Mutation in EGFP Domain of LDL Receptor-Related Protein 6 Impairs Cellular LDL Clearance

Wenzhong Liu, Sheida Mani, Nicole R. Davis, Nizal Sarrafzadegan, Paula B. Kavathas, Arya Mani

Abstract—Mutation in the EGFP domain of LDL receptor-related protein 6 (LRP6R611C) is associated with hypercholesterolemia and early-onset atherosclerosis, but the mechanism by which it causes disease is not known. Cholesterol uptake was examined in cells from LRP6+/− mice and LRP6R611C mutation carriers. Splenic B cells of LRP6+/− mice have significantly lower LRP6 expression and low-density lipoprotein (LDL) uptake than those of the wild-type littermates. Although similar levels of total LRP6 were found in lymphoblastoid cells (LCLs) of LRP6R611C mutation carriers and those of the unaffected family member, LDL uptake was significantly lower in the mutant cells. Mutant and wild-type receptors show similar affinities for apolipoprotein B at neutral pH. LRP6 colocalized with LDL and was communoprecipitated with NPC1 (Niemann–Pick disease type C1), an endocytic regulator of LDL trafficking. However, the cellular localization of LRP6 in the mutant cells shifted from cell surface to late endosomes/lysosomes. Plasma membrane expression levels of LRP6R611C were lower compared to wild-type receptor and declined to a greater extent in LDL-rich medium. Further examinations revealed lower efficacy of apolipoprotein B dissociation from LRP6R611C compared to wild-type receptor at an acidic pH. These studies identify LRP6 as a receptor for LDL endocytosis and imply that R611C mutation results in reduced LRP6 membrane expression and decreased LDL clearance. Based on our findings, we conclude that the increased affinity of the mutant receptor for LDL in acidic pH leads to their impaired dissociation in late endosomes, which compromises their recycling to the plasma membrane.

Key Words: atherosclerosis ■ cholesterol ■ hypercholesterolemia ■ receptors ■ genetics

The LDL receptor–related protein family includes LDL receptor (LDLR) and LDLR-related protein (LRP)1, 2 proteins that have been extensively studied for their role in cellular cholesterol uptake.1–3 LRP1 mediates endocytosis of apolipoprotein (apo)E-enriched very-low-density lipoprotein (VLDL)4–5 and binds and internalizes aggregated LDL in human vascular smooth muscle cells.1 With the exception of LDLR, no other member of this receptor family has been implicated in human disease. LRP6 is a distinct member of LRP family6–10 which is primarily known for its key role in mediating the transduction of signals from secreted Wnt proteins to β-catenin.11 LRP6 has been extensively studied for its function in regulating embryogenesis and cell proliferation.12–14 This protein has been localized to lipid rafts15 and functions as a coreceptor for endocytosis.16 It contains 3 LDLR-like binding domains,17 a cytoplasmic YXXL motif, and undergoes coated-pit-mediated internalization.18 Although the role of LRP6 in regulating cellular cholesterol uptake is unknown, we recently reported in a large kindred that LRP6 mutation results in early coronary artery disease19 and hypercholesterolemia. To investigate the function of LRP6 in LDL clearance, the present study used in vitro expression assays and examined LDL uptake of splenic B cells of LRP6+/− mice. To investigate the mechanisms of hypercholesterolemia in LRP6R611C mutation carriers, we examined LDL clearance of lymphoblastoid cells (LCLs) from LRP6 mutation carriers and their unaffected family members. Our studies suggest that LRP6R611C mutation alters its affinity for LDL and its subcellular localization and reduces cellular LDL clearance.

Materials and Methods

Proteins and Antibodies
Anti LRP6 antibodies were purchased from Novus (goat) and ZYMED Laboratories (rabbit), and their specificity was confirmed in vitro. Anti-apoB antibody was kindly provided by Dr Khosrow Adeli (Hospital for Sick Children, Toronto, Canada). All other antibodies were purchased and were as follows: anti-human SREBP1 (Fremont, Calif), mouse anti-human monoclonal anti-LDLR (C7; Santa Cruz Biotechnology), rabbit anti–Niemann–Pick disease type C (NPC)2 and NPC1 (Neomarkers and Novus Biologicals, respectively), rabbit polyclonal anti-EEA1 (ABR-Affinity BioReagents), LAMP2b antibody (Abcam), anti-apoE antibody (BD Transduction Laboratories), anti-PCSK9 (goat and rabbit polyclonal; Abcam), Cy2-labeled anti-mouse (GE Healthcare), anti-rabbit (Sigma), anti-goat (Dianova, Hamburg, Germany), and Cy3-labeled anti-rabbit (Chemicon). Hu-
man transferrin (Alexa Fluor 488–conjugated) was purchased from Invitrogen. Dil-LDL and purified human VLDL were purchased from Biomedical Technologies Inc (Boston, Mass). ApoE and apoE isoforms were purchased from Bioenza.

### Preparation of Splenic B Lymphocytes From LRP6+/−/− Mice
LRP6+/−/− knockout mice were generated from LST067 embryonic stem (ES) cells obtained from Bay Genomics. Spleens were obtained from 4 heterozygote and equal number littermate wild-type (WT) mice, and B cells were isolated according to the Alliance for Cell Signaling protocol (http://www.afcs.org).

### Preparation of Transfected Cells and Immortalized Human Lymphoblastoid Cell Line
NIH3T3 cells were transfected with plasmids encoding WT, mutant hemagglutinin-tagged LRP6, or empty vectors and plasmids containing green fluorescent protein to assess transfection rate as described earlier.19 Peripheral blood nucleated cells of 4 LRP6 mutation carriers and 4 unaffected family members were transformed with Epstein–Barr virus and cultured in RPMI 1640 medium supplemented with 10% FBS, 100 μM penicillin, 100 μg/mL streptomycin, and 2 mM/L glutamine and were maintained in an atmosphere of 5% CO₂ in a 37°C humidified incubator. Cell viability was assayed by trypan blue dye. Cell concentrations were kept close to 1×10⁶ cells/mL.

### Analysis of Dil-LDL Uptake and LRP6 Cell Surface Expression
All 3 cell types were placed in a 6-well plate containing 5% lipoprotein-deficient serum (LPDS) plus 10 μg/dL cholesterol and 1 μg/mL 25-hydroxycholesterol to downregulate the endogenous LDL. After 24 hours, cells were transferred to separate wells containing 100 μL of the same medium plus 10 μg/mL Dil-LDL and were incubated at 37°C for 1, 2, 3, 4, and 4 hours. Cells were washed 3 times with PBS containing 1% FBS and were fixed in 4% paraformaldehyde. Single-cell fluorescence intensities were measured by the FACScan instrument (Becton Dickinson Immunocytometry Systems).

Analysis of the cell surface expression of LRP6/LRP6_R611C of the LCLs in lipoprotein-free medium and those incubated for 1, 2, 3, 4, 12, and 24 hours in high-cholesterol medium (NCLP/PS plus 10 μg/dL cholesterol and 1 μg/mL 2-hydroxysterol) were carried out with flow cytometry using a rabbit anti-LRP6 antibody (Zymed) that recognizes the extracellular N-terminal region of mouse and human LRP6: its specificity has been examined by our group in vitro expression assays using hemagglutinin-tagged LRP6 constructs and by others.20 Nonpermeabilized cells were incubated with the primary antibody and fluorescein isothiocyanate–conjugated anti-rabbit IgG antibody (Sigma) and were fixed in 1% paraformaldehyde (pH 7.5) at 4°C and analyzed by flow cytometry. The experiments were carried out also at acidic pH to assess the affinity of the antibody for LRP6 for solid phase binding assays. To exclude toxic effect of LDL on LRP6 membrane expression, cells in high-cholesterol medium were washed and incubated in LPDS for 1 hour, followed by fluorescence-activated cell-sorting (FACS) analysis of LDLR membrane expression. A similar method was used for analysis of cell surface expression of LDLR using monoclonal (C7) mouse anti-human anti-LDL antibody (Santa Cruz). To exclude artifacts from Epstein–Barr virus immortalization, these experiments were repeated in lymphocytes of the mutation carriers and the unaffected family members.

### Solid-Phase Binding Assay
To immobilize LRP6, microtiter plates were coated with polyclonal rabbit anti-human LRP6 antibody (1:2000) at 4°C overnight. BSA was used as control. All wells were blocked with 3% BSA in PBS at room temperature for 2 hours. Equal amount of cell lysates from WT and LRP6_R611C LCLs were transferred to separate wells and were incubated overnight. Cells were washed 3 times with PBS. Sera containing different apoE isoforms (50 μg/mL), VLDL (50 μg/mL), and LDL extracted from sera of patients with LRP6 mutation were separately added to different wells at increasing concentrations. LDL was prepared by differential and density gradient ultracentrifugation as described.21 For studies at acidic pH, experiments were carried out in 25 mmol/L sodium acetate, pH 5.3, 150 mmol/L NaCl, and 0.1 mmol/L CaCl₂. Signals were detected using primary antibodies against apoE or apoB (1:4000, incubated for 1.2 hours at room temperature), followed by incubation with horseradish peroxidase–conjugated secondary antibody (1:4000) at room temperature for 1 hour and developed with 3,3,5,5-tetramethylbenzidine. Reactions were terminated by addition of 1 mmol/L H₂SO₄, and absorbance was measured at 450 nm using a microplate reader. Equal amounts of proteins were used for samples and controls. Data were analyzed by nonlinear regression analysis using SigmaPlot 9.0 software. Experiments were performed in quadruplicate using LCLs of 4 mutation carriers and 4 unaffected family members.

### Immunoprecipitation Studies
Immunoprecipitation studies (10×10⁶) were washed with PBS and harvested in 1 mL of lysis buffer containing 50 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, and 1% Nonidet P-40 with protease inhibitors. Cell lysates were subjected to high-speed centrifugation at 14 000 rpm for 20 minutes. The supernatants were then incubated with an antibody including rabbit anti-human LRP6 (ZYMED) or rabbit anti-human NPC1 antibodies (Novus) and 20 μL of G sepharose beads overnight at 4°C. The immune complexes were pelleted and washed 3 times with cold lysis buffer in the absence of protease and phosphatase inhibitors. The proteins were released from beads into 25 μL of SDS sample buffer by heating to 95°C for 5 minutes. For immunoblotting, equal protein concentrations were subjected to SDS-PAGE under reducing conditions. β-Actin was used as normalization control. The immunoreactive signals were detected by incubation with either rabbit anti-human LRP6 or rabbit anti-human NPC1 and NPC2 antibodies, depending on the antibody that was used for the precipitation and rabbit anti-PCSK9 antibody, followed by incubation with peroxidase-conjugated secondary antibodies. Labeled protein bands were detected using ECL detection reagents (PerkinElmer LAS Inc).

### Immunohistochemistry and Fluorescence Microscopy
Poly-L-lysine–coated cover glasses were placed in a 6-well plate, and 3 million lymphoblastoid cells in culture medium were added to each well and after overnight incubation were fixed by 4% paraformaldehyde. Adhered cells were permeabilized in 0.1% Triton X-100 for 20 minutes at room temperature. Cells were blocked with 3% BSA in PBS after appropriate washes and incubated with 1:100 diluted primary antibodies overnight at 4°C and 1:500 diluted fluorescence conjugated secondary antibodies at room temperature for 1 hour and were mounted with a drop of Vectashield. To localize LRP6 in the receptor-mediated endocytosis, cells were incubated with Alexa Fluor 488–conjugated human transferrin from Invitrogen (200 μg/mL) for 20 minutes at 37°C, were immediately washed, and were incubated for 4 hours in regular medium and fixed after 4 hours. Cells were fixed in 3.7% paraformaldehyde for 20 minutes. Specimens were then examined by a Zeiss LSM510 confocal microscope using excitation and emission filters at 488 nm and 522 nm, respectively, for Fluor 488 or 568 and 605 nm for Cy3. To differentiate LRP6 localization along the endocytic pathway, cells were labeled with an early endosomal marker anti-EEA1 and lysosomal marker anti-LAMP2b.

### Real-Time PCR Quantification of RNA
RNA was extracted using TRIzol (Invitrogen, San Diego, Calif), and cDNA was produced using SuperScript III Reverse transcription (Invitrogen) kits. The PCR primers of the target genes were designed using Primer3 program. Aliquots were mixed with IQ SYBR Green Supermix (Applied Biosystems). Temperature cycling and real-time
fluorescence measurements were performed using Eppendorf Mastercycler. β-Actin was used as the reference housekeeping gene. The relative quantification of gene expression was performed by using the comparative Ct (ΔΔCt) method.

Statistical Analysis
Statistical analyses of the data were performed by Student’s t test. Experiments were performed in quadruplicate using LCLs of 4 mutation carriers and 4 unaffected family members. Mean and SEM of experiments were calculated.

Results
LDL Uptake Is Reduced in the Mutant Cells
LDL uptake was examined in NIH3T3 cells overexpressing LRP6 and LRP6R611C receptors using Dil-LDL (low-density

![Figure 1](http://circres.ahajournals.org/)

Figure 1. Greater LDL uptake of NIH3T3 cells transfected with WT LRP6 compared to those of untransfected cells and cells transfected with LRP6R611C (a), reduced LDL uptake of splenic B lymphocytes of the LRP6+/− mice compared to those of the WT littermates (b), and increased LDL uptake of WT lymphocytes compared to LRP6R611C lymphocytes (c) despite equal membrane expression of the LDLR (d), total LRP6, LDLR, and SREBP (e and f). a, NIH3T3 cells were transfected with plasmids encoding hemagglutinin-tagged WT or mutant LRP6 or with empty plasmids. Cells were incubated with Dil-LDL at 37°C and subsequently fixed in 4% paraformaldehyde. Single-cell fluorescence intensities were measured using FACS. b, LDL uptake of splenic B lymphocytes of the LRP6+/− mice compared to those of the WT littermates was assessed using same technique. LRP6 R611C cells demonstrate lower LDL uptake (29±3.4%, P<0.005) compared to WT cells after 1 hour of incubation with Dil-LDL. Bottom, Expression levels of LRP6 in WT and LRP6+/− lymphocytes. c, LDL uptake of immortalized LCLs of the LRP6R611C mutation carriers (R611C) and their unaffected family members (WT) was explored using the same technique. LRP6R611C cells demonstrate lower LDL uptake (31±2.7%, P<0.005) compared to WT cells after 4 hours of incubation with Dil-LDL. d, Plasma membrane LDLR expression, assayed by FACS, is similar in LCLs of the LRP6R611C mutation carriers and their unaffected family members. e and f, mRNA (e) and protein (f) expression of LRP6, LDLR, and SREBP1 (sterol regulatory–binding protein 1) are roughly equal in the mutant and WT cells, whereas total LDLR mRNA expression in slightly higher in the mutant cells.
lipoprotein labeled with 1,1′-dioctadecyl-3,3′,3′,3′-tetramethylindolocarbocyanine perchlorate) analyzed by FACS analysis. Cells transfected with WT LRP6 but not those transfected with the mutant LRP6 showed higher LDL uptake compared to the cells transfected with empty vector (Δ=28.7±1.9%, P<0.005) after 4 hours incubation with Dil-LDL (Figure 1a). To further confirm that LDL uptake is mediated by LRP6, LDL uptake of the splenic B lymphocytes from LRP6<sup>−/−</sup> mice and their WT littermates was examined. The LRP6<sup>−/−</sup> lymphocytes demonstrated ≈40% less LRP6 expression and 30% reduced LDL uptake compared to the WT cells (Figure 1b and Figure I in the online data supplement, available at http://circres.ahajournals.org). Next, LDL uptake in LCLs from male nondiabetic heterozygote R611C mutation carriers were compared to those of age and sex-matched unaffected family members (n=4). LRP6<sub>R611C</sub> cells demonstrated significantly lower LDL uptake compared to WT cells when incubated more than 2 hours with Dil-LDL. The maximum difference was reached after 4 hours of incubation (Δ=31±2.7%, P<0.005) and remained relatively constant after this time point (Figure 1c). If LCLs were incubated for less than 1 hour with Dil-LDL, an opposite effect was observed (supplemental Figure II). LDLR cell surface expression is similar in both cell types in all conditions, suggesting that impaired LDL uptake of the mutant cells is largely LDLR-independent (Figure 1d). Total protein and mRNA expression levels of LDLRs were slightly increased in the mutant cells. No significant difference in mRNA or protein expression of the Sterol regulatory element-binding proteins (SREBP) was noted (Figure 1e and 1f).

Figure 2. LRP6 colocalizes with LDL and its cellular distribution is perturbed in the mutant cells. a, LDL colocalizes with LRP6 and is abnormally redistributed in the mutant cells. Top, LRP6 localization (Cy2) in the WT and mutant LCLs (R611C). Goat anti-LRP6 was used as the primary antibody. Middle, Dil-LDL uptake of the same cells. Bottom, Dil-LDL colocalizes with LRP6 in the WT and mutant cells. The colocalization is shifted away from cell surface seen in the WT cells to clusters within the cell in the mutant cells. The arrowhead shows clustering of LDL and arrows show colocalization of Dil-LDL with LRP6. b, LRP6 is localized evenly along the endocytotic pathway as seen by Alexa Fluor 488–transferrin in the WT cells (left images) but is shifted away from the cell surface to the late endocytotic compartments in the mutant cells (right images). Similarly, whereas a lesser amount of the mutant LRP6 localizes to early endosomes (right images) (c) compared to WT LRP6 (d), a significantly higher amount of the mutant LRP6 is localized to late endosomes/lysosomes labeled with LAMP2b (right) compared to the WT LRP6 (left).
LRP6 and LDL Are Colocalized and Their Distribution Is Altered in the Mutant Cells

We determined the subcellular localization of LRP6 by immunocytochemistry in both WT and mutant LCLs before and after incubation with Dil-LDL. In both cell types LRP6 colocalizes with Dil-LDL (Figure 2a), but there is significant difference in their intracellular distributions, which was most obvious after 4 hours of incubation with Dil-LDL. In the WT cells, the Dil-LDL and LRP6 colocalize throughout the cytoplasm in both punctuate and vesicular patterns. In the mutant cells, both LRP6 and Dil-LDL cluster in large cytoplasmic aggregates. Immunolocalization with Alexa Fluor 488–conjugated human transferrin revealed reduced cell surface expression of LRP6 in the mutant compared to WT cells. Furthermore, remarkable accumulation of LRP6R611C was observed in the late endocytic compartments within the cells (Figure 2b), whereas LRP6 in the WT cells appeared in the cell surface and early endocytic compartments. Consistent with these findings it was observed that WT LRP6 colocalizes with the early endosomal marker EEA1 in the cell periphery (Figure 2c), but the mutant receptor is shifted from cell surface and early endosomes to late endosomes and lysosomes that labeled with anti-LAMP2b (Figure 2d).

Affinity of WT and Mutant LRP6 for ApoB

Solid-phase binding assays were used to characterize binding of LRP6 to lipid-bound apoB and apoE (all 3 isoforms) at neutral pH as described by others.22 The assays showed a low affinity of LRP6 for apoB (Figure 3a) and undetectable affinity for apoE (data not shown). The affinity of the mutant LRP6 for apoB was slightly higher compared to WT receptor. OD indicates optic density. b, ApoB colocalizes with LRP6 in WT cells at the cell surface and within the cell (arrows, lower left). In contrast, mutant LRP6–apoB colocalization is less in the cell periphery and more in the juxtanuclear region (right images). Top, LRP6 localization (Cy3) in the WT and the mutant LCLs. The middle and lower images show cellular localization of apoB and its colocalization with LRP6, respectively (see arrows).

Membrane Expression of the Mutant LRP6 Is Reduced

Analysis of LRP6 surface expression by FACS in LCLs cultured in lipoprotein-deficient medium showed lower cell surface expression of LRP6R611C compared to WT LRP6 (Figure 4a). Membrane expression levels of WT and mutant LRP6 decreased in presence of high LDL in the medium. In the WT cells, this process is reversible (Figure 4b). The membrane expression of the mutant LRP6 in presence of high LDL continues to decline and the difference in expression levels of the mutant and WT receptors steadily increases. Moreover, the membrane expression levels of the mutant LRP6 does not return to baseline levels after it is exposed to LPDS for up to 4 hours, indicative of its degradation. These observations suggest that the impaired cellular LDL clearance of the mutant LRP6 is attributable to its reduced membrane expression caused by impaired membrane recycling.

LRP6 Is Associated With NPC1, a Regulator of Endosomal LDL Trafficking

NPC1 is a major regulator of the LDL endocytosis and has been localized to late endosomes and lipid rafts. In Niemann–Pick type C disease, NPC1 has impaired trafficking and clusters in late endosomes, a cellular redistribution that explained based on the binding affinity of the mutant LRP6. Immunolocalization of apoB shows colocalization of apoB with WT LRP6 in different stages of endocytosis (Figure 3b). In the mutant LCLs, juxtamembrane retention of LRP6 appears to be related to its reduced colocalization with apoB in the cell surface. We next examined the membrane expression levels of the mutant LRP6 by FACS analysis.
resembles the mutant LRP6. We therefore examined its binding to the mutant LRP6. After precipitation with rabbit anti-human anti-LRP6 antibody (ZYMED Laboratories), blotting with anti-NPC1 antibody revealed a 170-kDa band in mutant cell lysates, which corresponded to human NPC1 (Figure 5a). Similar results were obtained when goat anti-LRP6 antibody (Novus) was used for precipitation. Conversely, when cell lysates were precipitated with anti-NPC1 antibody, LRP6 coprecipitated (data not shown). Coimmunoprecipitation studies in WT cell lysates showed similar results (Figure 5a). In contrast, LRP6 did not bind NPC2 or PCSK9. The immunostaining of LRP6 and NPC1 revealed that these 2 proteins colocalize (Figure 5b). Although NPC1 and WT LRP6 colocalize to diffuse punctate structures throughout the cytoplasm, NPC1 colocalization with the mutant LRP6 was restricted to large clusters in the juxtanuclear region.

Increased Affinity of the Mutant LRP6 for ApoB in Acidic pH Suggests Impaired Ligand Dissociation in Late Endosomes

One possible explanation for the altered LDL trafficking in the mutant LRP6 is that impaired ligand dissociation from the mutant receptor in an acidic environment might trap the receptor in endosomes and hinder its recycling to the plasma membrane. To investigate this potential mechanism, we examined the apoB binding of WT and mutant LRP6 in an acidic pH. The affinity of the LRP6 for apoB was reduced in an acidic pH as predicted. However, the affinity for apoB of the mutant LRP6 is significantly higher than the WT (Figure 6). FACS analysis revealed that changing pH had no effect on the affinity of the LRP6 antibody for either the mutant or WT receptors (data not shown). The high affinity of the mutant receptor for its ligand in an acidic pH has likely important consequences. This characteristic is comparable to LDLRs with deletions within EGF-domain, in which strong ligand-binding affinities in acidic environments result in impaired ligand dissociation in late endosomes and reduced membrane recycling. The enhanced ligand binding affinity of the mutant LRP6 in acidic pH and the continuous decline of its membrane expression in LDL-rich medium imply that impaired membrane recycling of LRP6 is the likely mechanism for low cholesterol clearance and hyperlipidemia in LRP6 mutation carriers.

Discussion

Hypercholesterolemia is a major risk factor for atherosclerosis. We recently showed that mutations in LRP6 is associated with high-serum LDL and early-onset coronary artery disease. To date, only a few members of LRP receptor family have been shown to mediate cellular cholesterol uptake, and, except for LDLR, none has been associated with hyperlipidemia or coronary artery disease in human. On the other hand, the conventional wisdom has been that LRP6 and its homolog LRP5 act mainly as membrane coreceptors for soluble Wnt proteins, and their role in lipid homeostasis has not been considered.

We studied the role of LRP6 and the effect of R611C mutation on cholesterol uptake in the LCLs of the affected and unaffected family members. Use of LCLs for studies of lipid trafficking has been previously validated. Native cells were preferred to in vitro expression systems because of impaired glycosylation and function of LRP6 in the latter. The major findings of the present study were, however, reproduced in the native lymphocytes of the mutation carriers to exclude artifacts caused by Epstein–Barr virus immortalization.

In the present study, we demonstrate a previously undescribed function of LRP6 as a receptor for cellular LDL uptake and a component of LDL cholesterol trafficking complex. In our investigation, we found that LRP6 has affinity for apoB and is involved in direct LDL uptake, albeit to a much lesser degree than LDLR.

Our investigations also demonstrate that elevated serum LDL cholesterol in LRP6R611C mutation carriers is likely attributable to reduced LDL clearance. The binding affinity of the mutant LRP6 for LDL at neutral pH appears preserved and may be even slightly stronger compared to the WT LRP6.
This results initially in a slightly higher uptake of Dil-LDL when cells are incubated with Dil-LDL for a short period (<1 hour; Figure 1). Although this could potentially explain the mechanisms of foam cell formation in atherosclerosis, longer incubation time with Dil-LDL leads to opposite results. WT cells show persistently higher LDL uptake compared to the mutant cells when incubated >2 hours with Dil-LDL. Our results suggest that the defect in LDL uptake is caused, in part, by the lower cell membrane expression of the mutant receptor, which is shifted from cell surface to late endosomes and lysosomes as compared to the WT receptor. Higher affinity of the mutant receptor for LDL at acidic pH suggests that ligand dissociation and membrane recycling of the LRP6 is most likely less efficient, leading to its sequestration away from the plasma membrane. Based on mutation analysis in LDLR, this effect may be attributable to the perturbed function of the second EGFP domain in which R611C mutation resides. Studies of the LDLR have demonstrated an absolute requirement for the β-propeller EGF domain pair in low pH-mediated release of bound β-VLDL or LDL by the mutant receptor.
native LDLR. Investigations of the crystal structure of the LDLR ectodomain at endosomal pH has shown that the receptor folds back on itself, whereby the primary intramolecular interface in the structure brings the central lipid binding repeats in contact with the propeller domain. The propeller domain acts as an intramolecular ligand for the central lipoprotein binding repeats in place of LDL or β-VLDL at low pH. Receptors lacking the entire EGFP region fail to release bound β-VLDL or bound LDL at acidic endosomal pH, are not recycled to the cell membrane efficiently, and are degraded after ligand binding. Interestingly, replacing the native LDLR sequence with different propeller EGF domains of LRP6 has shown that the second propeller EGF is specifically needed to induce release of bound LDL at low pH. Lack of upregulation of LDLR in the mutant cells suggests impaired feedback regulation of LDL-receptor which may contribute to reduced LDL clearance in the mutant cells.

LRP6 in the mutant cells is largely shifted from cell membrane to the late endosomes, suggesting impaired vesicular trafficking of LDL/LRP6 in these cells. NPC1 is a putative integral membrane protein that is involved in late endosomal LDL trafficking. Late endocytic circuits are considered to be important for the regulation of the cellular cholesterol content as highlighted in cholesterol storage disorder Niemann–Pick type C (NPC) disease. The accumulation of unesterified cholesterol mainly in late endocytic organelles initiates a progressive neurodegenerative process. The mechanisms by which NPC1 orchestrate intracellular LDL trafficking has remained uncertain. Interestingly, NPC1 and LRP6 have been both localized to the lipid rafts. Our investigations revealed that LRP6 binds and colocalizes with NPC1. These findings suggest that LRP6 and NPC1 jointly regulate the vesicular trafficking of LDL, a process that is likely impaired by defect in LRP6 recycling and its retention in late endosomes. Whether paired recycling defect of the mutant LRP6 and NPC1 affects cholesterol trafficking similar to those in NPC disease is unclear. Further studies are needed to determine the fate of LDL and the direct effect of LRP6 on NPC1 function in the mutant cells.

The discovery of LRP6 as a mediator of LDL uptake and trafficking and its relationship with NPC1 in our study expands our understanding of the cellular cholesterol homeostasis and the role of the lipid raft in cholesterol transport. Such advances may provide opportunities for the development of novel therapeutic targets for atherosclerosis, Alzheimer disease, and disorders caused by impaired LDL trafficking.

Acknowledgments
We thank Dr Fred Gorelick (Yale University) for critical review of the manuscript.

Sources of Funding
This work was supported, in part, by an NIH K08 award and American Heart Association Grants-in-Aid (to A.M.) and NIH grant CA048115 (to P.B.K.).

Disclosures
None.

References


Mutation in EGFP Domain of LDL Receptor-Related Protein 6 Impairs Cellular LDL Clearance

Wenzhong Liu, Sheida Mani, Nicole R. Davis, Nizal Sarrafzadegan, Paula B. Kavathas and Arya Mani

*Circ Res.* 2008;103:1280-1288; originally published online October 23, 2008; doi: 10.1161/CIRCRESAHA.108.183863

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/103/11/1280

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2008/10/23/CIRCRESAHA.108.183863.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org//subscriptions/
Supplement Material

W Liu et al.

Online Figure I. mRNA expression levels of LRP6 in LRP6 +/- and wildtype mice B-lymphocytes. The expression levels in heterozygote knockout cells are roughly 60% of the wildtype cells.

Online Figure II. Increased LDL uptake of wildtype lymphocytes compared to LRP6<sub>R611C</sub> lymphocytes

LDL uptake of immortalized lymphoblastoid cells (LCL) of the LRP6<sub>R611C</sub> mutation carriers (R611C) and their unaffected family members (WT) were explored. LRP6<sub>R611C</sub> cells demonstrated higher LDL uptake compared to wildtype cells when cells when incubated with Dil-LDL at 37°C for 1 hour. Single cell fluorescence intensities were measured using fluorescence activated cell sorter (FACS) as described.
Online Figure II