

Niemann-Pick C1 protein regulates cholesterol transport to the *trans*-Golgi network and plasma membrane caveolae

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Abstract The Niemann-Pick C1 (NPC1) protein regulates cholesterol transport from late endosomes-lysosomes to other intracellular compartments. In this article, cholesterol transport to caveolin-1 and caveolin-2 containing compartments, such as the *trans*-Golgi network (TGN) and plasma membrane caveolae, was examined in normal (NPC^{+/+}), NPC heterozygous (NPC^{+/-}), and NPC homozygous (NPC^{-/-}) human fibroblasts. The expression and distribution of NPC1 in each cell type were similar, and characterized by a finely dispersed, granular staining pattern. The expression of caveolin-1 and caveolin-2 was increased in NPC^{+/-} and NPC^{-/-} fibroblasts, although the distribution in each cell type was similar and characterized by predominant staining of the TGN and plasma membrane. The TGN in NPC^{+/+} fibroblasts was relatively cholesterol-enriched, whereas the TGN in NPC^{+/-} and NPC^{-/-} fibroblasts was partially or completely cholesterol-deficient, respectively. Consistent with studies demonstrating the transport of cholesterol from the TGN to plasma membrane caveolae, the concentration of cholesterol in plasma membrane caveolae isolated from NPC^{+/-} and NPC^{-/-} fibroblasts was significantly decreased, even though the total concentration of plasma membrane cholesterol in each cell type was similar. **FIG** These studies demonstrate that NPC1 regulates cholesterol transport to caveolin-1 and caveolin-2 containing compartments such as the TGN and plasma membrane caveolae.—Garver, W. S., K. Krishnan, J. R. Gallagos, M. Michikawa, G. A. Francis, and R. A. Heidenreich. **Niemann-Pick C1 protein regulates cholesterol transport to the *trans*-Golgi network and plasma membrane caveolae.** *J. Lipid Res.* 2002. 43: 579–589.

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Niemann-Pick type C (NPC) disease is a rare neurodegenerative disorder characterized by the accumulation of cholesterol within most tissues (1, 2). The accumulation of cholesterol in tissues of NPC mice is proportional to the endocytosis of LDL through the clathrin coated-pit pathway (3). At the cellular level, NPC cells accumulate cholesterol in late endosomes-lysosomes and the *trans*-cis-

ternae of the Golgi apparatus, delaying cholesterol transport to other cellular compartments responsible for maintaining cholesterol homeostasis (4–6). Recent results suggest that endogenously synthesized and plasma membrane-derived cholesterol may also contribute to the accumulation of cholesterol in late endosomes-lysosomes of NPC cells, most likely as a result of constitutive plasma membrane internalization through the clathrin coated-pit pathway (7–10).

The human gene responsible for the first complementation group of NPC has been identified and shown to encode the Niemann-Pick C1 (NPC1) protein (11). The NPC1 protein contains several structural motifs that are required to regulate the mobilization of late endosomal-lysosomal cholesterol. Among these are the following: a unique cysteine-rich NPC1 domain that contains a leucine zipper, a sterol-sensing domain homologous to domains in 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the sterol-regulatory element binding protein (SREBP) cleavage-activating protein (SCAP), and a carboxy-terminal dileucine motif that mediates endocytosis to late endosomes-lysosomes (12–15). When normal (NPC^{+/+}) fibroblasts are incubated in the presence of LDL, the NPC1 protein localizes to a distinct subset of vesicles containing late endosome-lysosome markers, and to a lesser extent, the *trans*-Golgi network (TGN) (16–19). This NPC1-containing compartment is believed to function as a sterol-modulated late endosomal sorting organelle that facilitates the transport of LDL-derived and plasma membrane-derived cholesterol (20, 21). Studies have also demonstrated that particular Rab proteins (Rab7 and Rab9) colocalize with NPC1 and are involved in facilitating the

Abbreviations: MAPK, mitogen-activated protein kinase; NPC1, Niemann-Pick C1 protein; NPC, Niemann-Pick type C; SREBP, sterol-regulatory element binding protein; SCAP, SREBP cleavage-activating protein; TGN, *trans*-Golgi network.

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fission-fusion of vesicles transported between late endosomes-lysosomes and the TGN (18, 20, 22). Recent studies suggest that NPC1 may also function as a sterol-modulated transmembrane efflux pump that uses a proton-motive force to remove lipids, specifically fatty acids, from late endosomes-lysosomes (23).

Previous studies have shown that the expression of caveolin-1 is increased in NPC heterozygous (NPC^{+/-}) and NPC homozygous (NPC^{-/-}) fibroblasts and tissues (24, 25). Consistent with the hypothesis that increased caveolin-1 expression may participate in a compensatory mechanism involved in the transport of late endosome-lysosome cholesterol, a recent study has demonstrated that a caveolin dominant negative mutant induces intracellular cholesterol imbalance by promoting cholesterol accumulation in late endosomes-lysosomes (26). The treatment of these cells with U18666A, an amphiphile that induces a phenotype similar to NPC, acts synergistically with the caveolin dominant negative mutant to increase the accumulation of cholesterol in late endosomes-lysosomes (26). Both caveolin-1 and caveolin-2 have been shown to be integral membrane proteins of the TGN and TGN-derived vesicles (27–29). Caveolins also serve as coat-proteins for specialized flask-shaped domains of the plasma membrane called caveolae, previously proposed to regulate intracellular cholesterol homeostasis and transmembrane signaling events (30–33). Studies have characterized the caveolins as cholesterol-binding proteins involved in transporting endogenously synthesized cholesterol and LDL-derived cholesterol to plasma membrane caveolae (33–36). In the present report, NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} human fibroblasts were used to examine the role of NPC1 in regulating cholesterol transport to caveolin-1 and caveolin-2 containing compartments such as the TGN and plasma membrane caveolae.

MATERIALS AND METHODS

Materials

DMEM, PBS, FBS, trypsin-EDTA, and penicillin-streptomycin were purchased from Mediatech (Herndon, VA). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Germany). Antibodies generated against the carboxy-terminus of human NPC1 were generously provided by Peter G. Pentchev (National Institutes of Health, Bethesda, MD) and Shutish Patel (Veterans Administration Connecticut Healthcare System, Newington, CT). Mouse anti-caveolin-1 (clone 2297), rabbit anti-caveolin-1, and mouse anti-caveolin-2 (clone 65) were purchased from BD Biosciences (San Diego, CA). Rabbit anti-N-cadherin was generously provided by Ron L. Heimark (The University of Arizona, Tucson, AZ). Mouse anti-protein disulfide isomerase (PDI) was purchased from Affinity BioReagents (Golden, CO). Peroxidase-conjugated goat secondary antibodies were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Cy2- and Cy3-conjugated goat secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Supersignal substrate for Western blotting, Immopure IgG elution buffer, and bicinchoninic acid (BCA) protein assay kit were purchased from Pierce-Endogen (Rockford, IL). Nalco 2329 colloidal silica was purchased from Nalco Chem-

ical Company (Chicago, IL). Polyacrylic acid (250,000 Da) was purchased from Polysciences (Warrington, PA). Aluminum chlorohydroxide was purchased from Reheis Incorporated (Berkeley Heights, NJ). Nycodenz, filipin, morpholinoethanesulfonic acid (Mes), and mouse anti-Golgi 58 kDa protein were purchased from Sigma Chemical Company (St. Louis, MO).

Cell culture

NPC^{+/+} fibroblasts (CRL-2076 and CRL-2097) were purchased from American Type Culture Collection (Manassas, VA). Human NPC^{+/-} fibroblasts (H-2325 and H-2327) were generously provided by Wenda L. Greer and David M. Byers (Dalhousie University, Halifax, Nova Scotia, Canada). Human NPC^{-/-} fibroblasts (GM-03123A and GM-11094) were purchased from the Human Genetic Mutant Cell Repository (Camden, NJ). In most experiments conducted in this report, both cell lines from NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were used. The reported results are representative of the particular cell type. For all experiments, fibroblasts were grown to near confluence in DMEM containing 10% FBS and 1% penicillin-streptomycin.

Preparation of cationic colloidal silica

Cationic colloidal silica was prepared as previously described (37, 38). This was accomplished by adding 35 gm of the aluminum chlorohydroxide complex (50%, w/w) and 450 gm of Nalco 2329 colloidal silica into 300 ml distilled water and blending for 2 min at high speed. The resulting suspension was incubated in a water bath at 80°C for 30 min and then overnight at room temperature. The mixture was adjusted to pH 5.0 using 1 N NaOH until stable for at least 24 h.

Isolation of plasma membrane and caveolae

The plasma membrane and caveolae were isolated from human fibroblasts using cationic colloidal silica as previously described (37, 38). Fibroblasts were rinsed once with PBS, and then twice with MBS-1 (20 mM Mes, pH 5.5, 135 mM NaCl). The cells were incubated in MBS-1 containing 1.0% cationic colloidal silica for 10 min, and then rinsed once with MBS-1. The cells were incubated in MBS-2 (20 mM Mes, pH 6.0, 135 mM NaCl) containing 1.0 mg/ml polyacrylic acid (250,000 Da) for 10 min, and then rinsed twice with MBS-2. The cells were scraped from the plate using MBS-2 containing a protease inhibitor cocktail and then pelleted using centrifugation (1,000 g, 10 min). The cell pellet was resuspended in MBS-3 (10 mM Mes pH 6.5) containing a protease inhibitor cocktail and aspirated using a 23-G needle. The samples were homogenized using a type C Teflon-glass homogenizer and diluted with an equal volume of MBS-4 (10 mM Mes pH 6.5, 270 mM NaCl) containing 100% (w/v) Nycodenz. The resulting 50% Nycodenz mixture was layered on a 70–55% linear Nycodenz gradient prepared in MBS-5 (10 mM Mes pH 6.5, 135 mM NaCl) and centrifuged (60,000 g for 30 min) using a Beckman SW-40 Ti rotor. After centrifugation, the purified cationic colloidal silica-coated plasma membrane was recovered and rinsed twice with MBS-5. The plasma membrane was extracted using MBS-6 (20 mM Mes pH 6.0, 135 mM NaCl) containing 1.0% Triton X-100 and homogenized using a type AA Teflon-glass homogenizer. An equal volume of 40 mM KCl containing 80% sucrose was added to the resulting homogenate and overlaid with a discontinuous sucrose step gradient (1 ml each of 35%, 30%, 25%, 20%, 15%, 10%, 5%, and 0% sucrose dissolved in 20 mM KCl). The sample was centrifuged (85,000 g for 16 h) using a Beckman SW-40 Ti rotor. The resulting opalescent band present at the 10–15% sucrose interface representing purified caveolae was collected and concentrated using centricon-10 concentrators.

Determination of cholesterol concentration

To determine cholesterol concentration, samples were extracted with hexane-isopropanol (3:2, v/v) and the lipids were separated using conventional thin-layer chromatography with hexane-diethyl ether-glacial acetic acid (80:20:1, v/v/v). Cholesterol was identified by comigration using a cholesterol standard after staining plates with iodine vapors. Spots representing cholesterol were scraped from the plate and extracted with hexane-water (3:1, v/v). After evaporation of the hexane phase, the mass of cholesterol was determined using the cholesterol oxidase method (39).

Determination of caveolae cholesterol

The amount of caveolae cholesterol was determined by labeling fibroblasts with [^3H]cholesterol and incubating with cholesterol oxidase (33). Cholesterol oxidase specifically oxidizes plasma membrane caveolae cholesterol to cholestenone, where both separated using thin-layer chromatography. To perform this experiment, fibroblasts were seeded into 6-well plates and grown to approximately 50% confluence in DMEM-10% FBS. Cells were refed with media containing [^3H]cholesterol (5.0 $\mu\text{Ci}/\text{ml}$) and grown to near confluence (approximately 3 days). Cells were rinsed three times with PBS (37°C) and allowed to equilibrate for 24 h in media. Finally, the cells were incubated in either media alone, or media containing cholesterol oxidase (0.5 U/ml) for 1 h at 37°C. Cells were rinsed three times with PBS and the lipids were extracted to separate [^3H]cholesterol and [^3H]cholestenone using thin-layer chromatography. The amount of [^3H]cholesterol and [^3H]cholestenone was determined using liquid scintillation counting.

Immunoblot analysis

Protein samples were separated using 6% or 12% SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane. Immunoblot buffer (10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 5% non-fat dry milk) was used to block nonspecific sites for 2 h. Immunoblots were incubated overnight at 4°C with respective primary antibodies and then rinsed with PBS (3 \times 10 min). The appropriate peroxidase-conjugated goat secondary antibodies were incubated at room temperature for 1 h and then rinsed with PBS (3 \times 10 min). Enhanced chemiluminescence (ECL) was performed and images were obtained using film.

Immunofluorescence labeling

Cells were fixed for 30 min using PBS containing 3% paraformaldehyde. Cells were rinsed with PBS (3 \times 5 min) and quenched with PBS containing 50 mM NH_4Cl (15 min). Cells were rinsed with PBS (3 \times 5 min) and then blocked for 1 h with either PBS containing 10% goat serum and 0.05% saponin, or PBS containing 10% goat serum and 50 $\mu\text{g}/\text{ml}$ filipin. Coverslips were incubated for 1 h with primary antibodies. After rinsing with PBS (3 \times 5 min), coverslips were incubated for 1 h with the appropriate Cy2- and Cy3-conjugated goat secondary antibodies. Finally, the coverslips were rinsed with PBS (3 \times 5 min) and mounted onto slides with Moviol.

Confocal microscopy

Fluorescent images were obtained using a BioRad MCR-1024 ES laser scanning confocal microscope equipped with a Nikon 60X, NA 1.4 oil immersion objective (W. M. Keck Foundation Bioimaging Core Facility, Steele Memorial Children's Research Center, Arizona Health Sciences Center). Simultaneous two-channel recording was performed using excitation wavelengths of 488 and 568 nm, with fluorescein/Cy2 and Cy3 emission filters (522 DF 35 and HQ 598 40). All images were derived from a single optical section estimated to be 1 μm thick. For merged im-

ages, the separate Cy2 and Cy3 images were adjusted to similar intensities and then merged using Adobe Photoshop 6.0.

Statistical analysis

Quantitative data is represented as the mean \pm SD of four plates. Significant differences ($P \leq 0.05$) between groups of data were determined using the two-tailed Student's *t*-test assuming equal variance.

RESULTS

Relative expression of NPC1, caveolin-1, and caveolin-2

The relative expression of NPC1, caveolin-1, and caveolin-2 was determined in NPC $^{+/+}$, NPC $^{+/-}$, and NPC $^{-/-}$ fibroblasts using immunoblot analysis (Fig. 1). The results indicated that in each cell type the relative expression of NPC1 was similar and represented by a band that migrated to approximately 180 kDa. In contrast, the relative expression of caveolin-1 and caveolin-2 in NPC $^{+/-}$ and NPC $^{-/-}$ fibroblasts was increased compared with that in NPC $^{+/+}$ fibroblasts. This result is consistent with a previous report demonstrating that the relative expression of caveolin-1 was significantly increased in several NPC $^{+/-}$ and NPC $^{-/-}$ fibroblast cell lines when compared with NPC $^{+/+}$ fibroblasts (24). Using β -actin as a control, equivalent amounts of homogenate protein were determined to be analyzed. These results demonstrate that although the relative expression of NPC1 is similar in each cell type, the relative expression of both caveolin-1 and caveolin-2 is increased in NPC $^{+/-}$ and NPC $^{-/-}$ fibroblasts.

Association of NPC1, caveolin-1, and caveolin-2 with the plasma membrane and plasma membrane caveolae

To investigate the association of NPC1, caveolin-1, and caveolin-2 with the plasma membrane and plasma mem-

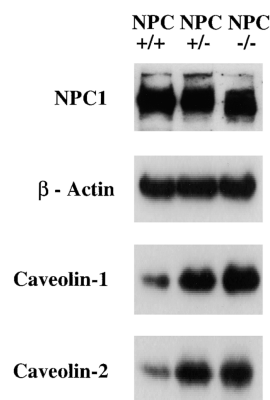


Fig. 1. Relative expression of Niemann-Pick C1 protein (NPC1), caveolin-1, and caveolin-2. Normal NPC $^{+/+}$, NPC heterozygous (NPC $^{+/-}$), and NPC homozygous (NPC $^{-/-}$) fibroblasts were plated and grown to near confluence in DMEM-10% FBS. Cell homogenates were prepared and an equivalent amount of protein (10 μg) from each cell type was used to conduct immunoblot analysis. The immunoblots were reprobbed for β -actin to assure that an equivalent amount of protein from each cell type was analyzed. Protein bands were visualized using enhanced chemiluminescence (ECL) and the resulting images were obtained using film.

brane caveolae, the plasma membrane and plasma membrane caveolae were isolated from NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts using cationic colloidal silica and examined using immunoblot analysis (Fig. 2). The sequential purification of the plasma membrane and plasma membrane caveolae in each cell type was monitored using organelle markers. These organelle markers included: β -actin, a cytoskeletal protein present in the cytoplasm and partially associated with the plasma membrane (40); N-cadherin, a plasma membrane cell-adhesion protein (41); the Golgi 58 kDa protein, a protein associated with the TGN (42); and protein disulfide isomerase (PDI), a protein in the endoplasmic reticulum and partially associated with the plasma membrane (43, 44). Caveolin-1 and caveolin-2 were used as markers for plasma membrane caveolae, although both proteins are also associated with the TGN and TGN-derived vesicles (28–30, 45).

The results indicated that β -actin and PDI were detected only in the homogenate and partially with the plasma membrane, but not plasma membrane caveolae. N-cadherin was enriched in the plasma membrane, but absent from plasma membrane caveolae. The Golgi 58 kDa protein was present in the homogenate, but absent

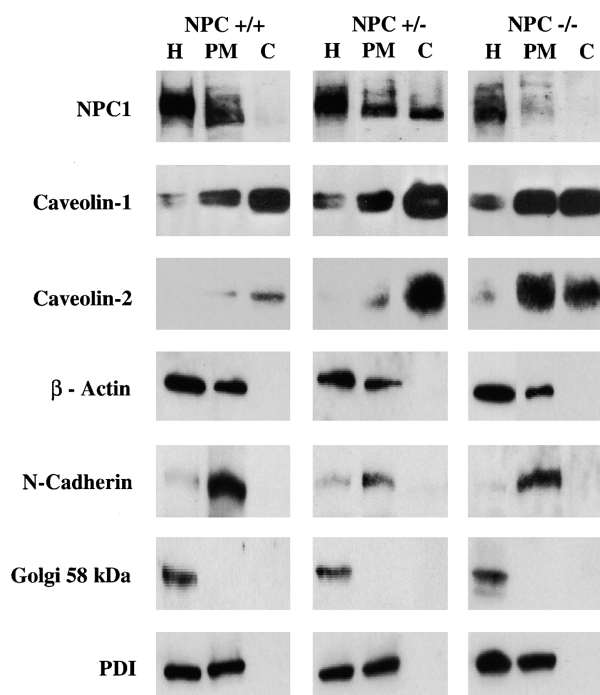


Fig. 2. Association of NPC1, caveolin-1, and caveolin-2 with the plasma membrane and plasma membrane caveolae. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated and grown to near confluence in DMEM-10% FBS. Cells were treated with cationic colloidal silica and polyacrylic acid to isolate the plasma membrane using Nycodenz density gradient centrifugation. Plasma membrane caveolae were isolated from the plasma membrane using detergent insoluble sucrose density gradient centrifugation. An equivalent amount of protein (10 μ g) from the homogenate (H), plasma membrane (PM), and plasma membrane caveolae (C) from each cell type was used to conduct immunoblot analysis. Protein bands were visualized using ECL and the resulting images were obtained using film.

from the plasma membrane and plasma membrane caveolae. This last result was especially important to demonstrate that plasma membrane caveolae had been successfully isolated using cationic colloidal silica, because it assured that other caveolin-1 and caveolin-2 containing detergent-insoluble membranes commonly associated with the TGN were not contaminating plasma membrane caveolae. With respect to plasma membrane caveolae, the only markers found enriched in this organelle were caveolin-1 and caveolin-2. Consistent with previous reports using cationic colloidal silica to isolate the plasma membrane and plasma membrane caveolae, there was an increasing enrichment of caveolin-1 and caveolin-2 in these compartments (40, 46). However, in contrast with NPC^{+/+} and NPC^{+/-} fibroblasts, in NPC^{-/-} fibroblasts there was a relatively larger amount of caveolin-1 and caveolin-2 associated with the plasma membrane, and not necessarily plasma membrane caveolae. With respect to the distribution of NPC1, results indicated that a fraction of NPC1 was associated with the plasma membrane in each cell type. Interestingly, in NPC^{+/-} fibroblasts, some of the plasma membrane-associated NPC1 resided with plasma membrane caveolae. The association of NPC1 with the plasma membrane in each cell type was confirmed by conducting biotinylation experiments using a membrane-impermeable biotinylating reagent and selective precipitation with streptavidin-conjugated agarose beads (results not shown).

The amount of protein recovered after isolation of the plasma membrane and plasma membrane caveolae in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts was also determined (Table 1). The results indicated that in NPC^{+/+} fibroblasts 2.18% of the homogenate protein was recovered in the plasma membrane, whereas in NPC^{+/-} and NPC^{-/-} fibroblasts, the amount of homogenate protein recovered in the plasma membrane was 3.79% and 3.43%, respectively. As a result of isolating plasma membrane caveolae, the amount of plasma membrane caveolae protein recovered from the plasma membrane of NPC^{+/+} fibroblasts was 4.33%, whereas the amount of plasma membrane ca-

TABLE 1. The amount of protein recovered after isolation of the plasma membrane and plasma membrane caveolae from NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts

Fibroblast Genotype	Plasma Membrane		Recovery %	Caveolae	
	Homogenate	Recovery		Recovery	Recovery
	mg	mg	%	mg	%
NPC ^{+/+}	41.20 \pm 7.20	0.90 \pm 0.06	2.18	0.039 \pm 0.007	4.33
NPC ^{+/-}	45.60 \pm 6.40	1.73 \pm 0.20	3.79	0.042 \pm 0.012	2.43
NPC ^{-/-}	74.80 \pm 4.00	2.57 \pm 0.20	3.43	0.039 \pm 0.002	1.52

NPC^{+/+} and NPC^{+/-} fibroblasts were plated into 15 150 mm plates and grown to near confluence in DMEM-10% FBS. NPC^{-/-} fibroblasts were plated into twice as many plates (30 150 mm plates) and grown to near confluence in the same media. The plasma membrane from each cell type was isolated using cationic colloidal silica and Nycodenz density gradient centrifugation. The plasma membrane caveolae from each cell type was isolated from the plasma membrane using detergent insoluble sucrose density gradient centrifugation. The protein obtained from each sample was determined in triplicate using the BCA protein method and the results are reported as the mean \pm SD.

veolae protein recovered from the plasma membrane of NPC^{+/-} and NPC^{-/-} fibroblasts was 2.43% and 1.52%, respectively. Because the isolation of plasma membrane caveolae using cationic colloidal silica is dependent on the sheering of these invaginated organelles from the plasma membrane, the decreasing amounts of plasma membrane caveolae protein recovered from NPC^{+/-} and NPC^{-/-} fibroblasts suggests that fewer of these organelles may have initially existed on the cell surface.

Cholesterol concentration of the plasma membrane and plasma membrane caveolae

The amount of cellular, plasma membrane, and plasma membrane caveolae cholesterol was determined in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts (Fig. 3). As predicted, the concentration of cellular cholesterol in NPC^{+/-} and NPC^{-/-} fibroblasts was significantly increased compared with that of NPC^{+/+} fibroblasts, as previously described (47). Determination of the plasma membrane cholesterol concentration indicated there was no significant difference among the different cell types. This result is consis-

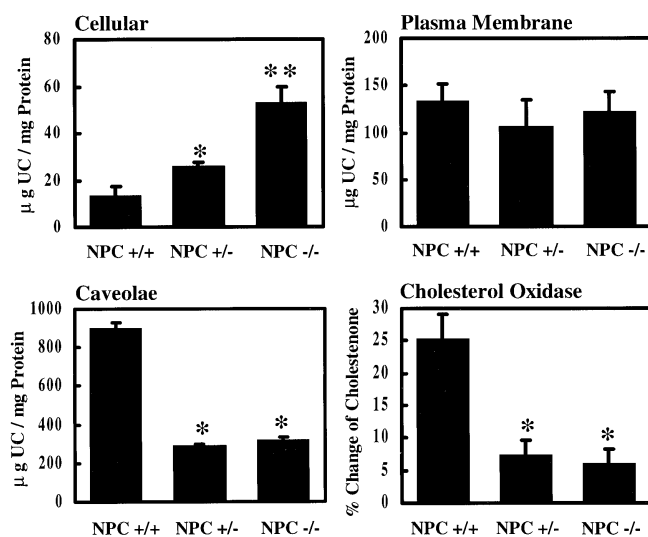


Fig. 3. Cholesterol concentration of the plasma membrane and plasma membrane caveolae. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated and grown to near confluence in DMEM-10% FBS. Cells were treated with cationic colloidal silica and polyacrylic acid to isolate the plasma membrane using Nycodenz density gradient centrifugation. Plasma membrane caveolae were isolated from the plasma membrane using detergent insoluble sucrose density gradient centrifugation. To determine the concentration of cholesterol associated with the homogenate, plasma membrane, and plasma membrane caveolae, an equivalent amount of protein (10 μg) from each cell type was analyzed. To determine the amount of cholesterol accessible to cholesterol oxidase, fibroblasts were plated and grown to near confluence in DMEM-10% FBS and labeled with [³H]cholesterol for 24 h. After rinsing with PBS, the cells were incubated with cholesterol oxidase for 1 h at 37°C to convert caveolae [³H]cholesterol to [³H]cholestenone. For each experiment, the values represent the mean ± SD cholesterol concentration (μg UC/mg protein) or the mean ± SD percent change of cholestenone (% change) from three plates. An asterisk represents a significant difference ($P \leq 0.05$) compared with NPC^{+/+}. Two asterisks represents a significant difference ($P \leq 0.05$) between NPC^{+/-} and NPC^{+/+}.

istent with a previous report indicating that the concentration of plasma membrane cholesterol is similar between NPC^{+/+} and NPC^{-/-} fibroblasts (48). However, the concentration of cholesterol measured in plasma membrane caveolae isolated from NPC^{+/-} and NPC^{-/-} fibroblasts was significantly decreased. The concentration of cholesterol measured in plasma membrane caveolae isolated from NPC^{+/-} and NPC^{-/-} fibroblasts was only 30–35% compared with the concentration of cholesterol measured in plasma membrane caveolae isolated from NPC^{+/+} fibroblasts. There was no significant difference in the concentration of cholesterol measured in plasma membrane caveolae between NPC^{+/-} and NPC^{-/-} fibroblasts. To verify these results, an independent method was conducted using cholesterol oxidase to specifically oxidize plasma membrane caveolae cholesterol. The results again indicated that the amount of cholesterol in plasma membrane caveolae from NPC^{+/-} and NPC^{-/-} fibroblasts was only 25–30% of that measured in plasma membrane caveolae from NPC^{+/+} fibroblasts. There was no significant difference in the amount of cholesterol measured in plasma membrane caveolae between NPC^{+/-} and NPC^{-/-} fibroblasts. With respect to the concentration of cholesterol associated with the plasma membrane and plasma membrane caveolae in NPC^{+/+} fibroblasts, it is important to emphasize that the values obtained using cationic colloidal silica was of similar magnitude and concentration as previously reported using independent techniques to isolate these cellular organelles (49, 50). Moreover, the relative concentration of cholesterol associated with plasma membrane caveolae that was measured in the different cell types were of similar magnitude using cholesterol oxidase, an independent method that specifically oxidizes plasma membrane caveolae cholesterol in unfixed cells (32).

Fluorescence microscopy of caveolin-1, caveolin-2, and the Golgi 58 kDa protein

The cellular distribution of caveolin-1, caveolin-2, and the Golgi 58 kDa protein was examined in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts using double-label fluorescence confocal microscopy (Fig. 4). The results indicated that in each cell type caveolin-1 and caveolin-2 (Fig. 4, left column) primarily colocalized with the Golgi 58 kDa protein (Fig. 4, middle column), as evident in the merged images (Fig. 4, right column). However, in each cell type there were additional caveolin-1 and caveolin-2 containing vesicles present in the cytoplasm that did not colocalize with the Golgi 58 kDa protein. Presumably, these additional caveolin-1 and caveolin-2 containing vesicles represented either TGN-derived vesicles or other cytoplasmic transport vesicles as previously described (28, 51).

Fluorescence microscopy of caveolin-1, caveolin-2, and PDI

The cellular distribution of caveolin-1, caveolin-2, and PDI were examined in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts using double-label fluorescence confocal mi-

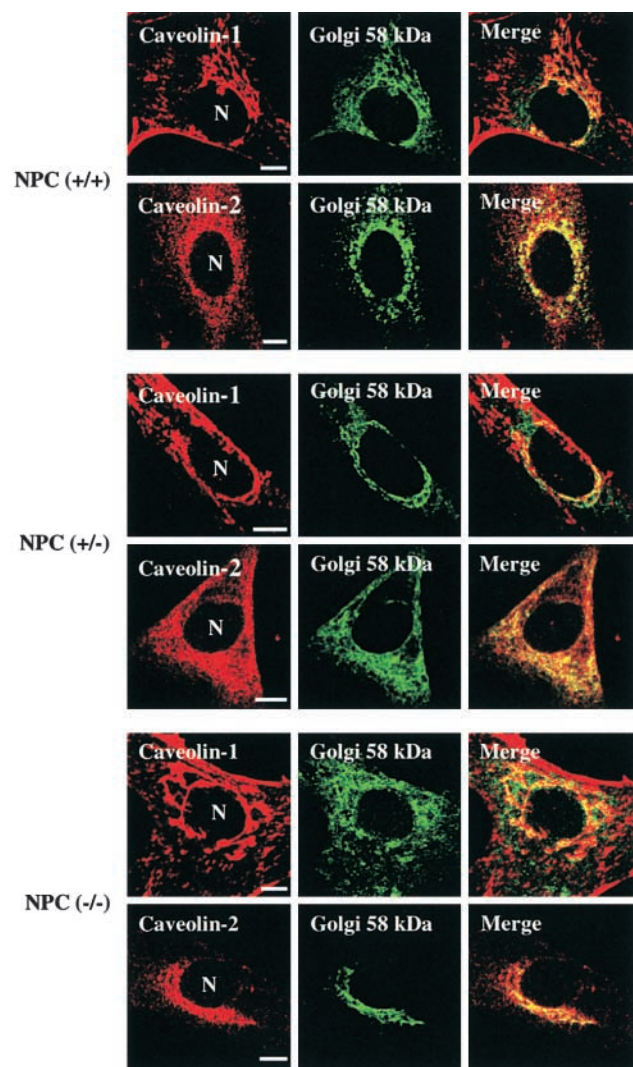


Fig. 4. Fluorescence microscopy of caveolin-1, caveolin-2, and the Golgi 58 kDa protein. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated onto coverslips and grown to near confluence in DMEM-10% FBS. The fibroblasts were stained for either caveolin-1 or caveolin-2 (left column), and the Golgi 58 kDa protein (middle column). Images were obtained for each cell type using double-label fluorescence confocal microscopy. For each cell type a merged image is provided (right column). The images were obtained using a single confocal section. N, nucleus. The bar represents 5 μ m.

scopy (Fig. 5). The results indicated that in each cell type caveolin-1 (Fig. 5, left column) did not colocalize with PDI (Fig. 5, middle column), as evident in the merged images (Fig. 5, right column). This result is consistent with two previous studies indicating that the amount of endogenous caveolin-1 associated with the endoplasmic reticulum is relatively small (26, 52). The finding that increased expression of caveolin-1 in NPC^{+/-} and NPC^{-/-} fibroblasts did not promote colocalization with the endoplasmic reticulum marker suggested that increased caveolin-1 expression may be due to NPC1 deficiency, and not to changes in the trafficking of caveolin-1 due to overexpression. The results also indicate that in each cell type caveolin-1 (Fig. 5, left column) and caveolin-2 (Fig. 5, middle column) colocalize at the TGN, as ev-

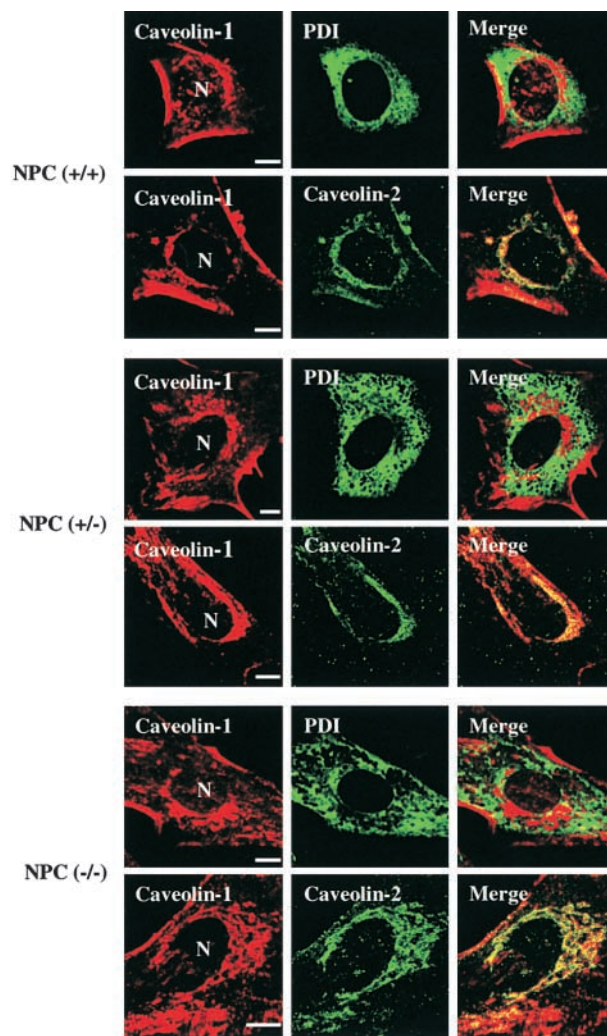


Fig. 5. Fluorescence microscopy of caveolin-1, caveolin-2, and PDI. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated onto coverslips and grown to near confluence in DMEM-10% FBS. The fibroblasts were stained for caveolin-1 (left column), and either caveolin-2 or the Golgi 58 kDa protein (middle column). Images were obtained for each cell type using double-label fluorescence confocal microscopy. For each cell type a merged image is provided (right column). The images were obtained using a single confocal section. N, nucleus. The bar represents 5 μ m.

ident in the merged images (Fig. 5, right column). These results are consistent with the colocalization of caveolin-1 and caveolin-2 at the TGN and TGN-derived vesicles as previously described (28, 45, 51).

Fluorescence microscopy of NPC1, caveolin-1, and caveolin-2

The cellular distribution of NPC1, caveolin-1, and caveolin-2 was examined in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts using double-label fluorescence confocal microscopy (Fig. 6). The results indicated that in each cell type the cellular distribution of NPC1 was similar and represented by a finely dispersed granular staining pattern within the perinuclear region (Fig. 6, left column). The distribution of caveolin-1 and caveolin-2 in each cell type was similar and primarily represented by staining of the

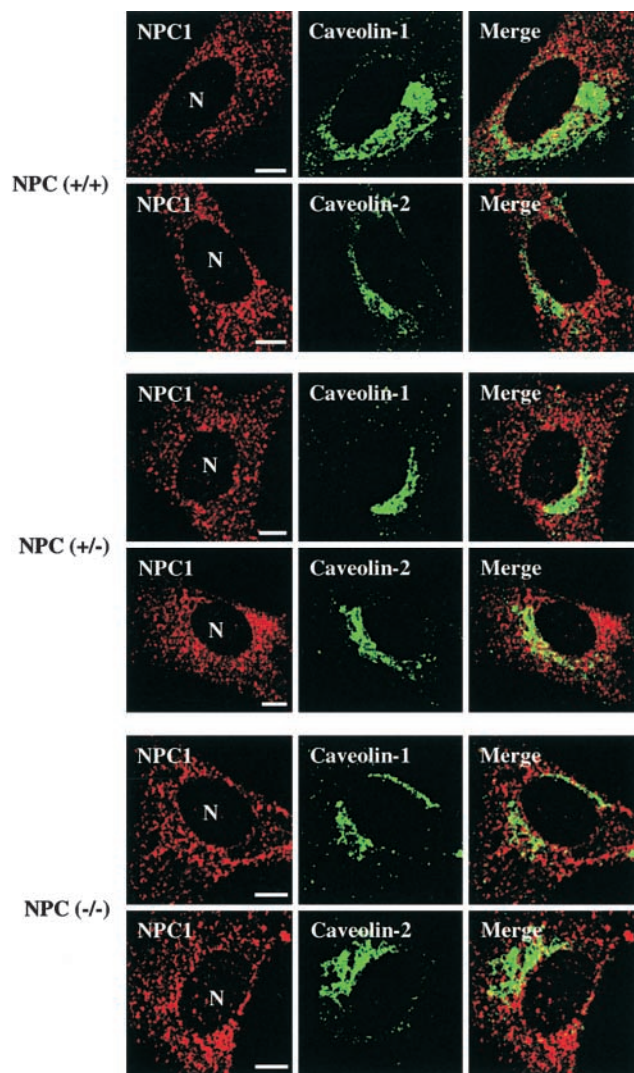


Fig. 6. Fluorescence microscopy of NPC1, caveolin-1, and caveolin-2. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated onto coverslips and grown to near confluence in DMEM-10% FBS. The fibroblasts were stained for NPC1 (left column), and either caveolin-1 or caveolin-2 (middle column). Images were obtained for each cell type using double-label fluorescence confocal microscopy. For each cell type a merged image is provided (right column). The images were obtained using a single confocal section. N, nucleus. The bar represents 5 μ m.

TGN and other caveolin-1 and caveolin-2 containing vesicles within the cytoplasm (Fig. 6, middle column). There was no extensive colocalization of NPC1 with either caveolin-1 and caveolin-2 at the TGN, or other caveolin-1 and caveolin-2 containing vesicles in the cytoplasm (Fig. 6, right column). This result is somewhat contradictory to previous studies describing partial colocalization of NPC1 with the TGN and caveolin-1 containing vesicles, but reconciled when considering that murine fibroblasts contain two distinct populations of NPC1 (18, 19). The larger of the two NPC1-containing vesicles in murine fibroblasts, which is apparently not detected in human fibroblasts, contains caveolin-1 and has an extensive network of internal membranes (19).

Fluorescence microscopy of caveolin-1, caveolin-2, and filipin

The cellular distribution of caveolin-1, caveolin-2, and filipin was examined in NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts using double-label fluorescence confocal microscopy (Fig. 7). The results indicated that in NPC^{+/+} fibroblasts the caveolin-1 and caveolin-2 containing TGN (Fig. 7, left column) colocalized with filipin (Fig. 7, middle column), as evident in the merged image (Fig. 7, right column). This suggested that in NPC^{+/+} fibroblasts the TGN was relatively cholesterol-enriched, as previously described (5). However, in NPC^{+/-} fibroblasts, the caveolin-1 and caveolin-2 containing TGN (Fig. 7, left column) only partially colocalized with filipin (Fig. 7, middle column), as evident in the merged image (Fig. 7, right column),

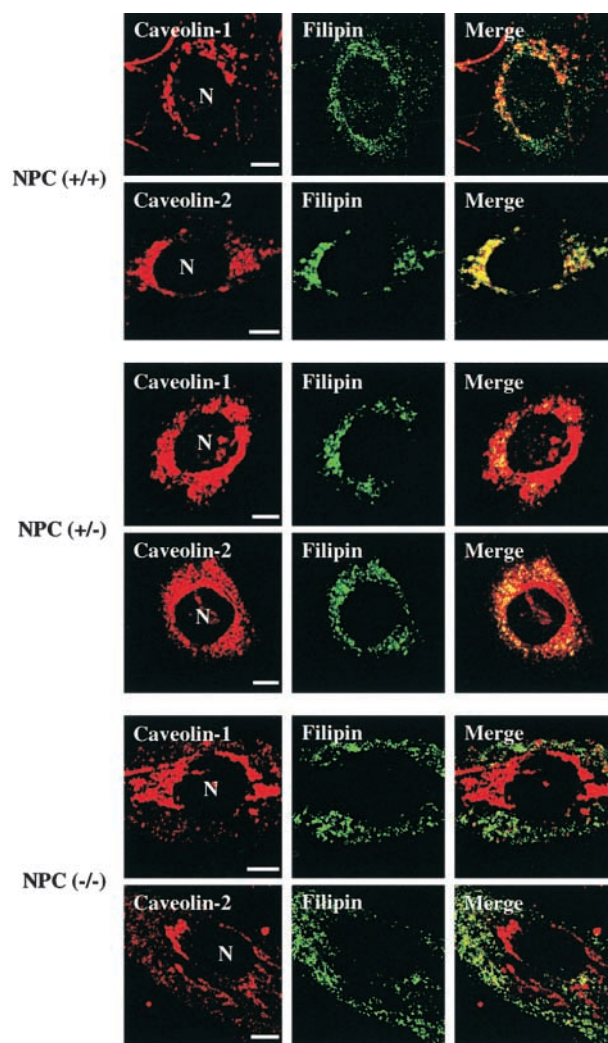


Fig. 7. Fluorescence microscopy of caveolin-1, caveolin-2, and filipin. NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} fibroblasts were plated onto coverslips and grown to near confluence in DMEM-10% FBS. The fibroblasts were stained for either caveolin-1 or caveolin-2 (left column), and cholesterol using filipin (middle column). Images were obtained for each cell type using double-label fluorescence confocal microscopy. For each cell type a merged image is provided (right column). The images were obtained using a single confocal section. N, nucleus. The bar represents 5 μ m.

suggesting that the TGN was partially cholesterol-deficient. Finally, in NPC^{-/-} fibroblasts, the caveolin-1 and caveolin-2 containing TGN (Fig. 7, left column) did not colocalize with filipin (Fig. 7, middle column), suggesting that the TGN was cholesterol-deficient and that a complete defect in the transport of cholesterol to the TGN was present in these cells.

DISCUSSION

The cholesterol transport pathway regulated by NPC1 is a subject of intense investigation. Previous studies suggest that NPC1 regulates cholesterol transport from late endosomes-lysosomes to the plasma membrane (4, 53, 54). The majority of this cholesterol is believed to flow through elements of the Golgi apparatus enroute to the plasma membrane, while the remaining cholesterol is transported to the endoplasmic reticulum in a pathway that is independent of the plasma membrane (55, 56). In contrast, other studies suggest that NPC1 regulates cholesterol transport only after reaching the plasma membrane (7–9). To further characterize the cholesterol transport pathway that is regulated by NPC1, NPC^{+/+}, NPC^{+/-}, and NPC^{-/-} human fibroblasts were used to examine cholesterol transport to caveolin-1 and caveolin-2 containing compartments such as the TGN and plasma membrane caveolae. First, the results indicated an increased expression of caveolin-1 and caveolin-2 in NPC^{+/-} and NPC^{-/-} fibroblasts, suggesting involvement of caveolin-1 and caveolin-2 containing compartments in the cholesterol transport defect. Second, compared with the accumulation of cellular cholesterol in NPC^{+/-} and NPC^{-/-} fibroblasts, there was an inverse amount of cholesterol associated with the caveolin-1 and caveolin-2 containing TGN. Third, the concentration of cholesterol associated with plasma membrane caveolae and accessible to cholesterol oxidase was significantly decreased in NPC^{+/-} and NPC^{-/-} fibroblasts.

Previous studies have described the increased expression of caveolin-1 mRNA and caveolin-1 protein in NPC^{+/-} and NPC^{-/-} fibroblasts (24). This same result has also been described in vivo using livers from NPC^{+/-} and NPC^{-/-} mice (25, 57). Consistent with the finding that NPC^{+/-} and NPC^{-/-} fibroblasts accumulate cholesterol, studies have shown that when cells are incubated in the presence of LDL, the amount of caveolin-1 mRNA and caveolin-1 protein increases (58, 59). The molecular mechanism responsible for increased expression of caveolin-1 mRNA and caveolin-1 protein has been shown to involve the SREBP-1 (60). The processing of SREBP-1 is mediated by SCAP, which is activated when the amount of cellular cholesterol decreases (61, 62). This being true, it may be assumed that a defect in the function of NPC1 would prevent cholesterol transport and thereby induce SCAP-mediated processing of SREBP-1 to cause decreased expression of caveolin-1. However, since the expression of caveolin-1 and caveolin-2 is increased in NPC^{+/-} and NPC^{-/-} fibroblasts, there must be an additional mechanism that accounts for the increased expression of these proteins.

The Golgi apparatus is a complex organelle consisting of discrete stacks of cisternae (*cis*, *medial*, and *trans*) and a *trans*-Golgi network having a heterogeneous distribution of cholesterol (63, 64). A number of studies have suggested that the Golgi apparatus has an important role in regulating intracellular cholesterol homeostasis. For instance, the Golgi apparatus contributes to the transport of endogenously synthesized cholesterol enroute to the plasma membrane (65). Other studies indicate that the Golgi apparatus is involved in apolipoprotein-mediated cholesterol efflux (66, 67). With respect to the transport of LDL-derived cholesterol, it has been shown that NPC^{+/+} fibroblasts incubated with LDL accumulate cholesterol in the *cis-medial*-Golgi cisternae and TGN, whereas NPC^{-/-} fibroblasts accumulate cholesterol in the *trans*-Golgi cisternae with the TGN remaining relatively cholesterol-deficient (5, 6). Finally, recent studies indicate that LDL-derived cholesterol is transported from clathrin-coated vesicles into intermediate density vesicles that contain markers for the TGN before reaching plasma membrane caveolae (35).

Consistent with other studies demonstrating that the TGN in NPC^{+/+} fibroblasts is relatively cholesterol-enriched, fluorescence microscopy of caveolin-1 and caveolin-2 with filipin indicated that the TGN colocalized with filipin (5, 63, 64). In contrast, the TGN in NPC^{+/-} fibroblasts only partially colocalized with filipin. This apparent disparity was further evident in NPC^{-/-} fibroblasts, which had no apparent colocalization of filipin with the TGN, suggesting that the TGN was cholesterol-deficient. It has recently been reported that cholesterol is required for the formation and regulated budding of TGN-derived vesicles, and that the formation of lipid rafts critically depends on the level of cholesterol residing within these membranes (68, 69). Indeed, it is well established that caveolin-1 and caveolin-2 serve as major components of the TGN that are responsible for facilitating protein and lipid transport to plasma membrane caveolae (28–30, 51). It is also within the TGN that sphingolipid and cholesterol-enriched rafts are generated for the transport of specific proteins and lipids to the cell surface (29, 70). The inability of cholesterol to gain access to the TGN, as evident in NPC^{+/-} and NPC^{-/-} fibroblasts, may therefore prevent the transport of cholesterol to plasma membrane caveolae, resulting in cholesterol-deficient caveolae.

A decrease in plasma membrane caveolae cholesterol may have adverse effects on cellular function since studies have demonstrated that plasma membrane caveolae participate in various signal transduction events (50, 71). With respect to NPC disease, results suggest that hyperactivation of mitogen-activated protein kinase (MAPK) in brains of NPC^{-/-} mice may contribute to neurodegeneration in NPC disease (72). The specific hyperactivation of MAPK has also been shown to occur in NPC^{-/-} fibroblasts (unpublished results). The inability to demonstrate MAPK hyperactivation in NPC^{+/-} fibroblasts, although plasma membrane caveolae cholesterol levels in these cells are also decreased is currently under investigation. It is possible, as suggested by recent studies, that additional

plasma membrane domains besides caveolae are cholesterol-deficient in NPC^{-/-} cells and therefore involved in the disease process (73).

In conclusion, the results from this report suggest that NPC1 regulates the transport of cholesterol to caveolin-1 and caveolin-2 compartments such as the TGN and plasma membrane caveolae. The results obtained from this study are consistent with the hypothesis that cholesterol obtained from late endosomes-lysosomes is transported to the TGN, either before or after reaching the plasma membrane, and that the TGN facilitates cholesterol transport to plasma membrane caveolae (35, 69). Although speculative, the increased expression of caveolin-1 and caveolin-2 in both NPC^{+/-} and NPC^{-/-} fibroblasts may represent a compensatory mechanism that is engaged to facilitate cholesterol transport to cholesterol-deficient caveolin-1 and caveolin-2 compartments due to a defect in NPC1. **FIG**

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