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APP, APLP2 and LRP1 interact with PCSK9 but are not required for PCSK9-mediated degradation of the LDLR *in vivo*

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Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that post-transcriptionally regulates the levels of hepatic low-density lipoprotein receptors (LDLRs). PCSK9 binds to the extracellular domain of the LDLR, and the PCSK9-LDLR complex is internalized through canonical clathrin-dependent endocytosis and then delivered to lysosomes for degradation. The mechanism by which PCSK9 blocks recycling of the LDLR has not been fully defined. Previous reports showed that amyloid precursor-like protein 2 (APLP2) interacts with PCSK9, but its role in PCSK9-mediated LDLR degradation remains controversial. Here we found that amyloid precursor protein (APP), APLP2 and LDL receptor-related protein 1 (LRP1) interact with PCSK9. To test whether any of these proteins are required for PCSK9-mediated LDLR degradation, we examined the effects of disrupting these proteins in mice. Infusion of PCSK9 into $App^{-/-}$, $Aplp2^{-/-}$, *Aplp2*-depleted $App^{-/-}$, or liver-specific $Lrp1^{-/-}$ mice resulted in similar reductions in the levels of hepatic LDLR as seen in wild-type (WT) mice. Infusion of PCSK9 into WT mice also had no effect on the levels of hepatic APP, APLP2 or LRP1. Thus, APP, APLP2 and LRP1 are not required for PCSK9-mediated LDLR degradation and are not regulated by PCSK9 *in vivo*.

Keywords

cholesterol; degradation; endocytosis; endosome; crosslinking

Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proteinase K subfamily (1). Gain-of-function mutations in *PCSK9*, including D374Y and S127R, cause hypercholesterolemia (2–4), and loss-of-function mutations result in lower plasma LDL-cholesterol in humans (5). Similarly, overexpression of PCSK9 in mice causes a dramatic increase in plasma LDL-cholesterol levels (6–8).

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Author contributions

Y.W. conceived the project and wrote the paper. Y.W., T.F., J.J.X., and Y.Y.G. conducted the experiments.

Most circulating LDL is removed via LDLRs in the liver (9). After LDL binds the LDLR on the cell surface, the LDL:LDLR complex is internalized through clathrin-dependent endocytosis (10). In the acidic milieu of the endosome, LDLR undergoes a conformational change and dissociates from the LDL particle (11). The receptor is then recycled back to the cell surface, and LDL is delivered to the lysosome. Each LDLR undergoes multiple rounds of internalization and recycling (12). PCSK9 reroutes LDLRs from the recycling pathway to lysosomes for degradation (13).

The mechanism whereby PCSK9 promotes degradation of LDLRs has not been fully elucidated. PCSK9 is a 692-amino-acid glycoprotein that contains a signal sequence followed by a prodomain, a catalytic domain, and a cysteine- and histidine-rich C terminal domain (1). PCSK9 undergoes autocatalytic cleavage in the endoplasmic reticulum that is required for PCSK9 secretion (6). After cleavage, the prodomain remains tightly associated with the catalytic domain and blankets the catalytic site as the protein traverses the secretory pathway and is secreted into the blood (1,7,14). PCSK9 binds the epidermal growth factor-like repeat (EGF)-A in the extracellular domain of the LDLR, and the PCSK9:LDLR complex is internalized by receptor-mediated endocytosis (15). The C-terminal domain of PCSK9 is not required for LDLR binding at the cell surface, although it is required for LDLR degradation (15–17).

After the PCSK9:LDLR complex is internalized by clathrin-dependent endocytosis, it is also delivered to the endosomal compartment (13). In contrast to LDL, the acidic environment of the endosome increases the affinity of PCSK9 for the LDLR by 50- to 150-fold (15,18). Subsequently, the PCSK9:LDLR complex is transported to lysosomes for degradation by a process that remains to be defined (13,15,19–22). One hypothesis is that PCSK9 interacts with other protein(s) that target the PCSK9:LDLR complex to the lysosome for degradation (13).

Amyloid precursor protein (APP) is a type I transmembrane protein that is ubiquitously expressed in humans and mice. Its cleavage by α- or β-secretase is a key regulatory process in the generation of the amyloid β peptide (Aβ). The deposition of Aβ is assumed to be the first event in the pathogenesis of Alzheimer's disease (AD), while the physiological function of APP remains obscure (23). Mice lacking APP alone have very subtle phenotypes, whereas APP and its mammalian homolog APLP2 double-knockout mice ($App^{-/-}/Aplp2^{-/-}$) are not viable, indicating that APP and APLP2 have partly redundant functions (24).

APP contains an endocytic motif of the NPXY class and cycles back and forth between the plasma membrane and endosome (25). This motif is also found in other type I membrane proteins such as LDLR and LRP1 (26,27). APLP2 is a ubiquitously expressed type I transmembrane protein. Its cytoplasmic tail contains two binding motifs of adaptor protein-2 (AP-2), NPXY and YXXØ, which are both required for APLP2 internalization (28). Tuli et al. (29) reported that APLP2 interacts with the MHC class I molecule K(d) on the cell surface and targets it to the lysosome for degradation through a canonical clathrin-dependent pathway. PCSK9 was previously shown to physically interact with APLP2 in a pH-dependent manner, but the importance of this interaction for PCSK9-mediated LDLR degradation remains controversial (30,31).

In the present study, we tested the hypothesis that PCSK9 interacts with another protein(s) to target the PCSK9:LDLR complex to lysosomes. To identify proteins that interact with PCSK9, we used crosslinking and affinity purification in HuH7 cells treated with exogenous PCSK9. We found that PCSK9, when added to the medium of cells in culture or into the circulation in mice, interacts with APP, APLP2 and LRP1 in a specific and reproducible fashion. Next, we tested whether genetic inactivation of these proteins inhibited PCSK9-mediated LDLR degradation. Finally, we tested whether PCSK9 promoted the degradation of any of these receptors in a similar fashion to that seen for LDLR.

Material and methods

Materials

Cell culture medium and phosphate buffered saline (PBS) were obtained from Meditech, Inc. (Herndon, VA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). EDTA-free protein inhibitor cocktail was purchased from Roche Applied Science. The crosslinkers DSP (dithiobis[succinimidylpropionate]) and SDA (succinimidyl 4,4-azipentanoate) were obtained from Thermo Scientific. Formulated anti-amyloid precursor-like protein 2 (APLP2) siRNA was provided by Alnylam. All other chemicals and reagents were obtained from Sigma unless otherwise specified.

The following antibodies were used in the experiments described herein: 3143, a rabbit polyclonal antibody (Ab) against the terminal 14 residues of mouse LDLR that also cross-reacts with human LDLR (32); 172C, home-made rabbit polyclonal anti-sera against full-length human PCSK9 (13); and 377, an anti-LRP1 antibody produced in rabbit against the 85 kDa domain of mouse LRP1 (27). Commercial antibodies were purchased to detect calnexin (StressGen), APP (Millipore) and APLP2 (EMD Biosciences).

Animals

Animal experiments were approved by the University of Texas Southwestern Medical Center Animal Care and Use Committee (IACUC) and were performed at UT Southwestern Medical Center in accordance with federal animal welfare policies and regulations. *App*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Aplp2*$^{-/-}$ mice were provided by Joachim Herz (UT Southwestern) (33), and liver-specific *Lrp1* KO mice were obtained from Michael Brown and Joseph Goldstein (UT Southwestern) (34). PCSK9 transgenic (overexpression), PCSK9 knockout (KO) and age- and gender-matched wild-type (WT) mice were obtained from Jay Horton (UT Southwestern) (35).

Purification of recombinant human PCSK9

C-terminal FLAG-tagged fusion proteins of WT PCSK9 (referred to as PCSK9 in this paper), PCSK9 containing the D374Y substitution (PCSK9-D374Y), the double substitution D374Y and S127R (PCSK9-D374Y and S127R), and the C-terminal domain (aa 425–692) of PCSK9 (PCSK9-C) were purified using anti-FLAG M2 beads (Sigma A2220) and size exclusion chromatography (Superdex 200 10/300 fast performance liquid chromatography (FPLC), GE Healthcare, Piscataway, NJ) (35).

Cell culture and crosslinking

For the crosslinking experiments, HuH7 cells were plated onto 60 dishes (150 mm; 1.5 X 10^6 cells/dish) in high-glucose DMEM (hDMEM) supplemented with 10% FCS and 100 units/ml penicillin G/streptomycin. After the cells reached confluence, the medium was replaced with hDMEM supplemented with 10% newborn calf lipoprotein-poor serum (NCLPPS) and 100 units/ml penicillin G/streptomycin. After 24 h, half of the cells (30 dishes) were treated with buffer only and the other half (30 dishes) were loaded with PCSK9 (10 μg/ml), PCSK9-D374Y (5 μg/ml), PSCK9-S127R and D374Y (5 μg/ml), or PSCK9-C (10 μg/ml) for 30 min at 37°C. Cells were washed twice with PBS and subjected to crosslinking. Two different crosslinkers were used for these experiments: the homobifunctional crosslinker dithiobis[succinimidylpropionate] (DSP) and the heterobifunctional crosslinker succinimidyl 4,4-azipentanoate (SDA). DSP was first dissolved in DMSO (20 mM) and diluted to 1.5 mM in PBS for crosslinking at room temperature (RT) for 1 h (20 ml/15-cm dish). The reaction was terminated by adding 50 mM Tris-Cl for 30 min. SDA was dissolved in DMSO (20 mM), diluted to 1.5 mM in PBS and incubated with PCSK9 (1.2 μg/mL) for 30 min at RT. Free crosslinker was removed using Zeba Spin Desalting Columns (Thermo). SDA-conjugated PCSK9 (10 μg/ml) was added to HuH7 cells for 30 min at 37°C. Cells were washed twice with PBS and exposed to 365 nm UV light for 15 min to activate the SDA crosslinker according to the manufacturer's instructions. For monensin treatment, cells were pretreated with monensin (30 μg/ml) for 15 min before PCSK9 was added.

After crosslinking, cells were washed twice with PBS and lysed in buffer containing Triton X-100 (1% v/v), 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA and protease inhibitor cocktail (Roche). Cell lysates were then incubated in 200 μl of anti-Flag M2 beads for 16 h at 4°C and then washed with 20 ml of wash buffer (1% v/v Triton X-100, 50 mM Tris-Cl, pH 7.5, 450 mM NaCl, and 5 mM EDTA). Bound proteins were eluted with 500 μl of elution buffer (0.05% v/v Triton X-100, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA plus 200 ng/ml Flag peptide). The eluted sample was concentrated and loaded onto SDS-polyacrylamide gradient gels (4–12%) under non-reducing conditions. Bands containing PCSK9 were identified by aligning the gel with an immunoblot of the same material. Bands excised from the gel were analyzed by mass spectrometry. Proteins identified in the PCSK9-treated and control samples, or proteins that did not achieve a score over 150 using the computer program Mascot (Matrix Sciences) were not considered further.

For crosslinking in mouse liver, each mouse was first injected with 32 μg of PCSK9 in the tail vein. Five minutes later, mice were sacrificed and livers were washed and perfused with DSP (1.2 mM in PBS) for 30 min. The crosslinking reaction was stopped by perfusion with 50 mM Tris-Cl for another 15 min. Livers were harvested and proteins exacted and subjected to immunoprecipitation with anti-Flag M2 beads as described above.

Immunoblot analysis

Liver, brain (cerebellum) and adrenal glands were homogenized in RIPA buffer containing complete protease inhibitor cocktail (Roche). Protein concentrations in the supernatants

were measured using a BCA kit (BioRad). Equal amounts of protein were analyzed by SDS-PAGE and immunoblot analysis as previously described (15).

To quantify the immunoblot signals, films were scanned using an HP Scanjet 5590 and quantified using ImageJ (http://rsbweb.nih.gov/ij/). The intensity of each band was corrected for background using a blank region from the same film and normalized using a loading control (calnexin).

PCSK9-mediated LDLR degradation in mice

For the experiments in which PCSK9 was infused into mice, recombinant PCSK9 (32 μg in 200 μl saline/mouse) was injected into the tail vein. After 1 h, mice were sacrificed, and the livers, brains and adrenal glands were harvested for immunoblot analysis (36).

Results

APP, APLP2 and LRP1 Interact with PCSK9

Circulating PCSK9 preferentially reduces liver LDLR in mice (36). In our pilot experiment, we first incubated purified Flag-tagged PCSK9 with mouse liver lysate, which was pulled down using anti-Flag resin. The immunoprecipitated proteins were subjected to SDS-PAGE and mass spectrometry analyses. However, this experiment failed to reveal any specific proteins that interact with PCSK9 (data not shown). Once PCSK9 binds with cell surface LDLR, the PCSK9:LDLR complex is quickly internalized into the early endosome and is then targeted to the lysosome for degradation (13). The transition from the early endosome to the lysosome is highly dynamic; the whole process only takes 10–20 minutes (37). We reasoned that the interaction between PCSK9 and potential lysosome-targeting proteins may be very weak or unstable; therefore, we used large-scale HuH7 cell culture together with chemical crosslinkers and gain-of-function PCSK9 mutants to enrich the PCSK9 complex.

HuH7 cells are human hepatic carcinoma cells that respond very well to PCSK9 stimulation (13). In each experiment, we used 30 dishes (150 mm) of HuH7 cells as starting material. To stabilize the interaction between PCSK9 and potential interacting proteins, two different chemical crosslinkers, DSP and SDA, were used. DSP is a membrane-permeable homobifunctional crosslinker that contains two N-hydroxysuccinimide (NHS) esters that crosslink closely related primary amines. SDA is a heterobifunctional crosslinker that contains an amine-reactive NHS ester and a photoactivatable diazirine ring. We first labeled purified PCSK9 with SDA and incubated labeled PCSK9 with HuH7 cells for uptake. The diazirine ring was then activated using UV light as the second crosslinking step. In this manner, only proteins that interact with PCSK9 directly will be crosslinked. SDA labeling does not interfere with PCSK9 function in promoting LDLR degradation (data not shown).

Monensin is a monovalent carboxylic ionophore that disrupts vesicle trafficking and LDLR recycling (38). In our pilot experiments, monensin treatment blocked PCSK9-mediated LDLR degradation. More importantly, monensin treatment significantly enriched the signals of higher molecular weight bands after DSP crosslinking. We reasoned that monensin treatment would enrich the potential PCSK9-interacting proteins; therefore, this drug was used in some of the experiments.

PCSK9-D374Y and S127R are two gain-of-function mutations found in individuals with hypercholesterolemia (2–4). These two mutations increase the binding affinity of PCSK9 to LDLR; PCSK9 containing either of these two mutations exhibits an increased ability to degrade LDLR (18,19,35). We reasoned that these gain-of-function PCSK9 mutants may form a more stable complex between PCSK9, LDLR and potential interacting proteins; therefore, these mutants in some of the experiments.

The C terminal domain of PCSK9 does not bind to LDLR directly at the cell surface. A number of studies have shown that it is required for PCSK9-mediated LDLR degradation (15–17). To test whether the potential lysosome-targeting proteins interact with PCSK9 through the C terminal domain, we also used the C terminal domain alone as bait in some of the crosslinking experiments.

As indicated in Table 1, LDLR was consistently crosslinked to PCSK9 in all of the experimental conditions tested, except when using the C terminal domain alone as bait. Since PCSK9 is known to bind LDLR, this finding provided an internal positive control for the experiments. All experiments with the DSP crosslinker were repeated, and approximately 20% of the identified proteins were reproducible, which of them were listed in Table 1. Moreover, APP, APLP2, LRP1, and protein tyrosine phosphatase, receptor type F (PTPRF) were also crosslinked to PCSK9-D374Y with DSP. LDLR, APP and APLP2 were crosslinked to PCSK9-D374Y and S127R with SDA. APP and APLP2 were crosslinked to the C-terminal domain alone of PCSK9 with SDA (Table 1), which suggests a direct interaction between APP, APLP2 and the C-terminal domain of PCSK9. APP, APLP2 and LRP1 are all type I transmembrane proteins that can be internalized via clathrin-coated pits, which resembles the endocytic pathway of LDLR and PCSK9. Thus, we hypothesized that they may play a role in PCSK9-mediated LDLR degradation.

We first confirmed the physical interaction between LDLR, APP, APLP2 and LRP1 with PCSK9 by co-immunoprecipitation and immunoblotting experiments. Since we only have antibodies to human APP, APLP2, and mouse LPR1, we confirmed the interactions between APP and APLP2 with PCSK9 in HuH7 cells and the interaction between LRP1 with PCSK9 in mouse liver (Fig. 2A, B). LRP1 was crosslinked to PCSK9-D374Y by DSP, but not to PCSK9-D374Y and S127R by SDA (Table 1), suggesting that LRP1 forms part of a complex that includes PCSK9 or that the association is an artifact of crosslinking.

PCSK9-mediated LDLR Degradation in the Liver Does Not Require APP and APLP2

APP and APLP2 are type I transmembrane proteins that are expressed in several tissues, including the liver (39). To determine whether APP or APLP2 is required for PCSK9-mediated LDLR degradation, we tested whether PCSK9 promotes the degradation of LDLR in $App^{-/-}$ or $Aplp2^{-/-}$ mice. As shown in Figure 3, intravenous injection of purified recombinant PCSK9 into the circulation in mice caused a rapid and substantial decrease in hepatic LDLR levels in both WT and $App^{-/-}$ mice. In contrast, PCSK9 infusion did not reduce LDLR levels in the brains of these animals (Fig. 3B), consistent with previous reports (40).

PCSK9-mediated LDLR degradation was also preserved in the livers of *Aplp2*$^{-/-}$ mice (Fig. 4A). Mice lacking APP or APLP2 alone have very subtle phenotypes, whereas *App*$^{-/-}$/*Aplp2*$^{-/-}$ double-knockout mice are not viable (24), indicating that APP and APLP2 have partly redundant functions. To determine whether APP compensates for APLP2 deficiency or vice versa, we tested the function of PCSK9 in APLP2-depleted *App*$^{-/-}$ mice. siRNA-mediated knockdown of APLP2 in the livers of *App*$^{-/-}$ mice reduced the levels of APLP2 by 90%; however, this did not affect PCSK9-mediated LDLR degradation (Fig. 4B).

Therefore, APP and APLP2 are not required for PCSK9-mediated LDLR degradation in mouse liver. Moreover, the presence of PCSK9 in the circulation was not associated with changes in the steady state levels of APP or APLP2 (Fig. 3, 4). Thus, we found no evidence that APP or APLP2 participates in PCSK9-mediated LDLR degradation or that circulating PCSK9 alters the levels of either of these two proteins.

PCSK9-mediated LDLR Degradation in the Liver Does Not Require LRP1

LRP1 is a type I transmembrane protein that belongs to the LDLR family (41). Liver-specific ablation of LRP1 in mice results in a 2-fold increase in hepatic levels of LDLR protein with no change in LDLR mRNA (34). This change is similar to that observed in *Pcsk9*$^{-/-}$ mice (42). To test whether PCSK9-mediaed LDLR degradation requires LRP1, we administered PCSK9 intravenously into liver-specific LRP1 knockout mice. Despite a >90% reduction in hepatic LRP1, PCSK9-mediated LDLR degradation was preserved in these animals (Fig. 5). Infusion of PCSK9 into these mice did not affect LRP1 levels in WT animals or levels of LDLR in the adrenals of either the WT or LRP1 knockout mice.

Discussion

In the present study, we used chemical crosslinking, immunoprecipitation and mass spectrometry to identify proteins that interact with PCSK9 and tested their potential roles in PCSK9-mediated LDLR degradation. LDLR, APP, APLP2 were repeatedly found to be crosslinked to PCSK9 and LPR1 was only crosslinked to PCSK9-D374Y with homobifunctional crosslinker DSP. Those interactions were also verified by immunoblot analysis. Despite these findings, PCSK9-mediated degradation of LDLR was preserved in the livers of *App*$^{-/-}$, *Aplp2*$^{-/-}$, APLP2-depleted *App*$^{-/-}$, and liver-specific *Lrp1*$^{-/-}$ mice. Based on these results, we conclude that APP, APLP2 and LRP1 are not required for PCSK9-medicated LDLR degradation.

Our finding that APLP2 interacts with PCSK9 is consistent with previous reports (30,31). Devay et al. (30) reported that PCSK9 bound APLP2 in a pH-dependent fashion and proposed that APLP2 participates in the post-endocytic delivery of PCSK9 to lysosomes in HepG2 cells. Subsequently, Butkinaree et al. (31) reported that APLP2 physically interacts with PCSK9 but is not required for PCSK9-mediated LDLR degradation in immortalized hepatocytes (HuH7 and HepG2) or primary hepatocytes (31). Using *Aplp2*$^{-/-}$, *App*$^{-/-}$ and APLP2-depleted *App*$^{-/-}$ mice, our data clearly indicate that neither APLP2 nor APP is required for PCSK9-mediated LDLR degradation *in vivo* (Fig. 3, 4).

To test the hypothesis that PCSK9 regulates the levels of APP and APLP2, we compared the levels of these receptors in PCSK9 KO and WT mice. No differences in APP or APLP2 were found in either the livers or brains of either of these mice (data not shown), consistent with previous reports (31,40). Moreover, infusion of PCSK9 into the circulation was not associated with any detectable change in the level of APP or APLP2 in the liver, where LDLR responds readily (Fig. 3, 4). Thus, the physiological significance of the interactions between PCSK9, APP, and APLP2 remains to be determined.

PCSK9 also crosslinked to LRP1 in the present study, but only when the homobifunctional crosslinker DSP was used (Table 1, Fig. 2B). This finding suggests that LRP1 does not bind to PCSK9 directly but may form part of a complex that includes PCSK9. LRP1 is also a type I transmembrane protein that can be internalized and recycled back to the cell surface (43). Dr. Herz and colleagues found that genetic deletion of LRP1 in the liver caused a 2-fold increase in the LDLR protein level without affecting the LDLR mRNA level (34). This post-translational increase in LDLR is very similar to that observed in *Pcsk9*$^{-/-}$ mice (42). However, PCSK9-mediated LDLR degradation was preserved in liver-specific LRP1 knockout mice (Fig. 5A) and PCSK9 infusion did not affect the level of LRP1 in the liver (Fig. 5A). These findings argue against a role for LRP1 in PCSK9-mediated degradation of LDLR.

Several proteins that were previously reported to interact with PCSK9 were not found in our crosslinking studies. PCSK9 has been reported to interact with VLDLR and ApoER2 and to promote the degradation of these cell surface proteins in HEK293 and NIH 3T3 cells (44,45). Overexpression of PCSK9 was found to be associated with a reduction in the levels of β-site APP-cleaving enzyme 1 (BACE1) in CHO cells (46). BACE is a membrane protease that cleaves APP to produce β-amyloid peptide (Aβ) (47). We did not find any evidence that PCSK9 directly interacts with BACE, VLDLR or ApoER2. It is possible that the expression levels of these proteins were too low or that their interaction with PCSK9 was too transient to detect the interaction using the materials and methods employed in this study. It is also possible that physiological concentration of PCSK9 does not interact with these proteins *in vivo*. Consistent with this notion, Gu et al. (48) reported that the physiological concentration of PCSK9 couldn't bind and degrade VLDLR and ApoER2 in cultured cells, and Liu et al. (40) found that changes in PCSK9 expression did not alter the level of VLDLR, ApoER2, or BACE1 in the mouse brain.

In conclusion, APP, APLP2 and LRP1 are not required for PCSK9-mediated LDLR degradation and are not regulated by PCSK9 *in vivo*. PCSK9 is a short-lived protein with a half-life of approximately 5 minutes in the blood. The concentration of PCSK9 in humans is approximately 0.25 μg/ml (36,49). One caveat of the current study is that a single-dose injection of PCSK9 (32 μg/mouse) may have led to a significantly higher level than the endogenous one, which could mask the requirement of APP, APLP2 or LRP1 in PCSK9-mediated LDLR degradation. However, one hour of continuous PCSK9 infusion in mice (32 μg/h) produces similar PCSK9 levels as the endogenous one (36), rendering this possibility less likely. In contrast, *pcsk9*$^{-/-}$ mice have a 2-fold increased LDLR protein level in their livers, while this change was not observed in *App*$^{-/-}$, *Aplp2*$^{-/-}$, or APLP2-depleted *App*$^{-/-}$ mice, which suggests that APP and APLP2 are not required for PCSK9-mediated LDLR

degradation. It remains possible that the interaction between PCSK9 and the putative lysosomal-targeting protein(s) is too transient to be captured by crosslinking. Alternatively, it is possible that our hypothesis that PCSK9 couples the LDLR to a protein that targets the complex for degradation is incorrect. Holla et al. (16) reported that the C-terminal domain of PCSK9 could be replaced with an unrelated domain (DsRed) of similar size and charge without disrupting PCSK9 activity. This finding is not compatible with a role for the C-terminal domain in coupling PCSK9 to a specific partner. It is possible that the C-terminal domain blocks (rather than mediates) the interaction between the LDLR and a protein(s) required for recycling to the cell surface.

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Abbreviations

PCSK9	proprotein convertase subtilisin/kexin type 9
LDLR	low-density lipoprotein receptor
APP	amyloid precursor protein
APLP2	amyloid precursor-like protein 2
LRP1	LDL receptor-related protein 1
DSP	dithiobis[succinimidylpropionate]
SDA (succinimidyl 4	4-azipentanoate)
WT	wild type

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Highlights

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a secreted protein that promotes the degradation of low density lipoprotein receptors (LDLRs) in lysosome. While how PCSK9 directs LDLR to lysosome for degradation is still poorly understood. APLP2 was reported interacts with PCSK9, but its role in PCSK9-mediated LDLR degradation remains controversial. Using chemical cross-linking, co-immunoprecipitation and mass spectrometry, we found that APP, APLP2 and LRP1 co-immunoprecipitated with PCSK9, and their interaction was confirmed by immune blot analysis. While PCSK9-mediated degradation of LDLR is preserved in *App*$^{-/-}$, *Aplp2*$^{-/-}$, *Aplp2*-depleted *App*$^{-/-}$, liver-specific *Lrp1*$^{-/-}$ mice. We provided strong evidence and showed for the first time that none of APP, APLP2 and LRP1 is required for PCSK9-mediated LDLR degradation *in vivo*, and none of them is regulated by PCSK9. PCSK9 is a novel cholesterol lowering drug target. We believe our results will have broad interest and provide valuable information for both scientific community and pharmaceutical companies.

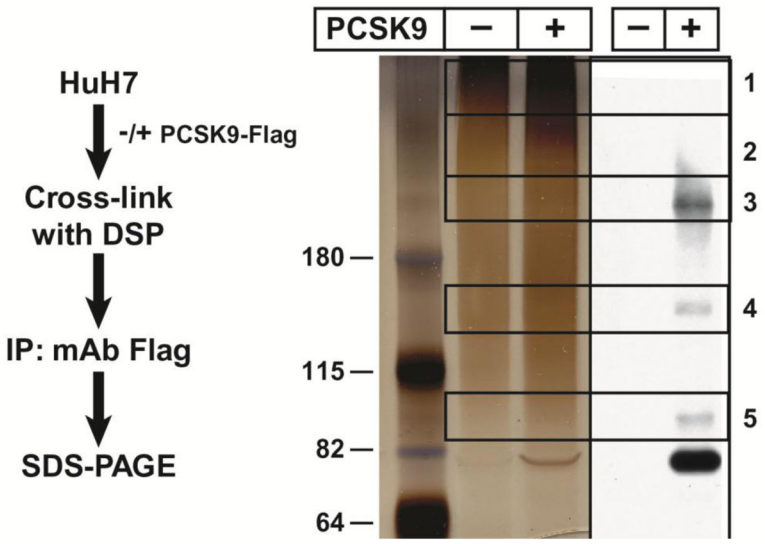

Figure 1. Crosslinking experiments in HuH7 cells

HuH7 cells (30 X 150 mm dishes) were treated with PCSK9-FLAG or control buffer and then crosslinked with DSP as described in the *Material and methods*. Cells were lysed, and proteins that were crosslinked to PCSK9 were isolated by precipitation with anti-Flag M2 beads. Gels containing 1% of the isolated protein were subjected to non-reducing immunoblot analysis with the anti-PCSK9 antibody (172C) to locate the PCSK9-containing bands. The remainder of the immuno-isolated material was run on the same non-reducing gel and visualized by silver staining. Bands corresponding to the immunoreactive bands were excised and identified by mass spectroscopy. The first line from the left contains the marker proteins with different molecular weights. This experiment was repeated multiple times, as indicated in Table 1.

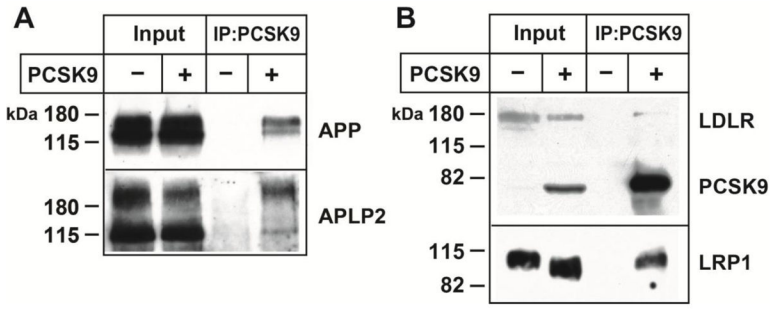

Figure 2. PCSK9 crosslinked to LDLR, APP, APLP2 and LRP1 in hepatocytes and mouse liver

A. Proteins crosslinked to PCSK9 in Fig. 1 were separated by SDS-PAGE under reducing conditions to cleave DSP. APP and APLP2 were detected by immunoblot analysis. *B.* Mouse livers were crosslinked with PCSK9 as described in the *Material and methods*. The crosslinked proteins were fractionated by reducing SDS-PAGE and blotted with antibodies to LDLR (3143), PCSK9 (172C) or LRP1 (377). Experiments were repeated with similar results.

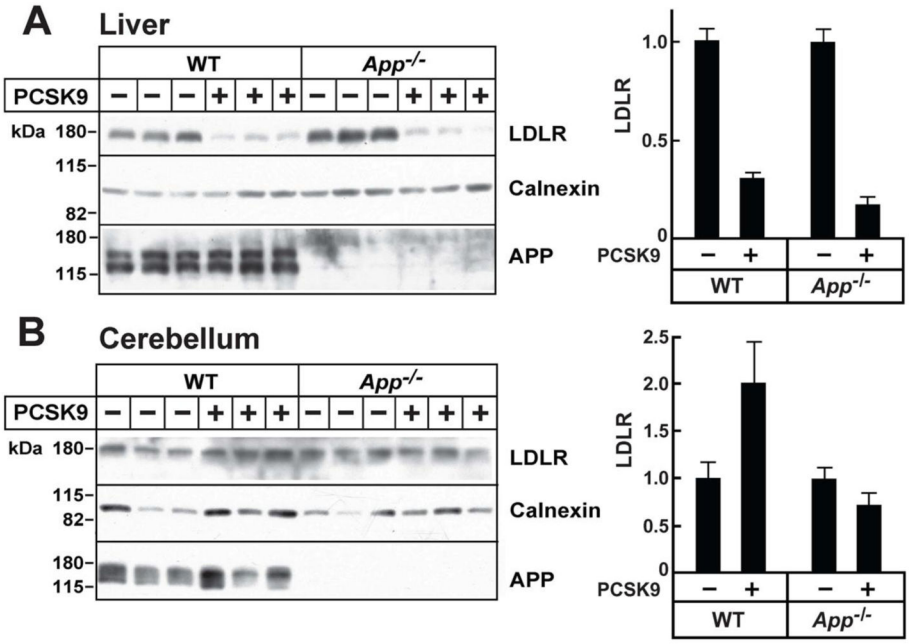

Figure 3. PCSK9-mediated LDLR degradation in *App*$^{-/-}$ mice

Wild-type or *App*$^{-/-}$ mice were injected with PCSK9 (32 μg) or saline for 1 hour, and the LDLR level in the liver (*A*) and cerebellum (*B*) was examined by immunoblot analysis (N=3 per treatment per genotype). Proteins were quantified as described in the *Material and methods*. Experiments were repeated with similar results. Graphs represent the means +/− SEM.

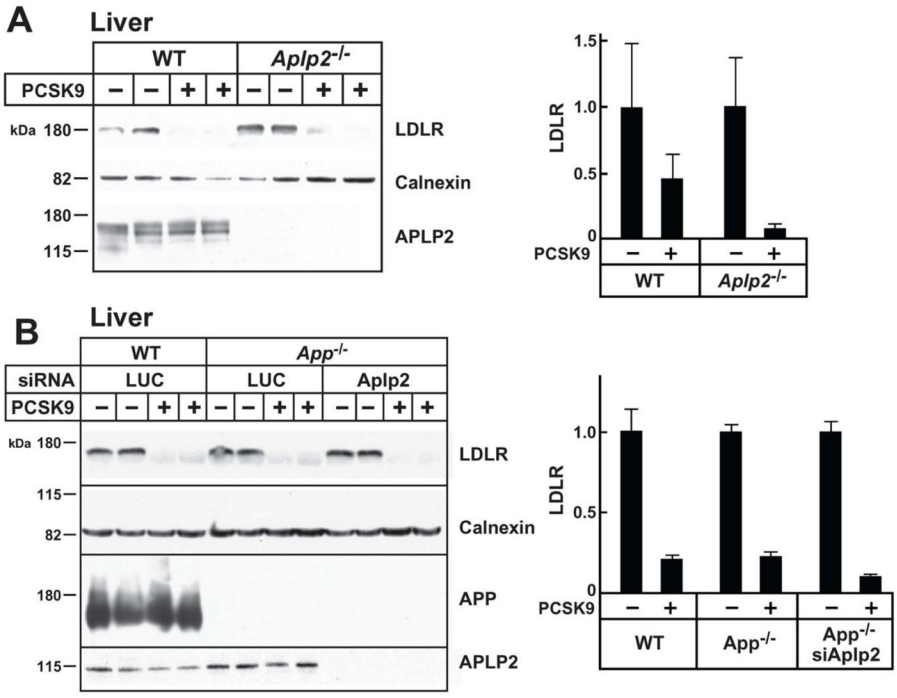

Figure 4. PCSK9-mediated LDLR degradation in the livers of *Aplp2*$^{-/-}$ mice and *App*$^{-/-}$, APLP2-depleted mice

A, Wild-type or *Aplp2*$^{-/-}$ mice were injected with PCSK9 (32 μg) or saline for 1 hour, and the LDLR level in the liver was examined by immunoblot analysis (N=2 per treatment per genotype). *B, Aplp2* was depleted from the livers of *App*$^{-/-}$ mice by intravenous injection of siRNAs (1 mg/kg) formulated in liposomes. siRNA against luciferase (LUC) was used as a negative control. Five days later, PCSK9 (32 μg) or saline was injected and PCSK9-mediated LDLR degradation in the liver was examined by immunoblot analysis (N=2 per treatment per genotype). Proteins were quantified as described in the *Material and methods*. Experiments were repeated with similar results. Graphs represent the means +/− SEM.

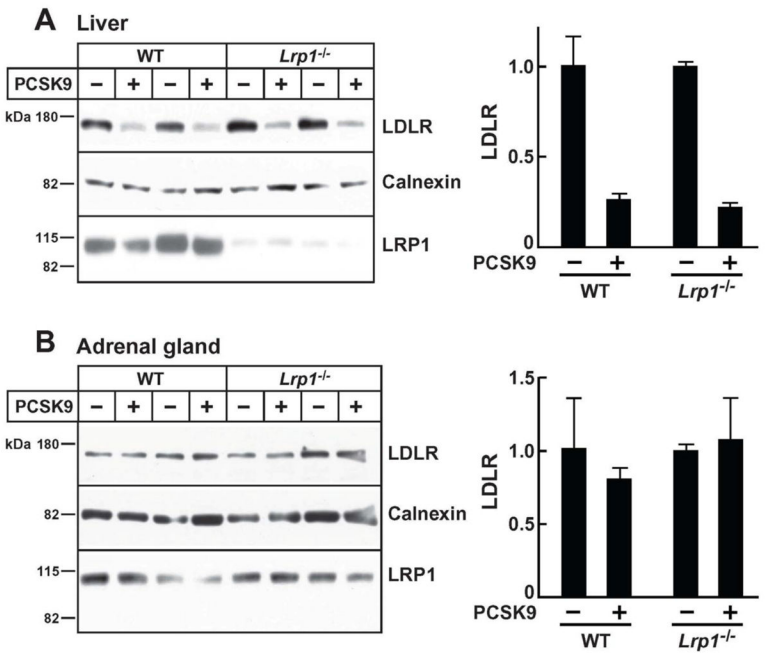

Figure 5. PCSK9-mediated LDLR degradation in liver-specific *Lrp1* knockout mice

Wild-type or liver-specific L*rp*$^{-/-}$ mice were injected with PCSK9 (32 μg) or saline for 1 hour, and the LDLR level in the liver and adrenal gland was examined by immunoblot analysis (N=2 per treatment per genotype). Proteins were quantified as described in the *Material and methods*. Experiments were repeated with similar results. Graphs represent the means +/− SEM.

Table 1

Summary of results from PCSK9 crosslinking experiments.

	Bait				
	PCSK9	**PCSK9 + Monensin**	**D374Y**	**S127R and D374Y**	**C terminal**
Crosslinker	**DSP**	**DSP**	**DSP**	**SDA**	**SDA**
# Exp	**2**	**2**	**2**	**1**	**1**
Protein ID	**Bands containing the indicated protein**				
PCSK9	2/3/4/5	2/3/4/5	1/2/3/4/5	2/3/4/5	2/3/4/5
LDLR	2/3	2/3	1/2/3/4	3/4	
APP			2/3/4	3/4	2/3/4
APLP2			3/4	3/4	2/3/4
LRP1			1		
Ubiquitin	5				
Fibrinogen		2			
PTPRF			1		
GCN1L1				3	
UBR5				3	
LRP2					2
FRAS1					2
Fibronectin					1
HSPG					1
Thrombospon din-1					2
EIF4G2					4
PRMT5					4

Crosslinking experiments were performed as described in the *Material and methods*. Four different types of PCSK9 proteins were used in the experiment (PCSK9 Wild Type, PCSK9-D374Y mutant, PCSK9-D374Y and S127R double mutant or PCSK9 C terminal domain alone). # Exp indicates the number of repeats for each experiment. Proteins shown in bold are candidates that were pursued for their function in PCSK9-mediated LDLR degradation. Band number indicates the number of bands that were found (see Fig. 1, right).

LDLR: low-density lipoprotein receptor; PCSK9: proprotein convertase subtilisin/kexin type 9; APP: amyloid-β precursor protein; APLP2: APP-like protein-2; LRP1: LDLR-related protein 1; PTPRF: protein tyrosine phosphatase, receptor type, F; GCN1L1: general control of amino-acid synthesis 1-like 1; UBR5: E3 ubiquitin-protein ligase; LRP2: LDL receptor-related protein 2; FRAS1: Fraser syndrome 1; HSPG: heparan sulfate proteoglycan; EIF4G2: eukaryotic translation initiation factor 4 gamma, 2; PRMT5: protein arginine N-methyltransferase 5.