *et al.*, (1962) *Nature* 144:945; David, *et al.*, (1974) *Biochemistry* 13:1014; Pain, *et al.*, (1981) *J. Immunol. Meth.* 40:219; and Nygren, J., (1982) *Histochem. and Cytochem.* 30:407.

Methods for conjugating antibodies are conventional and very well known in the art.

Screening Assays

The invention allows the discovery of selective agonists and antagonists of NPC1L1 (e.g., SEQ ID NO: 2, 4 or 12) that may be useful in treatment and management of a variety of medical conditions including elevated serum sterol (e.g., cholesterol) or 5 α -stanol. Thus, NPC1L1 of this invention can be employed in screening systems to identify agonists or antagonists. Essentially, these systems provide methods for bringing together NPC1L1, an appropriate, known ligand or agonist or antagonist, including a sterol (e.g., cholesterol, phytosterols (including, but not limited to, sitosterol, campesterol, stigmasterol and avenosterol)), a 5 α -stanol (including but not limited to cholestanol, 5 α -campestanol and 5 α -sitostanol), a cholesterol oxidation product, a substituted azetidinone (e.g., ezetimibe), BODIPY-ezetimibe (Altmann, *et al.*, (2002) *Biochim. Biophys. Acta* 1580(1):77-93) or 4", 6"-bis[(2-fluorophenyl)carbamoyl]-beta-D-cellobiosyl derivative of 11-ketotigogenin as described in DeNinno, *et al.*, (1997) (*J. Med. Chem.* 40(16):2547-54)

(Merck; L-166,143) or any substituted azetidinone, and a sample to be tested for the presence of an NPC1L1 agonist or antagonist.

The term “specific” when used to describe binding of, for example, a ligand or antagonist of NPC1L1 in a screening assay is a term of art which refers to the extent by which the ligand or antagonist (*e.g.*, detectably labeled substituted azetidinone, detectably labeled ezetimibe, detectably labeled sterol (*e.g.*, cholesterol) or detectably labeled 5 α -stanol) binds preferentially to NPC1L1 over that of other proteins in the assay system. For example, an antagonist or ligand of NPC1L1 binds specifically to NPC1L1 when the signal generated in the assay to indicate such binding exceeds, to any extent, a background signal in a negative control experiment wherein, for example, NPC1L1 or the antagonist or ligand is absent. Furthermore, “specific binding” includes binding of an antagonist or ligand either directly to NPC1L1 or indirectly, for example via another moiety, in a complex of which NPC1L1 is a part. The moiety to which an NPC1L1 ligand or antagonist binds can be another protein or a post-translational modification of NPC1L1 (*e.g.*, a lipid chain or a carbohydrate chain).

Non-limiting examples of suitable azetidinones include those disclosed in U.S. Patent Nos. RE37,721; 5,631,365; 5,767,115; 5,846,966; 5,688,990; 5,656,624; 5,624,920; 5,698,548 and 5,756,470 and U.S. Patent Application Publication No 2003/0105028-each of which is herein incorporated by reference in its entirety.

A convenient method by which to evaluate whether a sample contains an NPC1L1 agonist or antagonist is to determine whether the sample contains a substance which competes for binding between the known agonist or antagonist (*e.g.*, ezetimibe) and NPC1L1.

Ezetimibe can be prepared by a variety of methods well known to those skilled in the art, for example such as are disclosed in U.S. Patents Nos. 5,631,365, 5,767,115, 5,846,966, 6,207,822, U.S. Patent Application Publication No. 2002/0193607 and PCT Patent Application WO 93/02048, each of which is incorporated herein by reference in its entirety.

“Sample”, “candidate compound” or “candidate substance” refers to a composition which is evaluated in a test or assay, for example, for the ability to bind to, agonize or antagonize NPC1L1 (*e.g.*, SEQ ID NO: 2, 4 or 12) or a functional fragment thereof. The composition may small molecules, peptides, nucleotides, polynucleotides, subatomic particles (*e.g.*, α particles, β particles) or antibodies.

Two basic types of screening systems that can be used include, a labeled-ligand binding assay (*e.g.*, direct binding assay or scintillation proximity assay (SPA)) and a

“sterol (*e.g.*, cholesterol) or 5 α -stanol uptake” assay. A labeled ligand, for use in the binding assay, can be obtained by labeling a sterol (*e.g.*, cholesterol) or a 5 α -stanol or a known NPC1L1 agonist or antagonist with a measurable group (*e.g.*, ^{125}I or ^3H). Various labeled forms of sterols (*e.g.*, cholesterol) or 5 α -stanols are available commercially or can be generated using standard techniques (*e.g.*, Cholesterol- [1,2- ^3H (N)], Cholesterol- [1,2,6,7- ^3H (N)] or Cholesterol-[7- ^3H (N)]; American Radiolabeled Chemicals, Inc; St. Louis, MO). In a preferred embodiment, ezetimibe is fluorescently labeled with a BODIPY group (Altmann, *et al.*, (2002) *Biochim. Biophys. Acta* 1580(1):77-93) or labeled with a detectable group such as ^{125}I or ^3H .

Direct Binding Assay. Typically, a given amount of NPC1L1 of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) or a complex including NPC1L1 is contacted with increasing amounts of labeled ligand or known antagonist or agonist (discussed above) and the amount of the bound, labeled ligand or known antagonist or agonist is measured after removing unbound, labeled ligand or known antagonist or agonist by washing. As the amount of the labeled ligand or known agonist or antagonist is increased, a point is eventually reached at which all receptor binding sites are occupied or saturated. Specific receptor binding of the labeled ligand or known agonist or antagonist is abolished by a large excess of unlabeled ligand or known agonist or antagonist.

Preferably, an assay system is used in which non-specific binding of the labeled ligand or known antagonist or agonist to the receptor is minimal. Non-specific binding is typically less than 50%, preferably less than 15%, and more preferably less than 10% of the total binding of the labeled ligand or known antagonist or agonist.

A nucleic acid encoding an NPC1L1 polypeptide of the invention (*e.g.*, SEQ ID NO: 2, 4 or 12) can be transfected into an appropriate host cell, whereby the receptor will become incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of the receptor for assay. Alternatively, the whole cell expressing the receptor in the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or to a membrane fraction from an untransfected/untransformed host cell will be negligible.

In principle, a binding assay of the invention could be carried out using a soluble NPC1L1 polypeptide of the invention, *e.g.*, following production and refolding by standard methods from an *E. coli* expression system, and the resulting receptor-labeled ligand complex could be precipitated, *e.g.*, using an antibody against the receptor. The

precipitate could then be washed and the amount of the bound, labeled ligand or antagonist or agonist could be measured.

In the basic binding assay, the method for identifying an NPC1L1 agonist or antagonist includes:

5 (a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a subsequence thereof or a complex including NPC1L1, in the presence of a known amount of labeled sterol (*e.g.*, cholesterol) or 5 α -stanol or known antagonist or agonist (*e.g.*, labeled ezetimibe or labeled L-166,143) with a sample to be tested for the presence of an NPC1L1 agonist or antagonist; and

10 (b) measuring the amount of labeled sterol (*e.g.*, cholesterol) or 5 α -stanol or known antagonist or agonist directly or indirectly bound to NPC1L1.

An NPC1L1 antagonist or agonist in the sample is identified by measuring substantially reduced direct or indirect binding of the labeled sterol (*e.g.*, cholesterol) or 5 α -stanol or known antagonist or agonist to NPC1L1, compared to what would be
15 measured in the absence of such an antagonist or agonist. For example, reduced direct or indirect binding between [³H]-cholesterol and NPC1L1 in the presence of a sample might suggest that the sample contains a substance which is competing against [³H]-cholesterol for NPC1L1 binding.

This assay can include a control experiment lacking any NPC1L1-dependent
20 ligand (*e.g.*, sterol such as cholesterol or 5 α -stanol) binding. In this assay, for example, a whole cell or cell membrane lacking any functional NPC1L1, for example, a cell or membrane isolated or derived from a transgenic mutant *npc1l1*⁻ mouse of the invention, is assayed for ligand binding. When screening a sample for the presence of an NPC1L1 antagonist, it is useful to compare the level of binding observed in the presence of a
25 sample being tested with that of a control experiment, as described herein, which completely lacks NPC1L1-dependent binding. Ideally, though by no means necessarily, the level of binding seen in the presence of a sample containing an antagonist will be similar to that of the control experiment.

Alternatively, a sample can be tested directly for binding to NPC1L1 (*e.g.*, SEQ
30 ID NO: 2, 4 or 12). A basic assay of this type may include the following steps:

(a) contacting NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12), a subsequence thereof or a complex including NPC1L1 with a labeled candidate compound (*e.g.*, [³H]-ezetimibe); and

(b) detecting direct or indirect binding between the labeled candidate compound
35 and NPC1L1.

Again, these experiment can be performed along with a control experiment wherein NPC1L1-dependent binding is completely lacking. For example, the assay can be performed using a whole cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant *npc1l1*⁻ mouse as described
5 herein).

A candidate compound which is found to bind to NPC1L1 may function as an agonist or antagonist of NPC1L1 (*e.g.*, by inhibition of sterol (*e.g.*, cholesterol) or 5 α -stanol uptake).

SPA Assay. NPC1L1 antagonists or agonists may also be measured using
10 scintillation proximity assays (SPA). SPA assays are conventional and very well known in the art; see, for example, U.S. Patent No. 4,568,649. In SPA, the target of interest is immobilised to a small microsphere approximately 5 microns in diameter. The microsphere, typically, includes a solid scintillant core which has been coated with a polyhydroxy film, which in turn contains coupling molecules, which allow generic links
15 for assay design. When a radioisotopically labeled molecule binds to the microsphere, the radioisotope is brought into close proximity to the scintillant and effective energy transfer from electrons emitted by the isotope will take place resulting in the emission of light. While the radioisotope remains in free solution, it is too distant from the scintillant and the electron will dissipate the energy into the aqueous medium and therefore remain
20 undetected. Scintillation may be detected with a scintillation counter. In general, ³H and ¹²⁵I labels are well suited to SPA.

For the assay of receptor-mediated binding events, the lectin wheat germ agglutinin (WGA) may be used as the SPA bead coupling molecule (Amersham Biosciences; Piscataway, NJ). The WGA coupled bead captures glycosylated, cellular
25 membranes and glycoproteins and has been used for a wide variety of receptor sources and cultured cell membranes. The receptor is immobilized onto the WGA-SPA bead and a signal is generated on binding of an isotopically labeled ligand. Other coupling molecules which may be useful for receptor binding SPA assays include poly-L-lysine and WGA/polyethyleneimine (Amersham Biosciences; Piscataway, NJ). See, for
30 example, Berry, J.A., *et al.*, (1991) Cardiovascular Pharmacol. 17 (Suppl.7): S143-S145; Hoffman, R., *et al.*, (1992) Anal. Biochem. 203: 70-75; Kienhus, *et al.*, (1992) J. Receptor Research 12: 389-399; Jing, S., *et al.*, (1992) Neuron 9: 1067-1079.

The scintillant contained in SPA beads may include, for example, yttrium silicate (YSi), yttrium oxide (YOx), diphenyloxazole or polyvinyltoluene (PVT) which acts as a
35 solid solvent for diphenylanthracene (DPA).

SPA assays may be used to analyze whether a sample contains an NPC1L1 antagonist or agonist. In these assays, a host cell which expresses NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface or a membrane fraction thereof is incubated with and captured by SPA beads (*e.g.*, WGA coated YOx beads or WGA coated YSi beads). The beads bearing the NPC1L1 are incubated with labeled, known ligand or agonist or antagonist (*e.g.*, ^3H -cholesterol, ^3H -ezetimibe or ^{125}I -ezetimibe). The assay mixture further includes either the sample to be tested or a blank (*e.g.*, water). After an optional incubation, scintillation is measured using a scintillation counter. An NPC1L1 agonist or antagonist may be identified in the sample by measuring substantially reduced fluorescence, compared to what would be measured in the absence of such agonist or antagonist (blank). Measuring substantially reduced fluorescence may suggest that the sample contains a substance which competes for direct or indirect NPC1L1 binding with the known ligand, agonist or antagonist.

Alternatively, a sample may be identified as an antagonist or agonist of NPC1L1 by directly detecting binding in a SPA assay. In this assay, a labeled version of a candidate compound to be tested may be put in contact with the host cell expressing NPC1L1 or a membrane fraction thereof which is bound to the SPA bead. Fluorescence may then be assayed to detect the presence of a complex between the labeled candidate compound and the host cell or membrane fraction expressing NPC1L1 or a complex including NPC1L1. A candidate compound which binds directly or indirectly to NPC1L1 may possess NPC1L1 agonistic or antagonistic activity.

SPA Assays can also be performed along with a control experiment lacking any NPC1L1-dependent binding. The control experiment can be performed, for example, with a cell or cell membrane lacking any functional NPC1L1 (*e.g.*, cell or cell membrane derived from a transgenic, mutant *npc1l1* mouse as described herein). When the control experiment is performed, the level of binding observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

Host cells expressing NPC1L1 may be prepared by transforming or transfecting a nucleic acid encoding an NPC1L1 of the invention into an appropriate host cell, whereby the receptor becomes incorporated into the membrane of the cell. A membrane fraction can then be isolated from the cell and used as a source of the receptor for assay.

Alternatively, the whole cell expressing the receptor on the cell surface can be used in an assay. Preferably, specific binding of the labeled ligand or known antagonist or agonist to an untransfected/untransformed host cell or membrane fraction from an

untransfected/untransformed host cell will be negligible. Preferred host cells include Chinese Hamster Ovary (CHO) cells, murine macrophage J774 cells or any other macrophage cell line and human intestinal epithelial Caco2 cells.

Sterol/5 α -stanol Uptake Assay. Assays may also be performed to determine if a sample can agonize or antagonize NPC1L1 mediated sterol (*e.g.*, cholesterol) or 5 α -stanol uptake. In these assays, a host cell expressing NPC1L1 (*e.g.*, SEQ ID NO: 2 or 4 or 12) on the cell surface (discussed above) can be contacted with detectably labeled sterol (*e.g.*, ³H-cholesterol or ¹²⁵I-cholesterol) or 5 α -stanol along with either a sample or a blank. After an optional incubation, the cells can be washed to remove unabsorbed sterol or 5 α -stanol. Sterol or 5 α -stanol uptake can be determined by detecting the presence of labeled sterol or 5 α -stanol in the host cells. For example, assayed cells or lysates or fractions thereof (*e.g.*, fractions resolved by thin-layer chromatography) can be contacted with a liquid scintillant and scintillation can be measured using a scintillation counter.

In these assays, an NPC1L1 antagonist in the sample may be identified by measuring substantially reduced uptake of labeled sterol (*e.g.*, ³H-cholesterol) or 5 α -stanol, compared to what would be measured in the absence of such an antagonist and an agonist may be identified by measuring substantially increased uptake of labeled sterol (*e.g.*, ³H-cholesterol) or 5 α -stanol, compared to what would be measured in the absence of such an agonist.

Uptake assays can also be performed along with a control experiment lacking any NPC1L1-dependent uptake. The control experiment can be performed, for example, with a cell lacking any functional NPC1L1 (*e.g.*, cell derived from a transgenic, mutant *npc1l1* mouse as described herein). When the control experiment is performed, the level of uptake observed in the presence of sample being tested for the presence of an antagonist can be compared with that observed in the control experiment.

Mouse Assay. The present invention comprises a mutant, transgenic mouse which lacks any functional NPC1L1. This mouse may serve as a convenient control experiment in screening assays for identifying inhibitors of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption, preferably inhibitors of NPC1L1. Preferably, a mouse assay of the present invention would comprise the following steps:

- (a) feeding a sterol (*e.g.*, cholesterol) or 5 α -stanol-containing substance (*e.g.*, comprising radiolabeled cholesterol, such as ¹⁴C-cholesterol or ³H-cholesterol) to a first and second mouse comprising a functional *NPC1L1* gene and to a third, mutant mouse lacking a functional *NPC1L1*;

The sterol (*e.g.*, cholesterol) or 5 α -stanol containing substance preferably contains labeled cholesterol, such as a radiolabeled cholesterol, for example, ^3H or ^{14}C labeled cholesterol. The sterol (*e.g.*, cholesterol) or 5 α -stanol containing substance may also include cold, unlabeled sterol (*e.g.*, cholesterol) or 5 α -stanol such as in corn oil.

5 In these assays, the third *npc111* mutant mouse serves as a (+)-control experiment which exhibits low levels of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption and the second mouse serves as a (-)-control experiment which exhibits normal, uninhibited levels of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption. The second mouse is not administered the sample to be tested for an NPC1L1 antagonist. The
10 first mouse is the experiment.

(b) administering the sample to the first mouse comprising a functional *NPC1L1* but not to the second mouse;

(c) measuring the amount of sterol (*e.g.*, cholesterol) or 5 α -stanol absorption in the intestine of said first, second and third mouse;

15 Intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption may be measured by any method known in the art. For example, the level intestinal absorption can be assayed by measuring the level of serum sterol (*e.g.*, cholesterol) or 5 α -stanol.

(d) comparing the levels of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption in each mouse;

20 wherein the sample is determined to contain the intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption antagonist when the level of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption in the first mouse and in the third mouse are less than the amount of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption in the second mouse.

Preferably, if the sample contains an intestinal sterol (*e.g.*, cholesterol) or 5 α -
25 stanol absorption inhibitor (*e.g.*, an NPC1L1 inhibitor), the level of sterol (*e.g.*, cholesterol) or 5 α -stanol absorption in the first mouse will be similar to that of the third, *npc111* mutant mouse.

An alternative, (+)-control experiment which may be used in these screening assays is a mouse comprising functional NPC1L1 which is administered a known
30 antagonist of NPC1L1, such as ezetimibe.

Pharmaceutical Compositions

NPC1L1 agonists and antagonists discovered, for example, by the screening methods described above may be used therapeutically (*e.g.*, in a pharmaceutical

composition) to stimulate or block the activity of NPC1L1 and, thereby, to treat any medical condition caused or mediated by NPC1L1. In addition, the antibody molecules of the invention may also be used therapeutically (*e.g.*, in a pharmaceutical composition) to bind NPC1L1 and, thereby, block the ability of NPC1L1 to bind a sterol (*e.g.*, cholesterol) or 5 α -stanol. Blocking the binding of a sterol (*e.g.*, cholesterol) or 5 α -stanol would prevent absorption of the molecule (*e.g.*, by intestinal cells such as enterocytes). Blocking absorption of sterol (*e.g.*, cholesterol) or 5 α -stanol would be a useful way to lower serum sterol (*e.g.*, cholesterol) or 5 α -stanol levels in a subject and, thereby, reduce the incidence of, for example, hyperlipidemia, atherosclerosis, coronary heart disease, stroke or arteriosclerosis.

The term "subject" or "patient" includes any organism, preferably animals, more preferably mammals (*e.g.*, mice, rats, rabbits, dogs, horses, primates, cats) and most preferably humans.

The term "pharmaceutical composition" refers to a composition including an active ingredient and a pharmaceutically acceptable carrier and/or adjuvant.

Although the compositions of this invention could be administered in simple solution, they are more typically used in combination with other materials such as carriers, preferably pharmaceutically acceptable carriers. Useful, pharmaceutically acceptable carriers can be any compatible, non-toxic substances suitable for delivering the compositions of the invention to a subject. Sterile water, alcohol, fats, waxes, and inert solids may be included in a pharmaceutically acceptable carrier. Pharmaceutically acceptable adjuvants (buffering agents, dispersing agents) may also be incorporated into the pharmaceutical composition.

Preferably, the pharmaceutical compositions of the invention are in the form of a pill or capsule. Methods for formulating pills and capsules are very well known in the art. For example, for oral administration in the form of tablets or capsules, the active drug component may be combined with any oral, non-toxic pharmaceutically acceptable inert carrier, such as lactose, starch, sucrose, cellulose, magnesium stearate, dicalcium phosphate, calcium sulfate, talc, mannitol, ethyl alcohol (liquid forms) and the like. Moreover, when desired or needed, suitable binders, lubricants, disintegrating agents and coloring agents may also be incorporated in the mixture. Suitable binders include starch, gelatin, natural sugars, corn sweeteners, natural and synthetic gums such as acacia, sodium alginate, carboxymethylcellulose, polyethylene glycol and waxes. Among the lubricants there may be mentioned for use in these dosage forms, boric acid, sodium benzoate, sodium acetate, sodium chloride, and the like. Disintegrants include starch,

methycellulose, guar gum and the like. Sweetening and flavoring agents and preservatives may also be included where appropriate.

The pharmaceutical compositions of the invention may be administered in conjunction with a second pharmaceutical composition or substance. In preferred
5 embodiments, the second composition includes a cholesterol-lowering drug. When a combination therapy is used, both compositions may be formulated into a single composition for simultaneous delivery or formulated separately into two or more compositions (*e.g.*, a kit).

The formulations may conveniently be presented in unit dosage form and may be
10 prepared by any methods well known in the art of pharmacy. See, *e.g.*, Gilman *et al.* (eds.) (1990), The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, *supra*, Easton, Penn.; Avis *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and
15 Lieberman *et al.* (eds.) (1990), Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York.

The dosage regimen involved in a therapeutic application may be determined by a physician, considering various factors which may modify the action of the therapeutic substance, *e.g.*, the condition, body weight, sex and diet of the patient, the severity of any
20 infection, time of administration, and other clinical factors. Often, treatment dosages are titrated upward from a low level to optimize safety and efficacy. Dosages may be adjusted to account for the smaller molecular sizes and possibly decreased half-lives (clearance times) following administration.

An "effective amount" of an antagonist of the invention may be an amount that
25 will detectably reduce the level of intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption or detectably reduce the level of serum sterol (*e.g.*, cholesterol) or 5 α -stanol in a subject administered the composition.

Typical protocols for the therapeutic administration of such substances are well known in the art. Pharmaceutical composition of the invention may be administered, for
30 example, by any parenteral or non-parenteral route.

Pills and capsules of the invention can be administered orally. Injectable compositions can be administered with medical devices known in the art; for example, by injection with a hypodermic needle.

Injectable pharmaceutical compositions of the invention may also be administered
35 with a needleless hypodermic injection device; such as the devices disclosed in U.S.

Patent Nos. 5,399,163; 5,383,851; 5,312,335; 5,064,413; 4,941,880; 4,790,824 or 4,596,556.

Anti-Sense

5 The present invention also encompasses anti-sense oligonucleotides capable of specifically hybridizing to mRNA encoding NPC1L1 (*e.g.*, any of SEQ ID NOs: 1, 3, 5-11 or 13) having an amino acid sequence defined by, for example, SEQ ID NO: 2 or 4 or 12 or a subsequence thereof so as to prevent translation of the mRNA. Additionally, this invention contemplates anti-sense oligonucleotides capable of specifically hybridizing to
10 the genomic DNA molecule encoding NPC1L1, for example, having an amino acid sequence defined by SEQ ID NO: 2 or 4 or 12 or a subsequence thereof.

 This invention further provides pharmaceutical compositions comprising (a) an amount of an oligonucleotide effective to reduce NPC1L1-mediated sterol (*e.g.*, cholesterol) or 5 α -stanol absorption by passing through a cell membrane and binding
15 specifically with mRNA encoding NPC1L1 in the cell so as to prevent its translation and (b) a pharmaceutically acceptable carrier capable of passing through a cell membrane. In an embodiment, the oligonucleotide is coupled to a substance that inactivates mRNA. In another embodiment, the substance that inactivates mRNA is a ribozyme.

 Reducing the level of NPC1L1 expression by introducing anti-sense *NPC1L1*
20 RNA into the cells of a patient is a useful method reducing intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption and serum cholesterol in the patient.

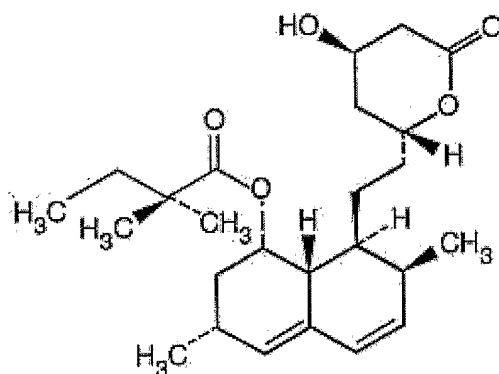
Kits

 Kits of the present invention include ezetimibe, preferably combined with a
25 pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. See for example, Gilman *et al.* (eds.) (1990), The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, *supra*, Easton, Penn.; Avis *et al.* (eds.) (1993)
30 Pharmaceutical Dosage Forms: Parenteral Medications Dekker, New York; Lieberman *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets Dekker, New York; and Lieberman *et al.* (eds.) (1990), Pharmaceutical Dosage Forms: Disperse Systems Dekker, New York. Preferably, the dosage form is a Zetia® tablet (Merck/Schering-Plough Corp.). Ezetimibe may be supplied in any convenient form. For example, tablets
35 including ezetimibe may be supplied in bottles of 30, 90 or 500.

The kits of the present invention also include information, for example in the form of a package insert, indicating that the target of ezetimibe is NPC1L1 (NPC3). The term “target of ezetimibe” indicates that ezetimibe reduces intestinal sterol (*e.g.*, cholesterol) or 5 α -stanol absorption, either directly or indirectly, by antagonizing NPC1L1. The form of the insert may take any form, such as paper or on electronic media such as a magnetically recorded medium (*e.g.*, floppy disk) or a CD-ROM.

The package insert may also include other information concerning the pharmaceutical compositions and dosage forms in the kit. Generally, such information aids patients and physicians in using the enclosed pharmaceutical compositions and dosage forms effectively and safely. For example, the following information regarding ezetimibe (*e.g.*, Zetia®) and/or simvastatin (*e.g.*, Zocor®) may be supplied in the insert: pharmacokinetics, pharmacodynamics, clinical studies, efficacy parameters, indications and usage, contraindications, warnings, precautions, adverse reactions, overdose, proper dosage and administration, how supplied, proper storage conditions, references and patent information.

The kits of the invention may also include simvastatin (



) preferably

combined with a pharmaceutically acceptable carrier, in a pharmaceutical formulation, more preferably in a pharmaceutical dosage form such as a pill, a powder, an injectable liquid, a tablet, dispersible granules, a capsule, a cachet or a suppository. Preferably, the dosage form of simvastatin is a Zocor® tablet (Merck & Co.; Whitehouse Station, NJ).

Tablets or pills comprising simvastatin may be supplied in any convenient form. For example, pills or tablets comprising 5mg simvastatin can be supplied as follows:

bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 10 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000. Pills or tablets comprising 20 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

Pills or tablets comprising 40 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100 or 1000. Pills or tablets comprising 80 mg simvastatin may be supplied as follows: bottles of 30, 60, 90, 100, 1000 or 10,000.

Ezetimibe and simvastatin may be supplied, in the kit, as separate compositions or combined into a single composition. For example, ezetimibe and simvastatin may be supplied within a single, common pharmaceutical dosage form (*e.g.*, pill or tablet) as in separate pharmaceutical dosage forms (*e.g.*, two separate pills or tablets).

npc11l⁻ Cells

The present invention provides any isolated mammalian cell, (*e.g.*, an isolated mouse cell, an isolated rat cell or an isolated human cell) which lacks an *NPC1L1* gene which encodes or can produce a functional NPC1L1 protein. Included within this embodiment are mutant *npc11l* genes comprising a point mutation, truncation or deletion of the genetic coding region or of any regulatory element (*e.g.*, a promoter).

For example, the cell can be isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein (*e.g.*, the mouse described below in Example 22). Moreover, the present invention comprises any cell, tissue, organ, fluid, nucleic acid, peptide or other biological substance derived or isolated from such a mutant mouse, particularly a mutant, transgenic mouse which does not produce any functional NPC1L1, wherein the region of endogenous, chromosomal *NPC1L1* deleted, in the mouse, corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ ID NO: 45.

The isolated cell can be isolated or derived, for example, from the duodenum, gall bladder, liver, small intestine or stomach of the mutant mouse. Further, the cell can be an enterocyte.

The *npc11l*⁻ mutant cells are useful, for example, for use in control experiments in screening assays (see *e.g.*, *supra*) since they lack any NPC1L1-dependent uptake or binding of sterol, 5 α -stanol or ezetimibe. The level of inhibition caused by a particular sample, in a screening assay, can be compared to that of an assay performed with the mutant cell. Ideally, though by no means necessarily, in a screening assay, for example, as described herein, the same amount of binding will be observed by a non-mutant cell or cell membrane, in the presence of an antagonist, as is observed in connection with a mutant *npc11l*⁻ cell or cell membrane alone.

EXAMPLES

The following examples are provided to more clearly describe the present invention and should not be construed to limit the scope of the invention in any way.

5

Example 1: Cloning and Expression of Rat, Mouse and Human NPC1L1.

Rat *NPC*, mouse *NPC1L1* or human *NPC1L1* can all conveniently be amplified using polymerase chain reaction (PCR). In this approach, DNA from a rat, mouse or human cDNA library can be amplified using appropriate primers and standard PCR
10 conditions. Design of primers and optimal amplification conditions constitute standard techniques which are commonly known in the art.

An amplified *NPC1L1* gene may conveniently be expressed, again, using methods which are commonly known in the art. For example, NPC1L1 may be inserted into a pET-based plasmid vector (Stratagene; La Jolla, CA), downstream of the T7 RNA
15 polymerase promoter. The plasmid may then be transformed into a T7 expression system (e.g., BL21DE3 E.coli cells), grown in a liquid culture and induced (e.g., by adding IPTG to the bacterial culture).

Example 2: Direct Binding Assay.

Membrane preparation: Caco2 cells transfected with an expression vector containing a polynucleotide encoding NPC1L1 (e.g., SEQ ID NO: 2, 4 or 12) are harvested by incubating in 5 mM EDTA/phosphate-buffered saline followed by repeated pipeting. The cells are centrifuged 5 min at 1000 x g. The EDTA/PBS is decanted and an equal volume of ice-cold 50mM Tris-HCl, pH 7.5 is added and cells are broken up with a
25 Polytron (PT10 tip, setting 5, 30 sec). Nuclei and unbroken cells are sedimented at 1000 x g for 10 min and then the supernatant is centrifuged at 50,000 x g for 10 min. The supernatant is decanted, the pellet is resuspended by Polytron, a sample is taken for protein assay (bicinchoninic acid, Pierce), and the tissue is again centrifuged at 50,000 x g. Pellets are stored frozen at -20°C.

Binding assay: For saturation binding, four concentrations of [³H]-ezetimibe (15 Ci/mmol) are incubated without and with 10⁻⁵ M ezetimibe in triplicate with 50 µg of membrane protein in a total volume of 200 µl of 50 mM Tris-HCl, pH 7.5, for 30 min at 30°C. Samples are filtered on GF/B filters and washed three times with 2 ml of cold Tris buffer. Filters are dried in a microwave oven, impregnated with Meltilex wax scintillant,
35 and counted at 45% efficiency. For competition binding assays, five concentrations of a

sample are incubated in triplicate with 18 nM [^3H]-ezetimibe and 70 μg of membrane protein under the conditions described above. Curves are fit to the data with Prism (GraphPad Software) nonlinear least-squares curve-fitting program and K_i values are derived from IC_{50} values according to Cheng and Prusoff (Cheng, Y. C., *et al.*, (1973) Biochem. Pharmacol. 22:3099-3108).

Example 3: SPA Assay.

For each well of a 96 well plate, a reaction mixture of 10 μg human, mouse or rat NPC1L1-CHO overexpressing membranes (Biosignal) and 200 μg /well YSi-WGA-SPA beads (Amersham) in 100 μl is prepared in NPC1L1 assay buffer (25 mM HEPES, pH 7.8, 2 mM CaCl_2 , 1mM MgCl_2 , 125 mM NaCl, 0.1% BSA). A 0.4 nM stock of ligand- [^{125}I]-ezetimibe- is prepared in the NPC1L1 assay buffer. The above solutions are added to a 96-well assay plate as follows: 50 μl NPC1L1 assay buffer, 100 μl of reaction mixture, 50 μl of ligand stock (final ligand concentration is 0.1 nM). The assay plates are shaken for 5 minutes on a plate shaker, then incubated for 8 hours before cpm/well are determined in Microbeta Trilux counter (PerkinElmer).

These assays will indicate that [^{125}I]-ezetimibe binds to the cell membranes expressing human, mouse or rat NPC1L1. Similar results will be obtained if the same experiment is performed with radiolabeled cholesterol (*e.g.*, ^{125}I -cholesterol).

Example 4: Cholesterol Uptake Assay.

CHO cells expressing either SR-B1 or three different clones of rat NPC1L1 or one clone of mouse NPC1L1 were starved overnight in cholesterol free media then dosed with [^3H]-cholesterol in a mixed synthetic micelle emulsion for 4 min, 8 min ,12 min or 24 min in the absence or presence of 10 μM ezetimibe. The cells were harvested and the lipids were organically extracted. The extracted lipids were spotted on thin-layer chromatography (TLC) plates and resolved within an organic vapor phase. The free cholesterol bands for each assay were isolated and counted in a scintillation counter.

The SR-B1 expressing cells exhibited an increase in [^3H]-cholesterol uptake as early as 4 min which was also inhibited by ezetimibe. The three rat clones and the one mouse clone appeared to give background levels of [^3H]-cholesterol uptake which was similar to that of the untransformed CHO cell.

These experiments will yield data demonstrating that CHO cells can perform mouse, rat and human NPC1L1-dependent uptake of [^3H]-cholesterol when more optimal experimental conditions are developed.

Example 5: Expression of Rat *NPC1L1* in Wistar Rat Tissue.

In these experiments, the expression of rat *NPC1L1* mRNA, in several rat tissues, was evaluated. The tissues evaluated were esophagus, stomach, duodenum, jejunum, ileum, proximal colon, distal colon, liver, pancreas, heart, aorta, spleen, lung, kidney, brain, muscle, testes, ovary, uterus, adrenal gland and thyroid gland. Total RNA samples were isolated from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using standard dual-labeled fluorogenic oligonucleotide probes. Typical probe design incorporated a 5' reporter dye (e.g., 6FAM (6-carboxyfluorescein) or VIC) and a 3' quenching dye (e.g., TAMRA (6-carboxytetramethyl-rhodamine)).

rat *NPC1L1*:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 14)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 15)

Probe: [6FAM]TGCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 16)

rat β -actin:

Forward: ATCGCTGACAGGATGCAGAAG (SEQ ID NO: 17)

Reverse: TCAGGAGGAGCAATGATCTTGA (SEQ ID NO: 18)

Probe: [VIC]AGATTACTGCCCTGGCTCCTAGCACCAT[TAMRA] (SEQ ID NO: 19)

PCR reactions were run in 96-well format with 25 μ l reaction mixture in each well containing: Platinum SuperMix (12.5 μ l), ROX Reference Dye (0.5 μ l), 50 mM magnesium chloride (2 μ l), cDNA from RT reaction (0.2 μ l). Multiplex reactions contained gene specific primers at 200 nM each and FAM labeled probe at 100 nM and gene specific primers at 100 nM each and VIC labeled probe at 50 nM. Reactions were run with a standard 2-step cycling program, 95^o C for 15 sec and 60^o C for 1 min, for 40 cycles.

The highest levels of expression were observed in the duodenum, jejunum and ileum tissue. These data indicate that NPC1L1 plays a role in cholesterol absorption in the intestine.

Example 6: Expression of Mouse *NPC1L1* in Mouse Tissue.

In these experiments, the expression of mouse *NPC1L1* mRNA, in several tissues, was evaluated. The tissues evaluated were adrenal gland, BM, brain, heart, islets of langerhans, LI, small intestine, kidney, liver, lung, MLN, PLN, muscle, ovary, pituitary

gland, placenta, Peyers Patch, skin, spleen, stomach, testes, thymus, thyroid gland, uterus and trachea. Total RNA samples were isolate from at least 3 male and 3 female animals and pooled. The samples were then subjected to real time quantitative PCR using Taqman analysis using the following primers and probes:

5 mouse *NPC1L1*:

Forward: ATCCTCATCCTGGGCTTTGC (SEQ ID NO: 20)

Reverse: GCAAGGTGATCAGGAGGTTGA (SEQ ID NO: 21)

Probe: [6FAM]CCCAGCTTATCCAGATTTTCTTCTTCCGC[TAMRA] (SEQ ID NO: 22)

- 10 The highest levels of expression were observed in the Peyer's Patch, small intestine, gall bladder and stomach tissue. These data are consistent with a cholesterol absorption role for NPC1L1 which takes place in the digestive system.

Example 7: Expression of Human *NPC1L1* in Human Tissue.

- 15 In these experiments, the expression level of human *NPC1L1* mRNA was evaluated in 2045 samples representing 46 normal tissues. Microarray-based gene expression analysis was performed on the Affymetrix HG-U95 GeneChip using a cRNA probe corresponding to base pairs 4192-5117 (SEQ ID NO: 43) in strict accordance to Affymetrix's established protocols. Gene Chips were scanned under low photo multiplier
20 tube (PMT), and data were normalized using either Affymetrix MAS 4.0 or MAS 5.0 algorithms. In addition "spike ins" for most samples were used to construct a standard curve and obtain RNA concentration values according Gene Logic algorithms and procedures. A summary of these results are indicated, below, in Table 2.

25 **Table 2. Expression level of *NPC1L1* mRNA in various human tissues.**

Tissue	Present	Absent	Lower 25%	Median	Upper 75%
Adipose	2 of 32	30 of 32	-2.45	1.16	12.23
Adrenal Gland	0 of 12	12 of 12	-23.54	-4.47	10.51
Appendix	0 of 3	3 of 3	-8.02	-6.69	38.19
Artery	0 of 3	3 of 3	-6.59	-4.67	9.68
Bladder	1 of 5	4 of 5	-22	-7.95	-1.99
Bone	0 of 3	3 of 3	-1.64	3.3	19.53
Breast	4 of 80	76 of 80	-4.07	3.13	14.67
Cerebellum	0 of 5	5 of 5	-3.04	3.24	15.38
Cervix	3 of 101	98 of 101	-7.56	-0.07	20.89
Colon	9 of 151	142 of 151	-10.19	0.31	18.36
Cortex Frontal Lobe	0 of 7	7 of 7	1.4	8.46	11.75
Cortex Temporal Lobe	0 of 3	3 of 3	7.1	8.5	15.87
Diaphragm	59 of 65	6 of 65	-5.62	12.72	110.37
Endometrium	0 of 21	21 of 21	-14.43	-6.39	2.79
Esophagus	1 of 27	26 of 27	-10.93	-4.97	12.48
Fallopian Tube	3 of 51	48 of 51	5.02	13.24	26.77
Gallbladder	3 of 3	0 of 3	-2.57	2.78	32.28
Heart	0 of 3	3 of 3	3.33	11.19	11.66
Hippocampus	0 of 5	5 of 5	8.25	9.11	19.83
Kidney	4 of 86	82 of 86	-8.36	3.41	16.46
Larynx	0 of 4	4 of 4	-13.76	-0.81	8.54
Left Atrium	2 of 141	139 of 141	-18.9	-4.58	6.84
Left Ventricle	0 of 15	15 of 15	-21.19	-9.59	17.7
Liver	2 of 93	91 of 93	-3.47	11.03	22.34
Lung	0 of 11	11 of 11	-1.78	-0.19	1.34
Lymph Node	0 of 39	39 of 39	-21.57	8.25	26.73
Muscles	8 of 106	98 of 106	-3.98	4.87	17.55
Myometrium	0 of 15	15 of 15	-14.25	-1.6	19.58
Omentum	1 of 74	73 of 74	0.5	17.51	38.28
Ovary	0 of 34	34 of 34	-87.08	-53.2	-24.14
Pancreas	0 of 5	5 of 5	-20.4	-3.44	18.91
Placenta	0 of 32	32 of 32	1.08	15.56	27.24
Prostate	1 of 43	42 of 43	-9.26	-1.49	9.8
Rectum	4 of 169	165 of 169	-19.32	-6.58	7.72
Right Atrium	1 of 160	159 of 160	-24.01	-6.49	10.06
Right Ventricle	0 of 59	59 of 59	-12.68	1.5	22.77
Skin	1 of 6	5 of 6	-1.99	2.6	5.32
Soft Tissues	0 of 31	31 of 31	-9.41	-0.31	9.5
Spleen	0 of 5	5 of 5	-4.51	1.22	11.2
Stomach	1 of 71	70 of 71	-6.26	2.51	11.67
Testis	1 of 18	17 of 18	-12.22	2.84	17.86
Thymus	0 of 58	58 of 58	-10.67	1.59	16.01
Thyroid Gland	3 of 40	37 of 40	-16.45	-0.72	25.18
Uterus					
WBC					

Shaded data corresponds to tissues wherein the highest levels of *NPC1L1* mRNA was detected. The "Present" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* mRNA was detected. The "Absent" column indicates the proportion of specified tissue samples evaluated wherein *NPC1L1* RNA was not detected.

5 The "lower 25%", "median" and "upper 75%" columns indicate statistical distribution of the relative *NPC1L1* signal intensities observed for each set of tissue evaluated.

Example 8: Distribution of Rat *NPC1L1*, Rat *IBAT* or Rat *SR-B1* mRNA in Rat Small Intestine.

10 In these experiments, the distribution of rat *NPC1L1* mRNA along the proximal-distal axis of rat small intestines was evaluated. Intestines were isolated from five independent animals and divided into 10 sections of approximately equal length. Total RNA was isolated and analyzed, by real time quantitative PCR using Taqman analysis, for localized expression levels of rat *NPC1L1*, rat *IBAT* (ileal bile acid transporter) or rat
15 *SR-B1* mRNA. The primers and probes used in the analysis were:

rat *NPC1L1*:

Forward: TCTTCACCCTTGCTCTTTGC (SEQ ID NO: 23)

Reverse: AATGATGGAGAGTAGGTTGAGGAT (SEQ ID NO: 24)

20 Probe: [6FAM]TGCCACCTTTGTTGTCTGCTACC[TAMRA] (SEQ ID NO: 25)

rat Villin:

Forward: AGCACCTGTCCACTGAAGATTTC (SEQ ID NO: 26)

Reverse: TGGACGCTGAGCTTCAGTTCT (SEQ ID NO: 27)

Probe: [VIC]CTTCTCTGCGCTGCCTCGATGGAA[TAMRA] (SEQ ID NO: 28)

25 rat *SR-B1*:

Forward: AGTAAAAGGGCTCGCAGGAT (SEQ ID NO: 29)

Reverse: GGCAGCTGGTGACATCAGAGA (SEQ ID NO: 30)

Probe: [6FAM]AGGAGGCCATGCAGGCCTACTCTGA[TAMRA] (SEQ ID NO: 31)

rat *IBAT*:

30 Forward: GAGTCCACGGTCAGTCCATGT (SEQ ID NO: 32)

Reverse: TTATGAACAACAATGCCAAGCAA (SEQ ID NO: 33)

Probe: [6FAM]AGTCCTTAGGTAGTGGCTTAGTCCCTGGAAGCTC[TAMRA] (SEQ ID NO: 34)

The mRNA expression levels of each animal intestinal section were analyzed
35 separately, then the observed expression level was normalized to the observed level of

villin mRNA in that intestinal section. The observed, normalized mRNA expression levels for each section were then averaged.

The expression level of *NPC1L1* and *SR-B1* were highest in the jejunum (sections 2-5) as compared to that of the more distal ileum sections. Since the jejunum is believed to be the site of cholesterol absorption, these data suggest such a role for rat NPC1L1. *IBAT* distribution favoring the ileum is well documented and served as a control for the experiment.

Example 9: *In situ* Analysis of Rat *NPC1L1* mRNA in Rat Jejunum Tissue.

The localization of rat *NPC1L1* mRNA was characterized by *in situ* hybridization analysis of rat jejunum serial sections. The probes used in this analysis were:

T7-sense probe: GTAATACGACTCACTATAGGGCCCTGACGGTCCTTCCTGAGGGAATCTTCAC (SEQ ID NO: 35)

T7-antisense probe: GTAATACGACTCACTATAGGGCCTGGGAAGTTGGTCATGGCCACTCCAGC (SEQ ID NO: 36)

The RNA probes were synthesized using T7 RNA polymerase amplification of a PCR amplified DNA fragment corresponding rat *NPC1L1* nucleotides 3318 to 3672 (SEQ ID NO 1). Sense and anti-sense digoxigenin-UTP labeled cRNA probes were generated from the T7 promoter using the DIG RNA Labeling Kit following the manufacturer's instructions. Serial cryosections rat jejunum were hybridized with the sense and antisense probes. Digoxigenin labeling was detected with the DIG Nucleic Acid Detection Kit based on previous methods. A positive signal is characterized by the deposition of a red reaction product at the site of hybridization.

The anti-sense probe showed strong staining of epithelium along the crypt-villus axis under low magnification (40X). The observed rat *NPC1L1* mRNA expression levels may have been somewhat greater in the crypts than in the villus tips. Under high magnification (200X), staining was observed in the enterocytes but not in the goblet cells. A lack of staining observed with the sense probe (control) confirmed the high specificity of the *NPC1L1* anti-sense signal. These data provided further evidence of the role of rat NPC1L1 in intestinal cholesterol absorption.

Example 10: FACS Analysis of Fluorescently Labeled Ezetimibe Binding to Transiently Transfected CHO Cells.

In these experiments, the ability of BODIPY-labeled ezetimibe (Altmann, *et al.*, (2002) Biochim. Biophys. Acta 1580(1):77-93) to bind to NPC1L1 and SR-B1 was evaluated. "BODIPY" is a fluorescent group which was used to detect the BODIPY-ezetimibe. Chinese hamster ovary (CHO) cells were transiently transfected with rat
5 *NPC1L1* DNA (rNPC1L1/CHO), mouse *NPC1L1* DNA (mNPC1L1/CHO), mouse *SR-B1* DNA (mSRB1/CHO) or *EGFP* DNA (EGFP/CHO). EGFP is enhanced green fluorescent protein which was used as a positive control. The transfected CHO cells or untransfected CHO cells were then stained with 100 nM BODIPY-labeled ezetimibe and analyzed by FACS. Control experiments were also performed wherein the cells were not labeled with
10 the BODIPY-ezetimibe and wherein untransfected CHO cells were labeled with the BODIPY-ezetimibe.

No staining was observed in the untransfected CHO, rNPC1L1/CHO or mNPC1L1/CHO cells. Fluorescence was detected in the positive-control EGFP/CHO cells. Staining was also detected in the mouse SR-B1/CHO cells. These data show that,
15 under the conditions tested, BODIPY-ezetimibe is capable of binding to SR-B1 and that such binding is not ablated by the presence of the fluorescent BODIPY group. When more optimal conditions are determined, BODIPY-ezetimibe will be shown to label the rNPC1L1/CHO and mNPC1L1/CHO cells.

20 **Example 11: FACS Analysis of Transiently Transfected CHO Cells Labeled with Anti-FLAG Antibody M2.**

In these experiments, the expression of FLAG-tagged NPC1L1 on CHO cells was evaluated. CHO cells were transiently transfected with mouse *NPC1L1* DNA, rat *NPC1L1* DNA, FLAG- rat *NPC1L1* DNA or FLAG- mouse *NPC1L1* DNA. The 8 amino
25 acid FLAG tag used was DYKDDDDK (SEQ ID NO: 37) which was inserted on the amino-terminal extracellular loop just past the secretion signal sequence. The cells were incubated with commercially available anti-FLAG monoclonal mouse antibody M2 followed by a BODIPY-tagged anti-mouse secondary antibody. The treated cells were then analyzed by FACS.

30 The M2 antibody stained the CHO cells transfected with FLAG-rat *NPC1L1* DNA and with FLAG-mouse NPC1L1. No staining was observed in the CHO cells transfected with mouse *NPC1L1* DNA and with rat *NPC1L1* DNA. These data showed that rat NPC1L1 and mouse NPC1L1 possess no significant, inherent fluorescence and are not bound by the anti-FLAG antibody. The observed, FLAG-dependent labeling of

the cells indicated that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are localized at the cell membrane of the CHO cells.

Example 12: FACS Analysis of FLAG-rat NPC1L1-EGFP Chimera in Transiently Transfected CHO Cells.

In these experiments, the surface and cytoplasmic localization of rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG- rat *NPC1L1* DNA or with FLAG-rat *NPC1L1*-EGFP DNA. In these fusions, the FLAG tag is at amino-terminus of rat NPC1L1 and EGFP fusion is at the carboxy-terminus of rat NPC1L1. The cells were then stained with the M2 anti-FLAG mouse (primary) antibody followed by secondary staining with a BODIPY-labeled anti-mouse antibody. In control experiments, cells were stained with only the secondary antibody and not with the primary antibody (M2). The stained cells were then analyzed by FACS.

In a control experiment, FLAG-rat NPC1L1 transfected cells were stained with BODIPY anti-mouse secondary antibody but not with the primary antibody. The data demonstrated that the secondary, anti-mouse antibody possessed no significant specificity for FLAG-rat NPC1L1 and that the FLAG-rat NPC1L1, itself, possesses no significant fluorescence.

In another control experiment, unlabeled FLAG-rat NPC1L1-EGFP cells were FACS analyzed. In these experiments, autofluorescence of the enhanced green fluorescent protein (EGFP) was detected.

FLAG-rat NPC1L1 cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that the cells were labeled with the secondary, BODIPY-labeled antibody which indicated expression of the FLAG-rat NPC1L1 protein on the surface of the CHO cells.

FLAG-rat NPC1L1-EGFP cells were stained with anti-FLAG mouse antibody M2 and with the BODIPY-labeled anti-mouse secondary antibody and FACS analyzed. The data from this analysis showed that both markers (BODIPY and EGFP) were present indicating surface expression of the chimeric protein. The data also indicated that a portion of the protein was located within the cells and may be associated with transport vesicles. These data supported a role for rat NPC1L1 in vesicular transport of cholesterol or protein expressed in subcellular organelles such as the rough endoplasmic reticulum.

Example 13: FACS Analysis and Fluorescent Microscopy of FLAG-rat NPC1L1-EGFP Chimera in a Cloned CHO Cell Line.

In these experiments, the cellular localization of rat NPC1L1 was evaluated by FACS analysis and by immunohistochemistry. CHO cells were transfected with FLAG-rat *NPC1L1*-EGFP DNA and stained with anti-FLAG mouse antibody M2 and then with a BODIPY-labeled anti-mouse secondary antibody. In the fusion, the FLAG tag is at the amino-terminus of rat NPC1L1 and the enhanced green fluorescent protein (EGFP) tag is located at the carboxy-terminus of the rat NPC1L1. The stained cells were then analyzed by FACS and by fluorescence microscopy.

Cells transfected with FLAG-rat *NPC1L1*-EGFP DNA were stained with the anti-FLAG mouse antibody M2 and then with the BODIPY-labeled anti-mouse secondary antibody. FACS analysis of the cells detected both markers indicating surface expression of the chimeric protein.

FLAG-rat NPC1L1-EGFP transfected cells were analyzed by fluorescent microscopy at 63X magnification. Fluorescent microscopic analysis of the cells indicated non-nuclear staining with significant perinuclear organelle staining. Resolution of the image could not confirm the presence of vesicular associated protein. These data indicated that the fusion protein was expressed on the cell membrane of CHO cells.

Example 14: Generation of Polyclonal Anti-rat NPC1L1 Rabbit Antibodies.

Synthetic peptides (SEQ ID NO: 39-42) containing an amino- or carboxy-terminal cysteine residue were coupled to keyhole limpet hemocyanin (KLH) carrier protein through a disulfide linkage and used as antigen to raise polyclonal antiserum in New Zealand white rabbits (range 3-9 months in age). The KLH-peptide was emulsified by mixing with an equal volume of Freund's Adjuvant, and injected into three subcutaneous dorsal sites. Prior to the 16 week immunization schedule a pre-immune sera sample was collected which was followed by a primary injection of 0.25 mg KLH-peptide and 3 scheduled booster injections of 0.1 mg KLH-peptide. Animals were bled from the auricular artery and the blood was allowed to clot and the serum was then collected by centrifugation

The anti-peptide antibody titer was determined with an enzyme linked immunosorbent assay (ELISA) with free peptide bound in solid phase (1 µg/well). Results are expressed as the reciprocal of the serum dilution that resulted in an OD₄₅₀ of 0.2. Detection was obtained using the biotinylated anti-rabbit IgG, horse radish peroxidase-streptavidin (HRP-SA) conjugate, and ABTS.

Example 15: FACS Analysis of Rat NPC1L1 Expression in CHO Cells Transiently Transfected with Rat *NPC1L1* DNA Using Rabbit Anti-rat NPC1L1 Antisera.

In these experiments, the expression of rat NPC1L1 on the surface of CHO cells was evaluated. CHO cells were transfected with rat *NPC1L1* DNA, then incubated with either rabbit preimmune serum or with 10 week anti-rat NPC1L1 serum described, above, in Example 14 (*i.e.*, A0715, A0716, A0867 or A0868). Cells labeled with primary antisera were then stained with a BODIPY-modified anti-rabbit secondary antibody followed by FACS analysis.

No antibody surface labeling was observed for any of the pre-immune sera samples. Specific cell surface labeling of rat NPC1L1 transfected cells was observed for both A0715 and A0868. Antisera A0716 and A0867 did not recognize rat NPC1L1 surface expression in this assay format. This indicates that the native, unfused rat NPC1L1 protein is expressed in the CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

Example 16: FACS Analysis of CHO Cells Transiently Transfected with FLAG-Mouse *NPC1L1* DNA or FLAG-rat *NPC1L1* DNA or Untransfected CHO Cells Using Rabbit Anti-rat NPC1L1 Antisera.

In these experiments, the expression of FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 in CHO cells was evaluated. CHO cells were transiently transfected with FLAG-mouse *NPC1L1* DNA or with FLAG-rat *NPC1L1* DNA. The FLAG-mouse *NPC1L1* and FLAG-rat *NPC1L1* transfected cells were labeled with either A0801, A0802, A0715 or A0868 sera (see Example 14) or with anti-FLAG antibody, M2. The labeled cells were then stained with BODIPY-labeled anti-rabbit secondary antibody and FACS analyzed. The untransfected CHO cells were analyzed in the same manner as the transfected cell lines.

Positive staining of the untransfected CHO cells was not observed for any of the antisera tested. Serum A0801-dependent labeling of FLAG-rat *NPC1L1* transfected cells was observed but such labeling of FLAG-mouse *NPC1L1* transfected cells was not observed. Serum A0802-dependent labeling of FLAG-mouse *NPC1L1* or FLAG-rat *NPC1L1* transfected cells was not observed. Strong serum A0715-dependent labeling of FLAG-rat *NPC1L1* transfected cells was observed and weak serum A0715-dependent labeling of FLAG-mouse *NPC1L1* transfected cells was observed. Weak serum A0868-

dependent labeling of rat *NPC1L1* and mouse *NPC1L1* transfected cells was observed. Strong Anti-FLAG M2 antibody-dependent labeling of FLAG-rat *NPC1L1* and FLAG-mouse *NPC1L1* transfected cells was observed. The strong M2 staining is likely to be due to the fact that M2 is an affinity-purified, monoclonal antibody of known concentration.

- 5 In contrast, the respective antisera are polyclonal, unpurified and contain an uncertain concentration of anti-rat NPC1L1 antibody. These data provide further evidence that the FLAG-mouse NPC1L1 and FLAG-rat NPC1L1 proteins are expressed in CHO cells and localized to the CHO cell membranes. Cell surface expression of NPC1L1 is consistent with a role in intestinal cholesterol absorption.

10

Example 17: Immunohistochemical Analysis of Rat Jejunum Tissue with Rabbit Anti-rat NPC1L1 Antisera A0715.

- In these experiments, the localization of rat NPC1L1 in rat jejunum was analyzed by immunohistochemistry. Rat jejunum was removed, immediately embedded in O.C.T. compound and frozen in liquid nitrogen. Sections (6µm) were cut with a cryostat microtome and mounted on glass slides. Sections were air dried at room temperature and then fixed in Bouin's fixative. Streptavidin-biotin-peroxidase immunostaining was carried out using Histostain-SP kit. Endogenous tissue peroxidase activity was blocked with a 10 minute incubation in 3% H₂O₂ in methanol, and nonspecific antibody binding was minimized by a 45 minute incubation in 10% nonimmune rabbit serum. Sections were incubated with a rabbit anti-rat NPC1L1 antisera A0715 or A0868 at a 1:500 dilution at 4°C, followed by incubation with biotinylated goat anti-rabbit IgG and with streptavidin-peroxidase. Subsequently, the sections were developed in an aminoethyl carbazole (AEC)-H₂O₂ staining system and counterstained with hematoxylin and examined by microscopy. A positive reaction using this protocol is characterized by the deposition of a red reaction product at the site of the antigen-antibody reaction. Nuclei appeared blue from the hematoxylin counterstain. Controls were performed simultaneously on the neighboring sections from the same tissue block. Control procedures consisted of the following: (1) substitute the primary antibody with the pre-immune serum, (2) substitute the primary antibody with the non-immune rabbit serum, (3) substitute the primary antibody with PBS, (4) substitute the second antibody with PBS.

- The example shows tissue stained with anti-rat NPC1L1 sera A0715 or with the preimmune sera analyzed at low magnification (40X) and at high magnification (200X). The A0715-stained tissue, at low magnification, showed positive, strong staining of the

villi epithelial layer (enterocytes). The A0715-stained tissue at high magnification showed positive, strong staining of the enterocyte apical membranes. No staining was observed in tissue treated only with preimmune sera. Similar results were obtained with sera A0868. These data indicate that rat NPC1L1 is expressed in rat jejunum which is consistent with a role in intestinal cholesterol absorption.

Example 18: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells stably transfected with rat *NPC1L1* to take up labeled cholesterol was evaluated. In these assays, cholesterol uptake, at a single concentration, was evaluated in a pulse-chase experiment. The data generated in these experiments are set forth, below, in Table 3.

Cells:

A. CHO cells stably transfected with rat *NPC1L1* cDNA

B. CHO background (no transfection)

Cells were seeded at 500,000 cells/ well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL “starve” media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS).

One plate of each cell line was starved overnight. The remaining 2 plates were designated “No Starve” (see below).

Pre-Incubation. Media was removed from all plates, rinsed with serum-free HAMS and replaced with starve media for 30 minutes.

³H-Cholesterol Pulse. The following was added directly to each well.

0.5μCi ³H-cholesterol (~1.1 X 10⁶ dpm/well) in 50μl of a mixed bile salt micelle.

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

Dispersed in “starve” media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 0, 4, 12 and 24 minutes. Triplicate wells for each treatment were prepared.

Wash. At the designated times, media was aspirated and the cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis. Cells were digested overnight with 0.2N NaOH, 2mL/well at room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting. Two additional 50µl aliquots from all wells are assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized by the quantity of protein in the cells.

Table 3. Uptake of ³H-cholesterol by CHO cells transfected with rat *NPC1L1* or mouse *SR-B1* or untransfected CHO cells.

Time, min	Total Cholesterol, dpm protein ± sem					Total Cholesterol, dpm/mg protein ± sem			
	NPC1L1		CHO			NPC1L1		CHO	
After ³ H-Cholesterol					No Starve				
0	2067	±46	4568	±1937		10754	±166	22881	±9230
4	2619	±130	2868	±193		15366	±938	15636	±1471
12	2868	±193	4459	±170		15636	±1471	24622	±966
24	7010	±89	7204	±173		41129	±685	39361	±1207
					Starve				
0	1937	±273	2440	±299		10909	±1847	12429	±1673
4	3023	±308	2759	±105		17278	±1650	14307	±781
12	2759	±105	4857	±186		14307	±781	26270	±1473
24	6966	±72	7344	±65		39196	±174	38381	±161

dpm=disintegrations per minute
sem=standard error of the mean

Example 19: Effect of Ezetimibe on Cholesterol Uptake.

The effect of ezetimibe on the ability of CHO cells stably transfected with mouse or rat *NPC1L1* or mouse *SR-B1* to take up ³H-labeled cholesterol was evaluated in pulse-chase experiments. One cDNA clone of mouse *NPC1L1* (C7) and three clones of rat

NPC1L1 (C7, C17 and C21) were evaluated. The ability of CHO cells stably transfected with mouse *SR-B1*, mouse *NPC1L1* and rat *NPC1L1* to take up labeled cholesterol, in the absence of ezetimibe, was also evaluated in the pulse-chase experiments. Data generated in these experiments are set forth, below, in Tables 4 and 5. Additionally, the quantity of total cholesterol taken up by transfected and untransfected CHO cells in the presence of four different unlabeled cholesterol concentrations was also evaluated. The data from these experiments is set forth, below, in Table 6.

Cells:

- 10 A. CHO cells stably transfected with rat or mouse *NPC1L1* cDNA
- B. CHO background (no transfection)
- C. *SR-B1* transfected CHO cells

Cells seeded at 500,000 cells / well (mL) in 12-well plates.

15 Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed and the cells were rinsed with serum-free HAMS media. The serum-free media was then replaced with 1 mL “starve” media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS)). The cells were then starved overnight.

Pre-Incubation/ pre-dose. Media was removed from all plates and replaced with fresh starve media and preincubated for 30 minutes. Half of the wells received media containing ezetimibe (stock soln in EtOH; final conc. = 10μM).

³H-Cholesterol Pulse. The following was added directly to each well:

- 25 0.5μCi ³H-cholesterol (~1.1 X 10⁶ dpm/well) in 50μl of a mixed bile salt micelle

4.8mM sodium taurocholate (2.581mg/mL)

0.6 mM sodium oleate (0.183mg/mL)

0.25 mM cholesterol (0.1 mg/mL)

- 30 Dispersed in “starve” media by ultrasonic vibration

Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 4, 12, 24 minutes and 4 hours.

Triplicate wells were prepared for each treatment.

Wash. At designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% bovine serum albumin (BSA), pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis.

5 A. 4, 12, 24 minute time points: Cells were digested overnight with 0.2N NaOH, 2mL/well, room temperature. One 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

10 Extracts were spotted onto TLC plates and run for 30 minutes in 2 ml hexane:isopropanol (3:2) mobile phase for 30 minutes, followed by a second run in 1ml hexane:isopropanol (3:2) mobile phase for 15 minutes.

C. Protein determination of cell extracts. Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled
15 into 12 X 75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50µl aliquots from all wells were assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

20 **Table 4. Total Cholesterol in Transfected CHO Cells in the Presence and Absence of Ezetimibe.**

Clones:	Total Cholesterol, dpm± sem				Total Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)		Vehicle		EZ (10μM)	
4 Min Pulse								
CHO Control	3413	±417	3222	±26	33443	±4070	31881	±483
SR-BI	14207	±51	10968	±821	118242	±1261	92474	±2902
mNPC1L1(C7)	4043	±419	4569	±222	30169	±3242	30916	±1137
rNPC1L1(C21)	3283	±288	3769	±147	23728	±2111	27098	±689
rNPC1L1(C17)	3188	±232	3676	±134	24000	±832	28675	±527
rNPC1L1(C7)	1825	±806	3268	±121	15069	±6794	27285	±968
12 Min Pulse								
CHO Control	4710	±246	4532	±165	44208	±2702	43391	±1197
SR-BI	16970	±763	12349	±298	140105	±6523	98956	±4447
mNPC1L1(C7)	6316	±85	6120	±755	45133	±342	41712	±4054
rNPC1L1(C21)	5340	±12	4703	±231	40018	±1181	33985	±1928
rNPC1L1(C17)	4831	±431	4579	±257	37378	±3461	34063	±1619
rNPC1L1(C7)	4726	±272	4664	±63	39100	±2350	38581	±784
24 Min Pulse								
CHO Control	7367	±232	6678	±215	65843	±1281	61764	±2131
SR-BI	39166	±2152	23558	±1310	324126	±11848	198725	±11713
mNPC1L1(C7)	10616	±121	9749	±482	77222	±1040	74041	±3670
rNPC1L1(C21)	9940	±587	8760	±293	76356	±9618	66165	±2181
rNPC1L1(C17)	8728	±721	8192	±237	70509	±5189	62279	±4352
rNPC1L1(C7)	8537	±148	7829	±204	72134	±1305	63482	±368

EZ = ezetimibe

Table 5. Cholesterol Ester in CHO cells in the Presence or Absence of Ezetimibe.

Clones:	Cholesteryl Ester, dpm ± sem				4 Hour Pulse	Cholesteryl Ester, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)			Vehicle		EZ (10μM)	
CHO Control	652	±13	208	±9		5647	±55	1902	±87
SR-BI	47608	±1292	9305	±401		391067	±14391	72782	±3181
mNPC1L1(C7)	732	±127	453	±118		4994	±827	3057	±776
rNPC1L1(C21)	2667	±90	454	±33		18655	±1032	3193	±265
rNPC1L1(C17)	751	±74	202	±10		5379	±481	1510	±62
rNPC1L1(C7)	462	±25	191	±54		3597	±193	1496	±403

	Free Cholesterol, dpm ± sem				4 Hour Pulse	Free Cholesterol, dpm/mg protein ± sem			
	Vehicle		EZ (10 μM)			Vehicle		EZ (10μM)	
CHO Control	61612	±1227	56792	±568		533876	±17770	519607	±16203
SR-BI	214678	±4241	194519	±474		1762873	±46607	1521341	±4185
mNPC1L1(C7)	79628	±793	77516	±1910		544661	±1269	523803	±10386
rNPC1L1(C21)	71352	±1343	69106	±711		498016	±8171	485460	±4410
rNPC1L1(C17)	78956	±3782	71646	±446		566456	±29204	536651	±7146
rNPC1L1(C7)	75348	±2093	70628	±212		586127	±13932	556855	±7481

EZ =ezetimibe

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10

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Table 6. Uptake of labeled cholesterol in the presence of increasing amounts of unlabeled cholesterol.

	Total Cholesterol, dpm $\pm$ sem				Total Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
Cold Cholesterol								
	24 Min Pulse							
3 μ g/mL	12271 $\pm$ 430	49603 $\pm$ 2428	14250 $\pm$ 1628	10656 $\pm$ 1233	108936 $\pm$ 5413	541562 $\pm$ 13785	140764 $\pm$ 14433	94945 $\pm$ 12916
10 μ g/mL	16282 $\pm$ 2438	79967 $\pm$ 8151	25465 $\pm$ 3037	13225 $\pm$ 4556	151283 $\pm$ 23345	880224 $\pm$ 82254	250985 $\pm$ 27481	123433 $\pm$ 34092
30 μ g/mL	14758 $\pm$ 1607	71925 $\pm$ 3863	19001 $\pm$ 1530	13218 $\pm$ 1149	135109 $\pm$ 12106	796236 $\pm$ 18952	180436 $\pm$ 12112	111522 $\pm$ 6941
100 μ g/mL	16458 $\pm$ 1614	58185 $\pm$ 4548	15973 $\pm$ 1665	11560 $\pm$ 1132	149559 $\pm$ 17977	630143 $\pm$ 3718	147717 $\pm$ 8261	101328 $\pm$ 7191
	Cholesteryl Ester, dpm $\pm$ sem				Cholesteryl Ester, dpm/mg protein $\pm$ sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
4 Hour Pulse								
3 μ g/mL	2737 $\pm$ 114	39596 $\pm$ 1241	1561 $\pm$ 1	4015 $\pm$ 47	22050 $\pm$ 978	382641 $\pm$ 5955	13684 $\pm$ 217	32020 $\pm$ 641
10 μ g/mL	1646 $\pm$ 76	17292 $\pm$ 362	998 $\pm$ 36	1866 $\pm$ 33	13323 $\pm$ 606	157914 $\pm$ 3400	8917 $\pm$ 467	14849 $\pm$ 127
30 μ g/mL	970 $\pm$ 46	6642 $\pm$ 153	537 $\pm$ 82	970 $\pm$ 49	7627 $\pm$ 325	63547 $\pm$ 1760	4885 $\pm$ 748	7741 $\pm$ 100
100 μ g/mL	895 $\pm$ 156	4777 $\pm$ 27	405 $\pm$ 7	777 $\pm$ 16	7135 $\pm$ 1230	45088 $\pm$ 1526	3663 $\pm$ 68	6005 $\pm$ 198
	Free Cholesterol, dpm $\pm$ sem				Free Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
4 Hour Pulse								
3 μ g/mL	89013 $\pm$ 3724	211783 $\pm$ 3268	104343 $\pm$ 2112	92244 $\pm$ 987	717308 $\pm$ 34130	2047695 $\pm$ 16213	914107 $\pm$ 5869	735498 $\pm$ 11209
10 μ g/mL	136396 $\pm$ 8566	278216 $\pm$ 10901	196173 $\pm$ 4721	125144 $\pm$ 877	1105118 $\pm$ 76074	2540130 $\pm$ 92471	1753072 $\pm$ 86578	996824 $\pm$ 27850
30 μ g/mL	131745 $\pm$ 2922	224429 $\pm$ 2556	149172 $\pm$ 19689	117143 $\pm$ 4976	1036195 $\pm$ 21142	2149315 $\pm$ 78068	1357136 $\pm$ 180264	934772 $\pm$ 43202
100 μ g/mL	79336 $\pm$ 4011	231470 $\pm$ 4221	114599 $\pm$ 2803	93538 $\pm$ 1588	632965 $\pm$ 29756	2182022 $\pm$ 36793	1035979 $\pm$ 30329	723225 $\pm$ 21694
	Cholesteryl Ester, dpm $\pm$ sem				Cholesteryl Ester, dpm/mg protein $\pm$ sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
24 Hour Pulse								
3 μ g/mL	57373 $\pm$ 2704	162296 $\pm$ 1644	22986 $\pm$ 940	59377 $\pm$ 953	357629 $\pm$ 14639	1248900 $\pm$ 18565	160328 $\pm$ 6565	401315 $\pm$ 5557
10 μ g/mL	33730 $\pm$ 1296	112815 $\pm$ 373	14836 $\pm$ 552	31797 $\pm$ 525	215004 $\pm$ 5942	830231 $\pm$ 12764	98594 $\pm$ 4205	200451 $\pm$ 5239
30 μ g/mL	19193 $\pm$ 100	58668 $\pm$ 1413	8878 $\pm$ 355	18963 $\pm$ 380	122071 $\pm$ 1271	446581 $\pm$ 3472	59091 $\pm$ 2697	119728 $\pm$ 2131
100 μ g/mL	16761 $\pm$ 398	31280 $\pm$ 1270	8784 $\pm$ 946	14933 $\pm$ 311	103235 $\pm$ 1739	272796 $\pm$ 13392	60670 $\pm$ 4597	96215 $\pm$ 1023
	Free Cholesterol, dpm $\pm$ sem				Free Cholesterol, dpm/mg protein $\pm$ sem			
	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)	CHO Control	SR-BI	mNPC1L1(C7)	rNPC1L1(C21)
24 Hour Pulse								
3 μ g/mL	248985 $\pm$ 4207	357819 $\pm$ 4519	285610 $\pm$ 5187	227244 $\pm$ 1016	1552637 $\pm$ 18954	2752957 $\pm$ 24984	1993256 $\pm$ 56968	1536023 $\pm$ 10304
10 μ g/mL	231208 $\pm$ 8927	269822 $\pm$ 5872	311777 $\pm$ 8227	231666 $\pm$ 6198	1477414 $\pm$ 85954	1984473 $\pm$ 18420	2069980 $\pm$ 25517	1461157 $\pm$ 58517
30 μ g/mL	203566 $\pm$ 6008	225273 $\pm$ 5932	279604 $\pm$ 6612	209372 $\pm$ 3386	1294878 $\pm$ 41819	1716066 $\pm$ 52581	1859476 $\pm$ 29507	1321730 $\pm$ 5452
100 μ g/mL	178424 $\pm$ 2379	167082 $\pm$ 2211	229832 $\pm$ 4199	182678 $\pm$ 7709	1099648 $\pm$ 25160	1455799 $\pm$ 9885	1599244 $\pm$ 76938	1177546 $\pm$ 51191

5 Example 20: Labeled Cholesterol Uptake Assay.

In this example, the ability of CHO cells transiently transfected with rat *NPC1L1* or mouse *SR-BI* to take up labeled cholesterol was evaluated. Also evaluated was the ability of rat NPC1L1 to potentiate the ability of CHO cells transfected with mouse *SR-BI* to take up labeled cholesterol. In these assays, cholesterol uptake, at a single concentration, was evaluated in pulse-chase experiments. The data generated in these experiments are set forth, below, in Table 7.

Cells:

A. CHO background cells (mock transfection).

15 B. CHO cells transiently transfected with mouse *SR-BI*.

C. CHO transiently transfected with rat *NPC1L1* cDNAs (n=8 clones).

Transiently transfected cells were seeded at 300,000 cells / well (mL) in 12-well plates.

Procedure:

All reagents and culture plates were maintained at 37°C unless otherwise noted.

5

Starve. The maintenance media (F12 HAMS, 1%Pen/Strep, 10%FCS) was removed from the cells and replaced with 1 mL “starve” media (F12 HAMS, Pen/Strep, 5% lipoprotein deficient serum (LPDS). Cells were starved for 1 hour.

³H-Cholesterol Pulse. The following was added directly to each well.

10 0.5μCi ³H-cholesterol (~1.1 X 10⁶ dpm/well) in 50μl of a mixed bile salt micelle.

 4.8mM sodium taurocholate (2.581mg/mL)

 0.6 mM sodium oleate (0.183mg/mL)

 0.25 mM cholesterol (0.1 mg/mL)

15 Dispersed in “starve” media by ultrasonic vibration

 Final media cholesterol concentration = 5μg/mL

Labeled cholesterol pulse time points were 24 Min and 4 hours. Triplicate wells for each treatment.

20 **Wash.** At the designated times, media was aspirated and cells were washed once with Hobbs Buffer A (50mM Tris, 0.9% NaCl, 0.2% BSA, pH 7.4) and once with Hobbs Buffer B (50mM Tris, 0.9% NaCl, pH 7.4 (no BSA)) at 37°C.

Processing/Analysis.

25 A. 24 minute time point: Cells were digested overnight with 0.2N NaOH, 2mL/well at room temp. One, 1.5 mL aliquot was removed from each well, neutralized & counted for radioactivity by scintillation counting.

 B. 4 hour time point: The digested cells were analyzed by thin-layer chromatography to determine the content of cholesterol ester in the cells.

30 The extracts were spotted onto thin layer chromatography plates and run in 2 ml hexane:isopropanol (3:2) containing mobile phase for 30 minutes, followed by a second run in 1ml hexane:isopropanol (3:2) containing mobile phase for 15min.

 C. Protein determination of cell extracts: Plates containing a sample of the cell extracts were placed on orbital shaker at 120 rpm for indicated times and then extracts are pooled into 12X75 tubes. Plates were dried and NaOH (2ml/well) added. The protein content of the samples were then determined. Two additional 50μl aliquots from all wells were

assayed for total protein by the Pierce micro BCA method. The quantity of labeled cholesterol observed in the cells was normalized to the quantity of protein in the cells.

5 **Table 7. Labeled cholesterol uptake in transiently transfected CHO cells.**

	Total Cholesterol, $\pm$ sem	
	dpm	dpm/mg protein
Transfection	24 Min Pulse	
CHO Control (mock)	4721 $\pm$ 436	49024 $\pm$ 4328
SR-BI(Transient)	5842 $\pm$ 82	59445 $\pm$ 1099
NPC1L1 (Transient)	4092 $\pm$ 377	47026 $\pm$ 2658
SR-BI/NPC1L1 (trans)	3833 $\pm$ 158	52132 $\pm$ 3071
	Cholesteryl Ester, $\pm$ sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	2132 $\pm$ 40	20497 $\pm$ 640
SR-BI(Transient)	5918 $\pm$ 237	51812 $\pm$ 1417
NPC1L1 (Transient)	1944 $\pm$ 93	19788 $\pm$ 642
SR-BI/NPC1L1 (trans)	4747 $\pm$ 39	58603 $\pm$ 1156
	Free Cholesterol, $\pm$ sem	
	dpm	dpm/mg protein
	4 Hour Pulse	
CHO Control (mock)	45729 $\pm$ 328	439346 $\pm$ 5389
SR-BI(Transient)	50820 $\pm$ 2369	444551 $\pm$ 9785
NPC1L1 (Transient)	39913 $\pm$ 1211	406615 $\pm$ 6820
SR-BI/NPC1L1 (trans)	37269 $\pm$ 1225	459509 $\pm$ 6195

Example 21: Expression of rat, mouse and human NPC1L1.

In this example, *NPC1L1* was introduced into cells and expressed. Species specific NPC1L1 expression constructs were cloned into the plasmid pCDNA3 using clone specific PCR primers to generate the ORF flanked by appropriate restriction sites compatible with the polylinker of the vector. For all three species of NPC1L1, small intestine total tissue RNA was used as a template for reverse transcriptase-polymerase chain reaction (RT-PCR) using oligo dT as the template primer. The rat *NPC1L1* was cloned as an EcoRI fragment, human *NPC1L1* was cloned as a XbaI/NotI fragment and

mouse *NPC1L1* was cloned as an EcoRI fragment. Forward and reverse strand sequencing of each clone was performed to confirm sequence integrity. Standard transient transfection procedures were used with CHO cells. In a 6-well plate CHO cells were plated 1 day before transfection at a plating density of 2×10^5 cells/well. The following day, cells were incubated with 2 μ g plasmid DNA and 6 μ L Lipofectamine for 5 hours followed a fresh media change. Forty-eight hours later, cells were analyzed for NPC1L1 expression using anti-NPC1L1 antisera by either FACS or western blot. To establish stable long term cell lines expressing NPC1L1, transfected CHO cells were selected in the presence of geneticin (G418, 0.8 mg/ml) as recommended by the manufacturer (Life Technologies). Following one month of selection in culture, the cell population was stained with anti-NPC1L1 antisera and sorted by FACS. Individual positive staining cells were cloned after isolation by limiting dilution and then maintained in selective media containing geneticin (0.5 mg/ml).

Other cell types less susceptible to transfection procedures have been generated using adenoviral vector systems. This system used to express NPC1L1 is derived from Ad 5, a type C adenovirus. This recombinant replication-defective adenoviral vector is made defective through modifications of the E1, E2 and E4 regions. The vector also has additional modifications to the E3 region generally affecting the E3b region genes RIDa and RIDb. NPC1L1 expression was driven using the CMV promoter as an expression cassette substituted in the E3 region of the adenovirus. Rat and mouse NPC1L1 were amplified using clone specific primers flanked by restriction sites compatible with the adenovirus vector. Adenovirus infective particles were produced from 293-D22 cells in titers of 5×10^{10} P/mL. Viral lysates were used to infect cells resistant to standard transfection methodologies. In Caco2 cells, which are highly resistant to heterologous protein expression, adenovirus mediated expression of NPC1L1 has been shown by western blot analysis to persist at least 21 days post-infection.

Example 22: NPC1L1 Knock-Out Transgenic Mouse.

NPC1L1 knockout mice were constructed via targeted mutagenesis. This methodology utilized a targeting construct designed to delete a specific region of the mouse *NPC1L1* gene. During the targeting process the *E. coli lacZ* reporter gene was inserted under the control of the endogenous *NPC1L1* promoter. The region in *NPC1L1* (SEQ ID NO: 45) being deleted is from nucleotide 790 to nucleotide 998. The targeting vector contains the *LacZ-Neo* cassette flanked by 1.9 kb 5' arm ending with nucleotide 789 and a 3.2 kb 3' arm starting with nucleotide 999. Genomic DNA from the

recombinant embryonic stem cell line was assayed for homologous recombination using PCR. Amplified DNA fragments were visualized by agarose gel electrophoresis. The test PCRs employed a gene specific primer, which lies outside of and adjacent to the targeting vector arm, paired with one of three primers specific to the *LacZ-Neo* cassette sequence. For 5' PCR reconfirmation, the *NPC1L1* specific oligonucleotide ATGTTAGGTGAGTCTGAACCTACCC (SEQ ID NO: 46) and for 3'PCR reconfirmation the *NPC1L1* specific oligonucleotide GGATTGCATTTCTTCAA GAAAGCC (SEQ ID NO: 47) were used. Genotyping of the F2 mice was performed by multiplex PCR using the *NPC1L1* specific forward primer TATGGCTCTGCCC TCTGCAATGCTC (SEQ ID NO: 48) the *LacZ-Neo* cassette specific forward primer TCAGCAGCCTCTGTTCCACATACTTC (SEQ ID NO: 49) in combination with the *NPC1L1* gene specific reverse primer GTTCCACAGGGTCTGTGGTGAGTTC (SEQ ID NO: 50) allowed for determination of both the targeted and endogenous alleles. Analysis of the PCR products by agarose gel electrophoresis distinguished the wild-type, heterozygote and homozygote null mouse from each other.

Example 23: Acute Cholesterol Absorption in *NPC1L1*-Deficient Mice.

To determine whether *NPC1L1* plays a role in cholesterol absorption, *NPC1L1* deficient mice were studied.

Mice deficient in *NPC1L1* (-/-) were generated by breeding heterozygote mice (+/-) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-). Non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) were weighed and grouped (n=2 -/- and n=4 +/+). All animals were gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1μCi ¹⁴C-cholesterol (New England Nuclear, [⁴⁻¹⁴C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, MO). Two hours later, blood was collected by heart puncture. The liver was removed, weighed, and three samples were placed into 20 ml counting vials. Tissues were digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests were acidified by addition of 250μl of 4N HCl prior to liquid scintillation counting (LSC). Plasma was isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100μl aliquots of plasma were taken for LSC.

Cholesterol absorption, evaluated by this acute technique and expressed as the total amount of radioactive cholesterol in the plasma and liver, demonstrated that the wild type mice (+/+) absorbed an average of 11,773 dpm and *NPC1L1* deficient mice absorbed 992 dpm of the ¹⁴C-cholesterol. These results indicate that the *NPC1L1*

deficient mice have a 92% reduction in cholesterol absorption. These data confirm the role of NPC1L1 in intestinal cholesterol absorption. Inhibition of NPC1L1-mediated cholesterol absorption, in a subject, by administering NPC1L1 antagonists, such as ezetimibe, to the subject, are a useful way to reduce serum cholesterol levels and the occurrence of atherosclerosis in the subject.

Example 24: Cholesterol Absorption in *NPC1L1* (*NPC3*) Knockout Mice (Fecal Ratio Method: Cholesterol/Sitostanol).

In this example, cholesterol absorption and the activity of ezetimibe was determined in the *NPC1L1* knockout mice (-/-), heterozygous mice (+/-), and age matched wild-type mice (+/+).

Cholesterol absorption in the mice was determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1):77-93 (2002)). Mice (n= 4-6/group) were fed a standard rodent chow diet and in some groups treated daily with a maximally effective dose of ezetimibe (10 mg/kg). Mice were gavaged with ^{14}C -cholesterol (1 μCi , 0.1mg unlabeled cholesterol) and ^3H -sitostanol (2 μCi) in 0.1ml corn oil. Feces were collected for 2 days and fecal ^{14}C -cholesterol and ^3H -sitostanol levels were determined by combustion in a Packard Oxidizer. The fraction of cholesterol absorbed, as evaluated by the fecal dual isotope technique, was similar in wild type (+/+) and heterozygous mice (+/-) fed a chow diet (heterozygous mice absorbed $46 \pm 5\%$ and age matched wild type mice absorbed $51 \pm 3\%$ of the dose of ^{14}C -cholesterol). The *NPC1L1* knockout mice (-/-) absorbed $15.6 \pm 0.4\%$ of the ^{14}C -cholesterol, which was similar to the wild type mice treated with a maximally effective dose of ezetimibe ($16.1 \pm 0.3\%$), and reduced by 69% compared to wild type mice ($p < 0.001$). In *NPC1L1* knockout treated with ezetimibe at 10 mg/kg/day, cholesterol absorption was similar to that seen in the untreated knockout mice ($16.2 \pm 0.6\%$ compared to $15.6\% \pm 0.4\%$, respectively). Thus, the majority of cholesterol absorption is dependent on the presence of *NPC1L1* and the residual cholesterol absorption in mice lacking *NPC1L1* is insensitive to ezetimibe treatment. These results indicate that *NPC1L1* is involved in the small intestinal enterocyte uptake and absorption of cholesterol and is in the ezetimibe sensitive pathway.

Example 25: Mouse Screening Assay (Acute Cholesterol Absorption).

The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Mice deficient in *NPC1L1* (-/-) are generated by breeding heterozygote mice (+/-) to obtain wild-type (+/+) and *NPC1L1* deficient mice (-/-).

5 In a first set of experiments, non-fasted mice (6.5-9 weeks old, mixed 129 and C57BL/6 background) are weighed and grouped (n=1 to 4 -/- and n=1 to 4 +/+). All animals are gavaged (Feeding needles, 24G x 1 inch, Popper and Sons, NY) with 0.1 ml corn oil (Sigma; St. Louis, MO) containing 1 μ Ci ¹⁴C-cholesterol (New England Nuclear, [⁴⁻¹⁴C] Cholesterol, NEC-018) and 0.1mg carrier cholesterol mass (Sigma; St. Louis, 10 MO).

In another set of experiments, 1 to 4 wild-type *NPC1L1* mice (+/+) are treated identically to the mice in the first set of experiments, above, except that the mice are additionally fed a sample to be tested for the presence of an NPC1L1 antagonist.

15 Two hours later, blood is collected from each mouse by heart puncture. The liver is removed, weighed, and three samples are placed into 20 ml counting vials. Tissues are digested in 1 ml of 1N NaOH at 60°C overnight. The tissue digests are acidified by addition of 250 μ l of 4N HCl prior to liquid scintillation counting (LSC). Plasma is isolated by centrifugation at 10,000 rpm for 5 minutes in a microfuge and duplicate 100 μ l aliquots of plasma are taken for LSC.

20 Cholesterol absorption, evaluated by this acute technique is expressed as the total amount of radioactive cholesterol in the plasma and liver. The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol absorption (as measured by the above described methods) in the wild-type NPC1L1 mouse (+/+) which was fed the sample and in the NPC1L1 deficient mouse (-/-) are less than the amount of 25 cholesterol absorption in the wild-type NPC1L1 mouse (+/+) which was not fed the sample.

Example 26: Mouse Screening Assay (Fecal Ratio Method: Cholesterol/Sitostanol).

30 The following screening assay is used to identify the presence of an NPC1L1 antagonist in a sample.

Cholesterol absorption in the mice is determined by the dual fecal isotope ratio method as described by Altmann *et al.* (Biochim. Biophys. Acta. 1580(1):77-93 (2002)).

Three groups of mice (n=1-6/group) are assembled. Two separate groups comprise wild-type NPC1L1 mice (+/+) and one group comprises NPC1L1 deficient mice 35 (-/-).

Each group is fed a standard rodent chow diet and in some groups treated daily. Mice are gavaged with ^{14}C -cholesterol (1 μCi , 0.1mg unlabeled cholesterol) and ^3H -sitostanol (2 μCi) in 0.1ml corn oil. One group of mice, which comprise wild-type NPC1L1 mice (+/+) are further fed a sample to be tested for the presence of an NPC1L1 antagonist. Feces are collected for 2 days and fecal ^{14}C -cholesterol and ^3H -sitostanol levels are determined by combustion in a Packard Oxidizer.

The sample tested is determined to contain an NPC1L1 antagonist when the level of cholesterol and/or sitostanol absorption (as measured by the above described methods) in the wild-type NPC1L1 mouse (+/+) which was fed the sample and in the NPC1L1 deficient mouse (-/-) are less than the amount of cholesterol and/or sitostanol absorption in the wild-type NPC1L1 mouse (+/+) which was not fed the sample.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Patents, patent applications, publications, product descriptions, Genbank Accession Numbers and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes.

We Claim:

1. A mutant transgenic mouse comprising a homozygous mutation of endogenous,
chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1
5 protein.
2. The mouse of claim 1 wherein the mouse exhibits a reduced serum sterol or 5 α -stanol
level, a reduced liver sterol or 5 α -stanol level or a reduced level of intestinal absorption
of sterol or 5 α -stanol.
10
3. The mouse of claim 1 wherein the region of endogenous, chromosomal *NPC1L1*
deleted corresponds to nucleotides 790-998 of the nucleotide sequence set forth in SEQ
ID NO: 45.
- 15 4. An offspring or progeny of the mouse of claim 1 wherein the offspring or progeny has
inherited a mutated *NPC1L1* allele of said mouse.
5. A method for screening a sample for an intestinal sterol or 5 α -stanol absorption
antagonist comprising:
20 (a) feeding a sterol or 5 α -stanol-containing substance to a first and second mouse
comprising a functional *NPC1L1* gene and to a third, mutant mouse of claim 1;
(b) administering the sample to the first mouse but not the second mouse;
(c) measuring the amount of sterol or 5 α -stanol absorption in the intestine of said first,
second and third mouse; and
25 (d) comparing the levels of intestinal sterol or 5 α -stanol absorption in said first, second
and third mouse;
wherein the sample is determined to contain the intestinal sterol or 5 α -stanol absorption
antagonist when the level of intestinal sterol or 5 α -stanol absorption in the first mouse
and third mouse are less than the amount of intestinal sterol or 5 α -stanol absorption in the
30 second mouse.
6. The method of claim 5 wherein the sterol is cholesterol.
7. The method of claim 6 wherein the cholesterol is radiolabeled.

8. The method of claim 5 wherein the level of sterol or 5 α -stanol cholesterol absorption is determined by measuring the level of serum sterol or 5 α -stanol in the mice.

5 9. A method for inhibiting NPC1L1 mediated sterol or 5 α -stanol uptake, in a subject, by administering, to the subject, a substance identified by a method for identifying an antagonist of NPC1L1 comprising the steps of:

10 (a) contacting a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of detectably labeled ezetimibe, with a sample to be tested for the presence of the antagonist; and

(b) measuring the amount of detectably labeled ezetimibe specifically bound, directly or indirectly, to the polypeptide;

15 wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced direct or indirect binding of the detectably labeled ezetimibe to the polypeptide, compared to what would be measured in the absence of such an antagonist.

20 10. A method for inhibiting NPC1L1 mediated sterol or 5 α -stanol uptake, in a subject, by administering, to the subject, a substance identified by a method for identifying an antagonist of NPC1L1 comprising the steps of:

(a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer, to which a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a cell surface are attached;

25 (b) adding, to the suspension, radiolabeled ezetimibe and a sample to be tested for the presence of the antagonist, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon direct or indirect binding of the ezetimibe to the polypeptide to produce light energy, whereas radiolabeled ezetimibe that does not directly or indirectly bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and

30 (c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such an antagonist.

35

11. A method for inhibiting NPC1L1 mediated sterol or 5 α -stanol uptake, in a subject, by administering, to the subject, a substance identified by a method for identifying an antagonist of NPC1L1 comprising the steps of:
- 5 (a) contacting a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a cell surface with a detectably labeled sterol or 5 α -stanol and with a sample to be tested for the presence of the antagonist; and
- 10 (b) measuring the amount of detectably labeled sterol or 5 α -stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol or 5 α -stanol within the host cell, compared to what would be measured in the absence of such an antagonist..
12. A method for inhibiting NPC1L1 mediated sterol or 5 α -stanol uptake, in a subject, by administering, to the subject, a substance identified by the method of claim 5.
13. A kit comprising:
- (a) ezetimibe in a pharmaceutical dosage form; and
- 20 (b) information indicating that NPC1L1 is a target of ezetimibe.
14. The kit of claim 13 wherein the dosage form is a tablet comprising 10 mg ezetimibe.
15. The kit of claim 13 further comprising simvastatin in a pharmaceutical dosage form.
- 25 16. The kit of claim 15 wherein the simvastatin in pharmaceutical dosage form comprises 5 mg, 10 mg, 20 mg, 40 mg or 80mg simvastatin.
17. The kit of claim 15 wherein the simvastatin in pharmaceutical dosage form and the ezetimibe in pharmaceutical dosage form are associated in a single pill or tablet.
- 30 18. A method for decreasing the level of intestinal sterol or 5 α -stanol absorption in a subject comprising reducing the level of expression of NPC1L1 in the subject.
- 35 19. The method of claim 18 wherein the subject is a mouse, rat or human.

20. The method of claim 18 wherein the level of expression of NPC1L1 in the subject is reduced by mutating *NPC1L1* in the subject.

5 21. The method of claim 18 wherein the sterol is cholesterol.

22. A method for identifying an antagonist of NPC1L1 comprising:

(a) contacting a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a
10 cell surface, in the presence of a known amount of a detectably labeled substituted azetidinone, with a sample to be tested for the presence of the antagonist; and

(b) measuring the amount of detectably labeled substituted azetidinone specifically bound, directly or indirectly, to the polypeptide;

wherein an NPC1L1 antagonist in the sample is identified by measuring
15 substantially reduced direct or indirect binding of the detectably labeled substituted azetidinone to the polypeptide, compared to what would be measured in the absence of such an antagonist.

23. A kit comprising:

20 (a) a substituted azetidinone in a pharmaceutical dosage form; and
(b) information indicating that NPC1L1 is a target of the substituted azetidinone.

24. An isolated mammalian cell which lacks a gene which encodes a functional NPC1L1 protein.

25

25. The cell of claim 24 isolated from a mutant mouse comprising a homozygous mutation of endogenous, chromosomal *NPC1L1* wherein the mouse does not produce any functional NPC1L1 protein.

30 26. The cell of claim 25 wherein the mutation is of a gene which, when unmutated, encodes an amino acid sequence of SEQ ID NO: 12.

27. The cell of claim 24 isolated or derived from the duodenum, gall bladder, liver, small intestine or stomach tissue.

35

28. The cell of claim 27 which is an enterocyte.

29. An isolated polypeptide comprising 42 or more contiguous amino acids from an amino acid sequence selected from SEQ ID NOs: 2 and 12.

5

30. An isolated polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12.

31. An isolated polynucleotide encoding a polypeptide of claim 29.

10

32. An isolated polynucleotide comprising a nucleotide sequence selected from SEQ ID NOs: 1, 3 and 11.

33. A recombinant vector comprising the polynucleotide of claim 31.

15

34. A host cell comprising the vector of claim 33.

35. An isolated antibody which specifically binds to a polypeptide comprising 42 or more contiguous amino acids from an amino acid sequence selected from SEQ ID NOs: 2 and 12 or to an isolated polypeptide comprising an amino acid of SEQ ID NO: 4.

20

36. An isolated antibody which specifically binds to a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 39-42.

25 37. A method for making a polypeptide comprising culturing a host cell of claim 34 under conditions in which the polynucleotide is expressed.

38. The method of claim 37 wherein the polypeptide is isolated from the culture.

30 39. A method for identifying an antagonist of NPC1L1 comprising:

(a) contacting a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a cell surface, in the presence of a known amount of detectably labeled ezetimibe, with a sample to be tested for the presence of the antagonist; and

(b) measuring the amount of detectably labeled ezetimibe specifically bound, directly or indirectly, to the polypeptide;

wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced direct or indirect binding of the detectably labeled ezetimibe to the polypeptide, compared to what would be measured in the absence of such an antagonist.

40. A method for identifying an antagonist of NPC1L1 comprising:

(a) placing, in an aqueous suspension, a plurality of support particles, impregnated with a fluorescer, to which a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a cell surface are attached;

(b) adding, to the suspension, radiolabeled ezetimibe and a sample to be tested for the presence of the antagonist, wherein the radiolabel emits radiation energy capable of activating the fluorescer upon direct or indirect binding of the ezetimibe to the polypeptide to produce light energy, whereas radiolabeled ezetimibe that does not directly or indirectly bind to the polypeptide is, generally, too far removed from the support particles to enable the radioactive energy to activate the fluorescer; and

(c) measuring the light energy emitted by the fluorescer in the suspension; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced light energy emission, compared to what would be measured in the absence of such an antagonist.

41. The method of claim 40 wherein the fluorescer is selected from yttrium silicate, yttrium oxide, diphenyloxazole and polyvinyltoluene.

42. A method of claim 39 wherein the ezetimibe is labeled with a radiolabel selected from ^3H and ^{125}I .

43. A method of claim 40 wherein the ezetimibe is labeled with a radiolabel selected from ^3H and ^{125}I .

44. A method for identifying an antagonist of NPC1L1 comprising:

(a) contacting a host cell expressing a polypeptide comprising an amino acid sequence selected from SEQ ID NOs: 2, 4 and 12 or a functional fragment thereof on a

cell surface with a detectably labeled sterol or 5 α -stanol and with a sample to be tested for the presence of the antagonist; and

(b) measuring the amount of detectably labeled sterol or 5 α -stanol in the cell; wherein an NPC1L1 antagonist in the sample is identified by measuring substantially reduced detectably labeled sterol or 5 α -stanol within the host cell, compared to what would be measured in the absence of such an antagonist.

45 . The method of claim 44 wherein the sterol or 5 α -stanol is detectably labeled with a radiolabel selected from ^3H , ^{14}C and ^{125}I .

46. The method of claim 44 wherein the sterol is cholesterol.

47. A method according to claim 39 wherein the host cell is selected from a chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell and a Caco2 cell.

48. A method according to claim 40 wherein the host cell is selected from a chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell and a Caco2 cell.

49. A method according to claim 44 wherein the host cell is selected from a chinese hamster ovary (CHO) cell, a J774 cell, a macrophage cell and a Caco2 cell.

SEQUENCE LISTING

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Asp	Lys	Phe	Gln	Val	Ala	Phe	Ser	Ala	Glu	Arg	Ser	Leu	Glu	Asp	Glu 620	
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Ile	Asn	Arg	Thr	Thr	Ile	Gln	Asp	Leu	Pro	Val 635	Phe	Ala	Val	Ser	Tyr 640	
att	atc	gtc	ttc	ctg	tac	atc	tcc	ctg	gcc	ctg	ggc	agc	tac	tcc	aga	1968
Ile	Ile	Val	Phe	Leu	Tyr	Ile	Ser	Leu	Ala 650	Leu	Gly	Ser	Tyr	Ser	Arg 655	
tgc	agc	cga	gta	gcg	gtg	gag	tcc	aag	gct	act	ctg	ggc	cta	ggg	ggg	2016
Cys	Ser	Arg	Val	Ala	Val	Glu	Ser	Lys	Ala 665	Thr	Leu	Gly	Leu	Gly	Gly 670	
gtg	att	gtt	gtg	ctg	gga	gca	gtt	ctg	gct	gcc	atg	ggc	ttc	tac	tcc	2064
Val	Ile	Val	Val	Leu	Gly	Ala	Val	Leu	Ala	Ala	Met	Gly	Phe	Tyr	Ser 685	
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Tyr	Leu	Gly	Val	Pro	Ser	Ser	Leu	Val	Ile	Ile	Gln	Val	Val	Pro	Phe 700	
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Leu	Val	Leu	Ala	Val	Gly	Ala	Asp	Asn	Ile	Phe	Ile	Phe	Val	Leu	Glu	

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Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile				
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Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu				
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Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe				
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Lys Leu Pro Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe				
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cgc aag ata tac gct cct ttc ctg ctg cac aga ttc atc cgc cct gtt				2544
Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val				
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Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met				
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Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp				
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val				
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Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser				
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Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe				
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Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser				
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Tyr Val Ala Ile Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp				
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Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His	
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Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys	
980 985 990	
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Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Ala Glu Gln	
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Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Pro Pro Asn Ile	
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His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg	
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Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys	
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Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu	
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Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala	
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Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val	
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Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg	
1175 1180 1185	

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 Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala
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 Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu
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 Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Gln Glu Glu
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 Lys Leu Ala Ser Glu Ala Ala Val Ala Pro Glu Pro Ser Cys Pro
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 Phe Ala Pro Glu Leu Ala His Gly Ala Asn Ala Ala Arg Ser Ser
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 35 40 45

Thr Ser Leu Ser Asn Val Ser Cys Leu Ser Asn Thr Pro Ala Arg His
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Val Thr Gly Glu His Leu Ala Leu Leu Gln Arg Ile Cys Pro Arg Leu
 65 70 75 80

Tyr Asn Gly Pro Asn Thr Thr Phe Ala Cys Cys Ser Thr Lys Gln Leu
 85 90 95

Leu Ser Leu Glu Ser Ser Met Ser Ile Thr Lys Ala Leu Leu Thr Arg
 100 105 110

Cys Pro Ala Cys Ser Asp Asn Phe Val Ser Leu His Cys His Asn Thr
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Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Glu
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Arg Gly Ala Gly Glu Pro Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
 145 150 155 160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Gln Val Arg
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Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
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Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
 195 200 205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
 210 215 220

Pro Gly Gln Ala Leu Pro Asp Gly Ile Gln Pro Leu Asn Gly Lys Ile
 225 230 235 240

Ala Pro Cys Asn Glu Ser Gln Gly Asp Asp Ser Ala Val Cys Ser Cys
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Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Glu Ala Leu
 260 265 270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile

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Leu	Arg	Val	Val	Ser	Asn	Arg	Asn	Lys	Asn	Lys	Ala	Glu	Gly	Pro	Gln				
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Leu	Thr	Val	Leu	Ala	Leu	Ser	Phe	Ile	Val	Val	Ile	Ala	Leu	Ala	Ala				
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Ala	Pro	Lys	Ser	Gln	Ala	Arg	Lys	Glu	Lys	Ser	Phe	His	Asp	Glu	His				
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Arg	Ser	Ser	Tyr	Lys	Tyr	Asp	Ser	Leu	Leu	Leu	Gly	Ser	Lys	Asn	Phe				
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Glu	Arg	Leu	Arg	His	Leu	Gln	Val	Trp	Ser	Pro	Glu	Ala	Glu	Arg	Asn				
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Ile	Ser	Leu	Gln	Asp	Ile	Cys	Tyr	Ala	Pro	Leu	Asn	Pro	Tyr	Asn	Thr				
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Ser	Leu	Ser	Asp	Cys	Cys	Val	Asn	Ser	Leu	Leu	Gln	Tyr	Phe	Gln	Asn				
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Asn	Arg	Thr	Leu	Leu	Met	Leu	Thr	Ala	Asn	Gln	Thr	Leu	Asn	Gly	Gln				
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Thr	Ser	Leu	Val	Asp	Trp	Lys	Asp	His	Phe	Leu	Tyr	Cys	Ala	Asn	Ala				
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Pro Leu Thr Phe Lys Asp Gly Thr Ser Leu Ala Leu Ser Cys Met Ala
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Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
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Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Leu
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Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala Gln Ala Lys Leu Trp
 580 585 590

Glu Glu Ala Phe Leu Lys Glu Met Glu Ser Phe Gln Arg Asn Thr Ser
 595 600 605

Asp Lys Phe Gln Val Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
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Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Val Ser Tyr
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Ile Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
 645 650 655

Cys Ser Arg Val Ala Val Glu Ser Lys Ala Thr Leu Gly Leu Gly Gly
 660 665 670

Val Ile Val Val Leu Gly Ala Val Leu Ala Ala Met Gly Phe Tyr Ser
 675 680 685

Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
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Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
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Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
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Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
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Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
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- Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Leu Asp Phe
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Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785 790 795 800

Arg Gln Glu Ala Ser Arg Pro Asp Val Leu Cys Cys Phe Ser Thr Arg
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Lys Leu Pro Pro Pro Lys Glu Lys Glu Gly Leu Leu Leu Arg Phe Phe
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Arg Lys Ile Tyr Ala Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
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Val Met Leu Leu Phe Leu Thr Leu Phe Gly Ala Asn Leu Tyr Leu Met
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Cys Asn Ile Asn Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
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Gly Pro Pro Val Tyr Phe Val Thr Thr Ser Gly Phe Asn Phe Ser Ser
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Glu Ala Gly Met Asn Ala Thr Cys Ser Ser Ala Gly Cys Lys Ser Phe
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Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asp Gln Ser
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Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp
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Leu Thr Pro Ser Ser Ser Cys Cys Arg Leu Tyr Ile Arg Gly Pro His
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Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys
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Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Ala Glu Gln
995 1000 1005

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Val Pro Gly Thr Asp Pro Asn Phe Glu Val Phe Pro Tyr Thr Ile
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Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly
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Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys
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Tyr Leu Leu Leu Gly Leu Asp Met Cys Ser Gly Ile Leu Asn Leu
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Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala
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Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val
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Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg
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Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys
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Asp Ala Thr Val Phe Met Gly Ser Ala Val Phe Ala Gly Val Ala
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Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala
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Gln Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr

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Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Gln Glu Glu
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Lys Leu Ala Ser Glu Ala Ala Val Ala Pro Glu Pro Ser Cys Pro
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Gln Tyr Pro Ser Pro Ala Asp Ala Asp Ala Asn Val Asn Tyr Gly
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Leu Pro Lys Ser Asp Gln Lys Phe
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Arg Leu Ala Gln Ser Glu Pro Tyr Thr Thr Ile His Gln Pro Gly Tyr
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tgc gcc ttc tat gac gaa tgt ggg aag aac cca gag ctg tct gga agc      144
Cys Ala Phe Tyr Asp Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Ser
      35      40      45

ctc atg aca ctc tcc aac gtg tcc tgc ctg tcc aac acg ccg gcc cgc      192

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Cys	Pro	Ala	Cys	Ser	Asp	Asn	Phe	Val	Asn	Leu	His	Cys	His	Asn	Thr	
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Thr	Gly	Asn	Gly	Leu	Ala	Pro	Leu	Asp	Ile	Thr	Phe	His	Leu	Leu	Glu	
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Asp	Ser	Thr	Phe	Tyr	Leu	Gly	Gln	Met	Pro	Gly	Ser	Leu	Val	Leu	Ile	
		275					280					285				
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Ile	Ile	Leu	Cys	Ser	Val	Phe	Ala	Val	Val	Thr	Ile	Leu	Leu	Val	Gly	

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ctt ggc cag ttc ttc cag ggc tgg ggc acg tgg gtg gct tcg tgg cct Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro 340 345 350			1056
ctg acc atc ttg gtg cta tct gtc atc ccg gtg gtg gcc ttg gca gcg Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala 355 360 365			1104
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gcc ccc aac agc caa gcc cgg agt gag aaa gct ttc cat gac cag cat Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His 385 390 395 400			1200
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cgg tcc agc tac agg tat gac tct ctg ctg ctg ggg ccc aag aac ttc Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe 420 425 430			1296
agc gga atc ctg gac ctg gac ttg ctg ctg gag ctg cta gag ctg cag Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln 435 440 445			1344
gag agg ctg cgg cac ctc cag gta tgg tcg ccc gaa gca cag cgc aac Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn 450 455 460			1392
atc tcc ctg cag gac atc tgc tac gcc ccc ctc aat ccg gac aat acc Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr 465 470 475 480			1440
agt ctc tac gac tgc tgc atc aac agc ctc ctg cag tat ttc cag aac Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn 485 490 495			1488
aac cgc acg ctc ctg ctg ctc aca gcc aac cag aca ctg atg ggg cag Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln 500 505 510			1536
acc tcc caa gtc gac tgg aag gac cat ttt ctg tac tgt gcc aat gcc Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala 515 520 525			1584
ccg ctc acc ttc aag gat ggc aca gcc ctg gcc ctg agc tgc atg gct Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala 530 535 540			1632

gac tac ggg gcc cct gtc ttc ccc ttc ctt gcc att ggg ggg tac aaa	1680
Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys	
545 550 555 560	
gga aag gac tat tct gag gca gag gcc ctg atc atg acg ttc tcc ctc	1728
Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu	
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aac aat tac cct gcc ggg gac ccc cgt ctg gcc cag gcc aag ctg tgg	1776
Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp	
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Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala	
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Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu	
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Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr	
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att gtc ata ttc ctg tac atc tct ctg gcc ctg ggc agc tat tcc agc	1968
Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser	
645 650 655	
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Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly	
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gtg gcc gtg gtc ctg gga gca gtc atg gct gcc atg ggc ttc ttc tcc	2064
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Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe	
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Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu	
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Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile	
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Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe	
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Leu	Leu	Gln	Met	Ser	Ala	Phe	Val	Ala	Leu	Leu	Ser	Leu	Asp	Ser	Lys	
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Arg	Gln	Glu	Ala	Ser	Arg	Leu	Asp	Val	Cys	Cys	Cys	Val	Lys	Pro	Gln	
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Glu	Leu	Pro	Pro	Pro	Gly	Gln	Gly	Glu	Gly	Leu	Leu	Leu	Gly	Phe	Phe	
			820					825					830			
caa	aag	gct	tat	gcc	ccc	ttc	ctg	ctg	cac	tgg	atc	act	cga	ggt	gtt	2544
Gln	Lys	Ala	Tyr	Ala	Pro	Phe	Leu	Leu	His	Trp	Ile	Thr	Arg	Gly	Val	
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gtg	ctg	ctg	ctg	ttt	ctc	gcc	ctg	ttc	gga	gtg	agc	ctc	tac	tcc	atg	2592
Val	Leu	Leu	Leu	Phe	Leu	Ala	Leu	Phe	Gly	Val	Ser	Leu	Tyr	Ser	Met	
	850					855					860					
tgc	cac	atc	agc	gtg	gga	ctg	gac	cag	gag	ctg	gcc	ctg	ccc	aag	gac	2640
Cys	His	Ile	Ser	Val	Gly	Leu	Asp	Gln	Glu	Leu	Ala	Leu	Pro	Lys	Asp	
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tcg	tac	ctg	ctt	gac	tat	ttc	ctc	ttt	ctg	aac	cgc	tac	ttc	gag	gtg	2688
Ser	Tyr	Leu	Leu	Asp	Tyr	Phe	Leu	Phe	Leu	Asn	Arg	Tyr	Phe	Glu	Val	
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ggg	gcc	ccg	gtg	tac	ttt	gtt	acc	acc	ttg	ggc	tac	aac	ttc	tcc	agc	2736
Gly	Ala	Pro	Val	Tyr	Phe	Val	Thr	Thr	Leu	Gly	Tyr	Asn	Phe	Ser	Ser	
			900					905					910			
gag	gct	ggg	atg	aat	gcc	atc	tgc	tcc	agt	gca	ggc	tgc	aac	aac	ttc	2784
Glu	Ala	Gly	Met	Asn	Ala	Ile	Cys	Ser	Ser	Ala	Gly	Cys	Asn	Asn	Phe	
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Ser	Phe	Thr	Gln	Lys	Ile	Gln	Tyr	Ala	Thr	Glu	Phe	Pro	Glu	Gln	Ser	
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tac	ctg	gcc	atc	cct	gcc	tcc	tcc	tgg	gtg	gat	gac	ttc	att	gac	tgg	2880
Tyr	Leu	Ala	Ile	Pro	Ala	Ser	Ser	Trp	Val	Asp	Asp	Phe	Ile	Asp	Trp	
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Leu	Thr	Pro	Ser	Ser	Cys	Cys	Arg	Leu	Tyr	Ile	Ser	Gly	Pro	Asn	Lys	
				965					970					975		
gac	aag	ttc	tgc	ccc	tcg	acc	gtc	aac	tct	ctg	aac	tgc	cta	aag	aac	2976
Asp	Lys	Phe	Cys	Pro	Ser	Thr	Val	Asn	Ser	Leu	Asn	Cys	Leu	Lys	Asn	
			980					985				990				
tgc	atg	agc	atc	acg	atg	ggc	tct	gtg	agg	ccc	tcg	gtg	gag	cag	ttc	3024
Cys	Met	Ser	Ile	Thr	Met	Gly	Ser	Val	Arg	Pro	Ser	Val	Glu	Gln	Phe	
		995				1000					1005					
cat	aag	tat	ctt	ccc	tgg	ttc	ctg	aac	gac	cgg	ccc	aac	atc	aaa		3069
His	Lys	Tyr	Leu	Pro	Trp	Phe	Leu	Asn	Asp	Arg	Pro	Asn	Ile	Lys		
	1010					1015					1020					
tgt	ccc	aaa	ggc	ggc	ctg	gca	gca	tac	agc	acc	tct	gtg	aac	ttg		3114

- Cys	Pro	Lys	Gly	Gly	Leu	Ala	Ala	Tyr	Ser	Thr	Ser	Val	Asn	Leu	
	1025					1030					1035				
act	tca	gat	ggc	cag	gtt	tta	gcc	tcc	agg	ttc	atg	gcc	tat	cac	3159
Thr	Ser	Asp	Gly	Gln	Val	Leu	Ala	Ser	Arg	Phe	Met	Ala	Tyr	His	
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Lys	Pro	Leu	Lys	Asn	Ser	Gln	Asp	Tyr	Thr	Glu	Ala	Leu	Arg	Ala	
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gct	cga	gag	ctg	gca	gcc	aac	atc	act	gct	gac	ctg	cgg	aaa	gtg	3249
Ala	Arg	Glu	Leu	Ala	Ala	Asn	Ile	Thr	Ala	Asp	Leu	Arg	Lys	Val	
	1070					1075					1080				
cct	gga	aca	gac	ccg	gct	ttt	gag	gtc	ttc	ccc	tac	acg	atc	acc	3294
Pro	Gly	Thr	Asp	Pro	Ala	Phe	Glu	Val	Phe	Pro	Tyr	Thr	Ile	Thr	
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Asn	Val	Phe	Tyr	Glu	Gln	Tyr	Leu	Thr	Ile	Leu	Pro	Glu	Gly	Leu	
	1100					1105					1110				
ttc	atg	ctc	agc	ctc	tgc	ctt	gtg	ccc	acc	ttc	gct	gtc	tcc	tgc	3384
Phe	Met	Leu	Ser	Leu	Cys	Leu	Val	Pro	Thr	Phe	Ala	Val	Ser	Cys	
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Leu	Leu	Leu	Gly	Leu	Asp	Leu	Arg	Ser	Gly	Leu	Leu	Asn	Leu	Leu	
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Ser	Ile	Val	Met	Ile	Leu	Val	Asp	Thr	Val	Gly	Phe	Met	Ala	Leu	
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Trp	Asp	Ile	Ser	Tyr	Asn	Ala	Val	Ser	Leu	Ile	Asn	Leu	Val	Ser	
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Ala	Val	Gly	Met	Ser	Val	Glu	Phe	Val	Ser	His	Ile	Thr	Arg	Ser	
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ttt	gcc	atc	agc	acc	aag	ccc	acc	tgg	ctg	gag	agg	gcc	aaa	gag	3609
Phe	Ala	Ile	Ser	Thr	Lys	Pro	Thr	Trp	Leu	Glu	Arg	Ala	Lys	Glu	
	1190					1195					1200				
gcc	acc	atc	tct	atg	gga	agt	gcg	gtg	ttt	gca	ggt	gtg	gcc	atg	3654
Ala	Thr	Ile	Ser	Met	Gly	Ser	Ala	Val	Phe	Ala	Gly	Val	Ala	Met	
	1205					1210					1215				
acc	aac	ctg	cct	ggc	atc	ctt	gtc	ctg	ggc	ctc	gcc	aag	gcc	cag	3699
Thr	Asn	Leu	Pro	Gly	Ile	Leu	Val	Leu	Gly	Leu	Ala	Lys	Ala	Gln	
	1220					1225					1230				
ctc	att	cag	atc	ttc	ttc	ttc	cgc	ctc	aac	ctc	ctg	atc	act	ctg	3744
Leu	Ile	Gln	Ile	Phe	Phe	Phe	Arg	Leu	Asn	Leu	Leu	Ile	Thr	Leu	
	1235					1240					1245				
ctg	ggc	ctg	ctg	cat	ggc	ttg	gtc	ttc	ctg	ccc	gtc	atc	ctc	agc	3789
Leu	Gly	Leu	Leu	His	Gly	Leu	Val	Phe	Leu	Pro	Val	Ile	Leu	Ser	

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Tyr Val Gly Pro Asp Val Asn Pro Ala Leu Ala Leu Glu Gln Lys			
1265	1270	1275	
cgg gct gag gag gcg gtg gca gca gtc atg gtg gcc tct tgc cca			3879
Arg Ala Glu Glu Ala Val Ala Ala Val Met Val Ala Ser Cys Pro			
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Asn His Pro Ser Arg Val Ser Thr Ala Asp Asn Ile Tyr Val Asn			
1295	1300	1305	
cac agc ttt gaa ggt tct atc aaa ggt gct ggt gcc atc agc aac			3969
His Ser Phe Glu Gly Ser Ile Lys Gly Ala Gly Ala Ile Ser Asn			
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Phe Leu Pro Asn Asn Gly Arg Gln Phe			
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<211> 1332

<212> PRT

<213> Homo sapiens

<400> 4

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		20					25						30		

Cys	Ala	Phe	Tyr	Asp	Glu	Cys	Gly	Lys	Asn	Pro	Glu	Leu	Ser	Gly	Ser
	35						40					45			

Leu	Met	Thr	Leu	Ser	Asn	Val	Ser	Cys	Leu	Ser	Asn	Thr	Pro	Ala	Arg
50					55						60				

Lys	Ile	Thr	Gly	Asp	His	Leu	Ile	Leu	Leu	Gln	Lys	Ile	Cys	Pro	Arg
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Leu	Tyr	Thr	Gly	Pro	Asn	Thr	Gln	Ala	Cys	Cys	Ser	Ala	Lys	Gln	Leu
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Val	Ser	Leu	Glu	Ala	Ser	Leu	Ser	Ile	Thr	Lys	Ala	Leu	Leu	Thr	Arg
		100						105						110	

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Cys Pro Ala Cys Ser Asp Asn Phe Val Asn Leu His Cys His Asn Thr
  115                                120                        125

Cys Ser Pro Asn Gln Ser Leu Phe Ile Asn Val Thr Arg Val Ala Gln
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Leu Gly Ala Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
  145                                150                        155                        160

Gln His Ser Phe Ala Glu Gln Ser Tyr Asp Ser Cys Ser Arg Val Arg
                        165                                170                        175

Val Pro Ala Ala Ala Thr Leu Ala Val Gly Thr Met Cys Gly Val Tyr
                        180                                185                        190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
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Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
  210                                215                        220

Pro Gly Gln Ala Val Gly Ser Gly Ile Gln Pro Leu Asn Glu Gly Val
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Ala Arg Cys Asn Glu Ser Gln Gly Asp Asp Val Ala Thr Cys Ser Cys
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Gln Asp Cys Ala Ala Ser Cys Pro Ala Ile Ala Arg Pro Gln Ala Leu
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Asp Ser Thr Phe Tyr Leu Gly Gln Met Pro Gly Ser Leu Val Leu Ile
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Ile Ile Leu Cys Ser Val Phe Ala Val Val Thr Ile Leu Leu Val Gly
  290                                295                        300

Phe Arg Val Ala Pro Ala Arg Asp Lys Ser Lys Met Val Asp Pro Lys
  305                                310                        315                        320

Lys Gly Thr Ser Leu Ser Asp Lys Leu Ser Phe Ser Thr His Thr Leu
                        325                                330                        335

Leu Gly Gln Phe Phe Gln Gly Trp Gly Thr Trp Val Ala Ser Trp Pro
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Leu Thr Ile Leu Val Leu Ser Val Ile Pro Val Val Ala Leu Ala Ala
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Gly Leu Val Phe Thr Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
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Ala Pro Asn Ser Gln Ala Arg Ser Glu Lys Ala Phe His Asp Gln His
  385                               390                       395                       400

Phe Gly Pro Phe Phe Arg Thr Asn Gln Val Ile Leu Thr Ala Pro Asn
  405                               410                       415

Arg Ser Ser Tyr Arg Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
  420                               425                       430

Ser Gly Ile Leu Asp Leu Asp Leu Leu Leu Glu Leu Leu Glu Leu Gln
  435                               440                       445

Glu Arg Leu Arg His Leu Gln Val Trp Ser Pro Glu Ala Gln Arg Asn
  450                               455                       460

Ile Ser Leu Gln Asp Ile Cys Tyr Ala Pro Leu Asn Pro Asp Asn Thr
  465                               470                       475                       480

Ser Leu Tyr Asp Cys Cys Ile Asn Ser Leu Leu Gln Tyr Phe Gln Asn
  485                               490                       495

Asn Arg Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Met Gly Gln
  500                               505                       510

Thr Ser Gln Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
  515                               520                       525

Pro Leu Thr Phe Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Met Ala
  530                               535                       540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Ile Gly Gly Tyr Lys
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Gly Lys Asp Tyr Ser Glu Ala Glu Ala Leu Ile Met Thr Phe Ser Leu
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Asn Asn Tyr Pro Ala Gly Asp Pro Arg Leu Ala Gln Ala Lys Leu Trp
  580                               585                       590

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Glu Glu Ala Phe Leu Glu Glu Met Arg Ala Phe Gln Arg Arg Met Ala
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Gly Met Phe Gln Val Thr Phe Thr Ala Glu Arg Ser Leu Glu Asp Glu
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Ile Asn Arg Thr Thr Ala Glu Asp Leu Pro Ile Phe Ala Thr Ser Tyr
625 630 635 640

Ile Val Ile Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Ser
645 650 655

Trp Ser Arg Val Met Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
660 665 670

Val Ala Val Val Leu Gly Ala Val Met Ala Ala Met Gly Phe Phe Ser
675 680 685

Tyr Leu Gly Ile Arg Ser Ser Leu Val Ile Leu Gln Val Val Pro Phe
690 695 700

Leu Val Leu Ser Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
705 710 715 720

Tyr Gln Arg Leu Pro Arg Arg Pro Gly Glu Pro Arg Glu Val His Ile
725 730 735

Gly Arg Ala Leu Gly Arg Val Ala Pro Ser Met Leu Leu Cys Ser Leu
740 745 750

Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Pro Met Pro Ala
755 760 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Val Ile Leu Asp Phe
770 775 780

Leu Leu Gln Met Ser Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
785 790 795 800

Arg Gln Glu Ala Ser Arg Leu Asp Val Cys Cys Cys Val Lys Pro Gln
805 810 815

Glu Leu Pro Pro Pro Gly Gln Gly Glu Gly Leu Leu Leu Gly Phe Phe
820 825 830

Gln Lys Ala Tyr Ala Pro Phe Leu Leu His Trp Ile Thr Arg Gly Val

835	840	845
Val Leu Leu Leu Phe Leu Ala Leu Phe Gly Val Ser Leu Tyr Ser Met 850	855	860
Cys His Ile Ser Val Gly Leu Asp Gln Glu Leu Ala Leu Pro Lys Asp 865	870	875 880
Ser Tyr Leu Leu Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Phe Glu Val 885	890	895
Gly Ala Pro Val Tyr Phe Val Thr Thr Leu Gly Tyr Asn Phe Ser Ser 900	905	910
Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Asn Asn Phe 915	920	925
Ser Phe Thr Gln Lys Ile Gln Tyr Ala Thr Glu Phe Pro Glu Gln Ser 930	935	940
Tyr Leu Ala Ile Pro Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp 945	950	955 960
Leu Thr Pro Ser Ser Cys Cys Arg Leu Tyr Ile Ser Gly Pro Asn Lys 965	970	975
Asp Lys Phe Cys Pro Ser Thr Val Asn Ser Leu Asn Cys Leu Lys Asn 980	985	990
Cys Met Ser Ile Thr Met Gly Ser Val Arg Pro Ser Val Glu Gln Phe 995	1000	1005
His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Arg Pro Asn Ile Lys 1010	1015	1020
Cys Pro Lys Gly Gly Leu Ala Ala Tyr Ser Thr Ser Val Asn Leu 1025	1030	1035
Thr Ser Asp Gly Gln Val Leu Ala Ser Arg Phe Met Ala Tyr His 1040	1045	1050
Lys Pro Leu Lys Asn Ser Gln Asp Tyr Thr Glu Ala Leu Arg Ala 1055	1060	1065
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Phe	Met	Leu	Ser	Leu	Cys	Leu	Val	Pro	Thr	Phe	Ala	Val	Ser	Cys
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Leu	Leu	Leu	Gly	Leu	Asp	Leu	Arg	Ser	Gly	Leu	Leu	Asn	Leu	Leu
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<213> Rattus sp.

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<211> 4484

<212> DNA

<213> Rattus sp.

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Ser Ala Gln Gly Glu Leu Tyr Thr Pro Thr His Lys Ala Gly Phe Cys	
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acc ttt tat gaa gag tgt ggg aag aac cca gag ctt tct gga ggc ctc	144
Thr Phe Tyr Glu Glu Cys Gly Lys Asn Pro Glu Leu Ser Gly Gly Leu	
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aca tca cta tcc aat atc tcc tgc ttg tct aat acc cca gcc cgc cat	192
Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His	
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Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu	
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Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu	
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Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg	
100 105 110	
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Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr	
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Cys	Ser	Pro	Asp	Gln	Ser	Leu	Phe	Ile	Asn	Val	Thr	Arg	Val	Val	Gln	
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Gln	Arg	Ser	Phe	Ala	Glu	Lys	Ala	Tyr	Glu	Ser	Cys	Ser	Arg	Val	Arg	
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Ile	Pro	Ala	Ala	Ala	Ser	Leu	Ala	Val	Gly	Ser	Met	Cys	Gly	Val	Tyr	
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Gly	Ser	Ala	Leu	Cys	Asn	Ala	Gln	Arg	Trp	Leu	Asn	Phe	Gln	Gly	Asp	
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Thr	Gly	Asn	Gly	Leu	Ala	Pro	Leu	Asp	Ile	Thr	Phe	His	Leu	Leu	Glu	
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Pro	Gly	Gln	Ala	Leu	Ala	Asp	Gly	Met	Lys	Pro	Leu	Asp	Gly	Lys	Ile	
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Thr	Pro	Cys	Asn	Glu	Ser	Gln	Gly	Glu	Asp	Ser	Ala	Ala	Cys	Ser	Cys	
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Gln	Asp	Cys	Ala	Ala	Ser	Cys	Pro	Val	Ile	Pro	Pro	Pro	Pro	Ala	Leu	
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Ile	Ile	Phe	Thr	Ala	Val	Phe	Val	Leu	Leu	Ser	Val	Val	Leu	Val	Tyr	
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ctc	cga	gtg	gct	tcc	aac	agg	aac	aag	aac	aag	aca	gca	ggc	tcc	cag	960
Leu	Arg	Val	Ala	Ser	Asn	Arg	Asn	Lys	Asn	Lys	Thr	Ala	Gly	Ser	Gln	
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gaa	gcc	ccc	aac	ctc	cct	cgt	aag	cgc	aga	ttc	tca	cct	cac	act	gtc	1008
Glu	Ala	Pro	Asn	Leu	Pro	Arg	Lys	Arg	Arg	Phe	Ser	Pro	His	Thr	Val	
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Leu	Gly	Arg	Phe	Phe	Glu	Ser	Trp	Gly	Thr	Arg	Val	Ala	Ser	Trp	Pro	
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ctc	act	gtc	ttg	gca	ctg	tcc	ttc	ata	gtt	gtg	ata	gcc	ttg	tca	gta	1104
Leu	Thr	Val	Leu	Ala	Leu	Ser	Phe	Ile	Val	Val	Ile	Ala	Leu	Ser	Val	
		355					360					365				
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Gly	Leu	Thr	Phe	Ile	Glu	Leu	Thr	Thr	Asp	Pro	Val	Glu	Leu	Trp	Ser	

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Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg	
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Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe	
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Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu	
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Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala	
755 760 765	
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Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe	
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Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe	
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Gln	Leu	Ile	Gln	Ile	Phe	Phe	Phe	Arg	Leu	Asn	Leu	Leu	Ile	Thr	
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Lys	Leu	Ala	Thr	Glu	Ala	Ala	Met	Val	Ser	Glu	Pro	Ser	Cys	Pro	
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Gln	Tyr	Pro	Phe	Pro	Ala	Asp	Ala	Asn	Thr	Ser	Asp	Tyr	Val	Asn	
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Tyr	Gly	Phe	Asn	Pro	Glu	Phe	Ile	Pro	Glu	Ile	Asn	Ala	Ala	Ser	
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Thr Ser Leu Ser Asn Ile Ser Cys Leu Ser Asn Thr Pro Ala Arg His
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Val Thr Gly Asp His Leu Ala Leu Leu Gln Arg Val Cys Pro Arg Leu
 65 70 75 80

Tyr Asn Gly Pro Asn Asp Thr Tyr Ala Cys Cys Ser Thr Lys Gln Leu
 85 90 95

Val Ser Leu Asp Ser Ser Leu Ser Ile Thr Lys Ala Leu Leu Thr Arg
 100 105 110

Cys Pro Ala Cys Ser Glu Asn Phe Val Ser Ile His Cys His Asn Thr
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Cys Ser Pro Asp Gln Ser Leu Phe Ile Asn Val Thr Arg Val Val Gln
 130 135 140

Arg Asp Pro Gly Gln Leu Pro Ala Val Val Ala Tyr Glu Ala Phe Tyr
 145 150 155 160

Gln Arg Ser Phe Ala Glu Lys Ala Tyr Glu Ser Cys Ser Arg Val Arg
 165 170 175

Ile Pro Ala Ala Ala Ser Leu Ala Val Gly Ser Met Cys Gly Val Tyr
 180 185 190

Gly Ser Ala Leu Cys Asn Ala Gln Arg Trp Leu Asn Phe Gln Gly Asp
 195 200 205

Thr Gly Asn Gly Leu Ala Pro Leu Asp Ile Thr Phe His Leu Leu Glu
 210 215 220

Pro Gly Gln Ala Leu Ala Asp Gly Met Lys Pro Leu Asp Gly Lys Ile
 225 230 235 240

Thr Pro Cys Asn Glu Ser Gln Gly Glu Asp Ser Ala Ala Cys Ser Cys
 245 250 255

Gln Asp Cys Ala Ala Ser Cys Pro Val Ile Pro Pro Pro Pro Ala Leu
 260 265 270

Arg Pro Ser Phe Tyr Met Gly Arg Met Pro Gly Trp Leu Ala Leu Ile
 275 280 285

Ile Ile Phe Thr Ala Val Phe Val Leu Leu Ser Val Val Leu Val Tyr
 290 295 300

Leu Arg Val Ala Ser Asn Arg Asn Lys Asn Lys Thr Ala Gly Ser Gln
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 325 330 335

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Leu Thr Val Leu Ala Leu Ser Phe Ile Val Val Ile Ala Leu Ser Val
 355 360 365

Gly Leu Thr Phe Ile Glu Leu Thr Thr Asp Pro Val Glu Leu Trp Ser
 370 375 380

Ala Pro Lys Ser Gln Ala Arg Lys Glu Lys Ala Phe His Asp Glu His
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Phe Gly Pro Phe Phe Arg Thr Asn Gln Ile Phe Val Thr Ala Lys Asn
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Arg Ser Ser Tyr Lys Tyr Asp Ser Leu Leu Leu Gly Pro Lys Asn Phe
 420 425 430

Ser Gly Ile Leu Ser Leu Asp Leu Leu Gln Glu Leu Leu Glu Leu Gln
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Ser Leu Thr Asp Cys Cys Val Asn Ser Leu Leu Gln Tyr Phe Gln Asn
485 490 495

Asn His Thr Leu Leu Leu Leu Thr Ala Asn Gln Thr Leu Asn Gly Gln
500 505 510

Thr Ser Leu Val Asp Trp Lys Asp His Phe Leu Tyr Cys Ala Asn Ala
515 520 525

Pro Leu Thr Tyr Lys Asp Gly Thr Ala Leu Ala Leu Ser Cys Ile Ala
530 535 540

Asp Tyr Gly Ala Pro Val Phe Pro Phe Leu Ala Val Gly Gly Tyr Gln
545 550 555 560

Gly Thr Asp Tyr Ser Glu Ala Glu Ala Leu Ile Ile Thr Phe Ser Ile
565 570 575

Asn Asn Tyr Pro Ala Asp Asp Pro Arg Met Ala His Ala Lys Leu Trp
580 585 590

Glu Glu Ala Phe Leu Lys Glu Met Gln Ser Phe Gln Arg Ser Thr Ala
595 600 605

Asp Lys Phe Gln Ile Ala Phe Ser Ala Glu Arg Ser Leu Glu Asp Glu
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Ile Asn Arg Thr Thr Ile Gln Asp Leu Pro Val Phe Ala Ile Ser Tyr
625 630 635 640

Leu Ile Val Phe Leu Tyr Ile Ser Leu Ala Leu Gly Ser Tyr Ser Arg
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Trp Ser Arg Val Ala Val Asp Ser Lys Ala Thr Leu Gly Leu Gly Gly
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Val Ala Val Val Leu Gly Ala Val Val Ala Ala Met Gly Phe Tyr Ser
 675 680 685

Tyr Leu Gly Val Pro Ser Ser Leu Val Ile Ile Gln Val Val Pro Phe
 690 695 700

Leu Val Leu Ala Val Gly Ala Asp Asn Ile Phe Ile Phe Val Leu Glu
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Tyr Gln Arg Leu Pro Arg Met Pro Gly Glu Gln Arg Glu Ala His Ile
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Gly Arg Thr Leu Gly Ser Val Ala Pro Ser Met Leu Leu Cys Ser Leu
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Ser Glu Ala Ile Cys Phe Phe Leu Gly Ala Leu Thr Ser Met Pro Ala
 755 760 765

Val Arg Thr Phe Ala Leu Thr Ser Gly Leu Ala Ile Ile Phe Asp Phe
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Leu Leu Gln Met Thr Ala Phe Val Ala Leu Leu Ser Leu Asp Ser Lys
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Arg Gln Glu Ala Ser Arg Pro Asp Val Val Cys Cys Phe Ser Ser Arg
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Asn Leu Pro Pro Pro Lys Gln Lys Glu Gly Leu Leu Leu Cys Phe Phe
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Arg Lys Ile Tyr Thr Pro Phe Leu Leu His Arg Phe Ile Arg Pro Val
 835 840 845

Val Leu Leu Leu Phe Leu Val Leu Phe Gly Ala Asn Leu Tyr Leu Met
 850 855 860

Cys Asn Ile Ser Val Gly Leu Asp Gln Asp Leu Ala Leu Pro Lys Asp
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Ser Tyr Leu Ile Asp Tyr Phe Leu Phe Leu Asn Arg Tyr Leu Glu Val
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Gly Pro Pro Val Tyr Phe Asp Thr Thr Ser Gly Tyr Asn Phe Ser Thr
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Glu Ala Gly Met Asn Ala Ile Cys Ser Ser Ala Gly Cys Glu Ser Phe

915	920	925
Ser Leu Thr Gln Lys Ile Gln Tyr Ala Ser Glu Phe Pro Asn Gln Ser 930	935	940
Tyr Val Ala Ile Ala Ala Ser Ser Trp Val Asp Asp Phe Ile Asp Trp 945	950	955
960		
Leu Thr Pro Ser Ser Ser Cys Cys Arg Ile Tyr Thr Arg Gly Pro His 965	970	975
Lys Asp Glu Phe Cys Pro Ser Thr Asp Thr Ser Phe Asn Cys Leu Lys 980	985	990
Asn Cys Met Asn Arg Thr Leu Gly Pro Val Arg Pro Thr Thr Glu Gln 995	1000	1005
Phe His Lys Tyr Leu Pro Trp Phe Leu Asn Asp Thr Pro Asn Ile 1010	1015	1020
Arg Cys Pro Lys Gly Gly Leu Ala Ala Tyr Arg Thr Ser Val Asn 1025	1030	1035
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His Lys Pro Leu Arg Asn Ser Gln Asp Phe Thr Glu Ala Leu Arg 1055	1060	1065
Ala Ser Arg Leu Leu Ala Ala Asn Ile Thr Ala Glu Leu Arg Lys 1070	1075	1080
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Ser Asn Val Phe Tyr Gln Gln Tyr Leu Thr Val Leu Pro Glu Gly 1100	1105	1110
Ile Phe Thr Leu Ala Leu Cys Phe Val Pro Thr Phe Val Val Cys 1115	1120	1125
Tyr Leu Leu Leu Gly Leu Asp Ile Arg Ser Gly Ile Leu Asn Leu 1130	1135	1140
Leu Ser Ile Ile Met Ile Leu Val Asp Thr Ile Gly Leu Met Ala 1145	1150	1155

Val Trp Gly Ile Ser Tyr Asn Ala Val Ser Leu Ile Asn Leu Val
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Thr Ala Val Gly Met Ser Val Glu Phe Val Ser His Ile Thr Arg
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Ser Phe Ala Val Ser Thr Lys Pro Thr Arg Leu Glu Arg Ala Lys
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Met Thr Asn Phe Pro Gly Ile Leu Ile Leu Gly Phe Ala Gln Ala
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Gln Leu Ile Gln Ile Phe Phe Phe Arg Leu Asn Leu Leu Ile Thr
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Leu Leu Gly Leu Leu His Gly Leu Val Phe Leu Pro Val Val Leu
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Ser Tyr Leu Gly Pro Asp Val Asn Gln Ala Leu Val Leu Glu Glu
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Lys Leu Ala Thr Glu Ala Ala Met Val Ser Glu Pro Ser Cys Pro
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Gln Tyr Pro Phe Pro Ala Asp Ala Asn Thr Ser Asp Tyr Val Asn
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Tyr Gln Arg Leu
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Ile Arg Cys

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Leu	Ala	Gln	Ser	Glu	Pro	Tyr	Thr	Thr	Ile	His	Gln	Pro	Gly	Tyr	Cys	
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Ala	Phe	Tyr	Asp	Glu	Cys	Gly	Lys	Asn	Pro	Glu	Leu	Ser	Gly	Ser	Leu	
	35					40				45						

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Ile	Thr	Gly	Asp	His	Leu	Ile	Leu	Leu	Gln	Lys	Ile	Cys	Pro	Arg	Leu	

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Tyr	Thr	Gly	Pro	Asn	Thr	Gln	Ala	Cys	Cys	Ser	Ala	Lys	Gln	Leu	Val																																																								
			85				90						95																																																										
tca	ctg	gaa	gcg	agt	ctg	tcg	atc	acc	aag	gcc	ctc	ctc	acc	cgc	tgc		395																																																						
Ser	Leu	Glu	Ala	Ser	Leu	Ser	Ile	Thr	Lys	Ala	Leu	Leu	Thr	Arg	Cys																																																								
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cca	gcc	tgc	tct	gac	aat	ttt	gtg	aac	ctg	cac	tgc	cac	aac	acg	tgc		443																																																						
Pro	Ala	Cys	Ser	Asp	Asn	Phe	Val	Asn	Leu	His	Cys	His	Asn	Thr	Cys																																																								
			115				120						125																																																										
agc	ccc	aat	cag	agc	ctc	ttc	atc	aat	gtg	acc	cgc	gtg	gcc	cag	cta		491																																																						
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/40113

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01K 67/00, 033

US CL : 800/8

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
east medline biosis caplus**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ERICKSON et al. Studies on neuronal death in the mouse model of Niemann-pick C disease. J. Neurosci. Res. 2002. Vol. 68, pg 738-744. See entire article.	1-49
X	ERICKSON et al. mdr1a deficiency corrects sterility in Niemann-Pick C1 protein deficient female mice. Mol. Reprod. Develop. 2002. Vol. 62, pg 167-173. See entire article.	1-49
A T	ALTMANN et al. Niemann-Pick C1 like 1 protein is critical for intestinal cholesterol absorption. Science. 20 February 2004. Vol. 303, pg 1201-1204. See entire article.	1-49
Y	ERICKSON et al Pharmacological and genetic modifications of somatic cholesterol do not substantially alter the course of CNS disease in Niemann-Pick C mice. J. Inher. Metab. Dis. 2000. Vol. 23, Pg 54-62. See entire article.	1-49
Y	DAVIES et al. Evidence for a Niemann-Pick C (NPC) gene family: Identification and characterization of NPC1L1. Genomics. 15 April 2000. Vol. 65, No. 2, pg 137-145. See entire article.	1-49



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T"

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X"

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y"

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"

document member of the same patent family

Date of the actual completion of the international search

19 March 2004 (19.03.2004)

Date of mailing of the international search report

07 JUN 2004

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