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ORIGINAL RESEARCH ARTICLE

Ablation of Plasma Prekallikrein Decreases Low-Density Lipoprotein Cholesterol by Stabilizing Low-Density Lipoprotein Receptor and Protects Against Atherosclerosis

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BACKGROUND: High blood cholesterol accelerates the progression of atherosclerosis, which is an asymptomatic process lasting for decades. Rupture of atherosclerotic plaques induces thrombosis, which results in myocardial infarction or stroke. Lowering cholesterol levels is beneficial for preventing atherosclerotic cardiovascular disease.

METHODS: Low-density lipoprotein (LDL) receptor (LDLR) was used as bait to identify its binding proteins in the plasma, and the coagulation factor prekallikrein (PK; encoded by the *KLKB1* gene) was revealed. The correlation between serum PK protein content and lipid levels in young Chinese Han people was then analyzed. To investigate the effects of PK ablation on LDLR and lipid levels in vivo, we genetically deleted *Klkb1* in hamsters and heterozygous *Ldlr* knockout mice and knocked down *Klkb1* using adeno-associated virus-mediated shRNA in rats. The additive effect of PK and proprotein convertase subtilisin/kexin 9 inhibition also was evaluated. In addition, we applied the anti-PK neutralizing antibody that blocked the PK and LDLR interaction in mice. Mice lacking both PK and apolipoprotein e (*Klkb1*$^{-/-}$*Apoe*$^{-/-}$) were generated to assess the role of PK in atherosclerosis.

RESULTS: PK directly bound LDLR and induced its lysosomal degradation. The serum PK concentrations positively correlated with LDL cholesterol levels in 198 young Chinese Han adults. Genetic depletion of *Klkb1* increased hepatic LDLR and decreased circulating cholesterol in multiple rodent models. Inhibition of proprotein convertase subtilisin/kexin 9 with evolocumab further decreased plasma LDL cholesterol levels in *Klkb1*-deficient hamsters. The anti-PK neutralizing antibody could similarly lower plasma lipids through upregulating hepatic LDLR. Ablation of *Klkb1* slowed the progression of atherosclerosis in mice on *Apoe*-deficient background.

CONCLUSIONS: PK regulates circulating cholesterol levels through binding to LDLR and inducing its lysosomal degradation. Ablation of PK stabilizes LDLR, decreases LDL cholesterol, and prevents atherosclerotic plaque development. This study suggests that PK is a promising therapeutic target to treat atherosclerotic cardiovascular disease.

Key Words: atherosclerosis ■ blood coagulation ■ cardiovascular diseases ■ cholesterol ■ prekallikrein ■ receptors, LDL

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of death globally.[1,2] It encompasses an umbrella of disorders of the heart and blood vessels that initiate from a buildup of atherosclerotic plaques and the resultant narrowing of the arteries. In response to endothelial lesions, blood monocytes/macrophages migrate into the tunica intima, take up cholesterol-rich low-density lipoprotein (LDL) particles from

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Clinical Perspective

What Is New?

- The coagulation factor prekallikrein (PK) binds low-density lipoprotein receptor and induces its degradation.
- Inhibition of proprotein convertase subtilisin/kexin 9 and PK has an additive effect on stabilizing low-density lipoprotein receptor and lowering plasma cholesterol.
- Ablation of PK elevates hepatic low-density lipoprotein receptor protein, decreases blood cholesterol levels, and slows atherosclerosis progression.

What Are the Clinical Implications?

- PK absence, similar to proprotein convertase subtilisin/kexin 9 inhibition, can decrease the plasma cholesterol level by increasing low-density lipoprotein receptor.
- PK depletion can effectively prevent atherosclerosis development.

Nonstandard Abbreviations and Acronyms

Apo	apolipoprotein
aPTT	activated partial thromboplastin time
ASCVD	atherosclerotic cardiovascular disease
CARDIoGRAM	Coronary Artery Disease Genome-Wide Replication and Meta-Analysis
FIX	coagulation factor IX
FXII	coagulation factor XII
FXIIa	active factor XII
HCD	high-cholesterol diet
IDOL	inducible degrader of the low-density lipoprotein receptor
IgG	immunoglobulin G
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
LDLR	low-density lipoprotein receptor
M-PCSK9	mature form of proprotein convertase subtilisin/kexin 9
PCSK9	proprotein convertase subtilisin/kexin 9
PK	prekallikrein
PKa	plasma kallikrein
TC	total cholesterol
WT	wild-type

circulation, and form atherosclerotic plaques.[3,4] Atherosclerosis typically develops for years and even decades, with high levels of LDL cholesterol (LDL-C) drastically accelerating its progression. A major strategy for preventing and treating atherosclerosis is to lower LDL-C. The widely prescribed lipid-lowering medicines include statins, ezetimibe, and proprotein convertase subtilisin/kexin 9 (PCSK9) inhibitor. All can reduce plasma cholesterol levels, although through different mechanisms. However, regardless of how well cholesterol is controlled, at least 20% of patients experience recurrent myocardial infarction within 3 years after the first clinical manifestation.[5] Other treatments are urgently needed to reduce the morbidity and mortality associated with atherosclerosis.

ASCVD is more than a lipid-driven disorder. Factors such as inflammation and thrombosis also contribute to ASCVD.[6–8] Atherosclerotic plaques contain a variety of potent thrombogenic materials such as polyphosphates, including DNA and RNA derived from necrotic cells, collagen, and tissue factor, which are released on plaque rupture. Moreover, the microplaques are constantly ruptured or eroded, provoking thrombosis followed by repair. These repeated cycles of rupture, thrombosis, and healing of plaques promote the rapid progression of atherosclerotic lesions and vessel stenosis. Rupture of a large atherosclerotic plaque causes arterial occlusions that eventually lead to myocardial infarction or ischemic stroke.[6] Therefore, antithrombosis should be taken under consideration when strategies against ASCVD are being developed.[9]

Coagulation cascades are activated by tissue factor–initiated extrinsic pathway and contact system–initiated intrinsic pathway, both of which converge into the common pathway (Figure S1A).[9,10] Plasma kallikrein (PKa) is a serine protease playing a crucial regulatory role in the intrinsic pathway. PKa is generated from the liver-expressed prekallikrein (PK; aka Fletcher factor or plasma prekallikrein), a proenzyme encoded by the *KLKB1* gene and activated by active factor XII (FXIIa) through cleavage of the R371-L372 peptide bond. PKa in turn promotes the generation of FXIIa from FXII, thereby amplifying the coagulation cascade. FXIIa then cleaves factor XI to produce active factor XI, and active factor XI next converts factor IX (FIX) to active FIX. PKa can also directly cleave FIX to generate active FIX.[11] Active FIX then cleaves factor X to active factor X.[12,13] In the extrinsic pathway, exposure of subendothelial tissue factor activates factor X through factor VII. The active factor X proteolytically cleaves prothrombin to thrombin, which ultimately yields a fibrin and platelet cross-linked clot.[14]

The LDL receptor (LDLR) critically regulates plasma LDL-C levels by mediating its internalization into the cells, primarily hepatocytes.[15–17] It serves as a target of statins and PCSK9 inhibitors. While inhibiting cholesterol biosynthesis, statins can activate the sterol regulatory element-binding protein pathway and thus upregulate the expression of LDLR. The monoclonal PCSK9 antibodies (eg, alirocumab and evolocumab) act by blocking

PCSK9 binding to LDLR. PCSK9 is first synthesized as a precursor and undergoes proteolytic maturation before secretion into the blood, where it binds and causes LDLR to degrade in the lysosomes instead of recycling back to the cell surface. Besides increasing LDLR levels and promoting LDL-C clearance, the roles of the PCSK9 antibodies in preventing atherosclerosis and thrombosis are gradually being appreciated.[18,19] On the other hand, the existing anticoagulants, including active FX inhibitors and thrombin inhibitors, should be used with caution for treating ASCVD because of the side effect of bleeding.[20]

In our study, we identify that the plasma coagulation factor PK interacts with LDLR and induces its degradation in the lysosomes. In young Chinese Han adults, serum PK concentrations positively correlate with LDL-C levels. In hamsters, genetic ablation of *Klkb1* decreases plasma lipid levels through upregulating LDLR in an additive manner to the PCSK9 inhibitor evolocumab. Injections of the anti-PK neutralizing antibody in mice and knockdown of *Klkb1* in rats also increase hepatic LDLR levels and reduce plasma cholesterol levels. Furthermore, in mice with the apolipoprotein (apo) E–deficient background, PK absence arrests the progression of atherosclerotic lesions. These results suggest that PK regulates both LDL and thrombosis and that PK inhibition can be an attractive therapeutic strategy to lower plasma cholesterol levels and to prevent thrombosis simultaneously.

METHODS

The authors declare that all supporting data and methods are available in the article (and its Supplemental Material).

Animals

All animal care and use procedures followed the guidelines of the Institutional Animal Care and Use Committee of Wuhan University and were carried out under protocol WDSKY0201408.

Klkb1 whole-body knockout (*Klkb1*$^{-/-}$) golden hamsters and *Klkb1* whole-body knockout mice on C57BL/6J background were generated by Ex&InVivo (Shijiazhuang, China) and GemPharmatech (Nanjing China), respectively, using CRISPR/Cas9–based technology. In brief, the sgRNAs targeting the exon 3 (hamsters) or the sites flanking exon 3 (mice) were coinjected with the Cas9 mRNA into zygotes, resulting in the frameshift variation of *Klkb1* in hamsters and depletion of exon 3 and early stop of *Klkb1* in mice. For studies evaluating the effect of PK on plasma cholesterol, 5-month-old hamsters were fed a high-cholesterol diet (HCD; Research Diets, D12109C) for 3 to 4 weeks. *Ldlr* whole-body knockout (*Ldlr*$^{-/-}$) and apo E whole-body knockout (*Apoe*$^{-/-}$) mice were purchased from GemPharmatech. *Klkb1*$^{-/-}$ mice were crossed with *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice to generate *Klkb1*$^{+/+}$*Apoe*$^{-/-}$, *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ and *Klkb1*$^{+/+}$*Ldlr*$^{+/-}$, *Klkb1*$^{-/-}$*Ldlr*$^{+/-}$ mice. Male *Ldlr*$^{-/-}$ mice were crossed with female wild-type (WT) mice to get *Ldlr*$^{+/-}$ mice.

Male Sprague-Dawley rats (8 weeks of age) were purchased from the Center for Disease Control (Hubei, China) and randomly grouped. Rats were fed a chow diet (Research Diets, D10001) for 7 days and intravenously injected with 3×10^{12} viral genomes of adeno-associated virus 2/9–shcontrol or –sh*Klkb1*. Rats were kept on a chow diet for 2 additional weeks and then euthanized to analyze lipids in serum and LDLR proteins in the liver.

The animals generated by the CRISPR/Cas9 technology were backcrossed with the WT animals for at least 2 generations before experiments. All animals were housed in a pathogen-free environment with the ambient temperature maintained at 21°C to 23°C and relative humidity at 50% to 60%, with a 12 hour:12 hour light:dark cycle. Animals were allowed ad libitum access to water and food unless otherwise indicated. For experiments that measured circulating lipids and LDLR protein levels, animals were fasted at daytime (8 AM–8 PM), fed again at nighttime (8 PM–8 AM), and then fasted for 2 additional hours before euthanasia.

Study Participants and Measurement of Plasma PK in Humans

The human study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. Enrollment included 198 healthy, first-year college students (Chinese Han, 86 male and 112 female, 17–25 years of age). The basic demographic information is given in Table S1. None received lipid-lowering medications or were hospitalized. The information was collected during a routine examination, with written informed consent obtained from all participants in advance. PK levels in plasma were quantified by a human prekallikrein ELISA kit (Novus Biologicals).

Materials

We obtained DMEM (C11995500BT) from Thermo Fisher and FBS (10099141) from Gibco. Ammonium chloride (A9434), sodium mevalonate (41288), paraformaldehyde (P6148), and Sudan IV (198102) were from Sigma-Aldrich. Doxycycline hyclate (A600889) was from Sangon Biotech. Phenylmethylsulfonyl fluoride (HY-B0496), avoralstat (HY-16735), and MG132 (HY-13259) were from MCE. ALLN (208719) and pepstatin A (516481 mol/L) were from Calbiochem. Dithiothreitol (T5370) was from Targetmol. Evolocumab was from Amgen. PBS (120539) was from Monad. Rat control serum immunoglobulin G (IgG) was from DIA-AN (Wuhan, China). We prepared lipoprotein-deficient serum (density >1.215 g mL/L) from FBS using the ultracentrifugation system in our laboratory.

Cell Culture

Huh-7 and HEK293T cells were cultured in DMEM containing 100 U/mL penicillin and 100 µg/mL streptomycin sulfate and supplemented with 10% FBS (medium A) in 37°C incubator with 5.5% CO_2. Cholesterol-depleted medium comprises DMEM supplemented with 5% lipoprotein-deficient serum, 1 µmol/L lovastatin, and 50 µmol/L sodium mevalonate.

Identification of LDLR Binding Protein From Plasma

HEK293T cells were set up (8×10^6 cells per 15-cm dish) in medium A and transfected with the plasmid expressing

LDLR-Flag (20 μg DNA per dish). Twenty-four hours later, culture medium was replaced with DMEM containing 5% lipoprotein-deficient serum. After 24 hours, cells were harvested, lysed with 1 mL lysis buffer (PBS, 1% NP-40, 10 μmol/L MG132, 10 μg/mL leupeptin, 5 μg/mL pepstatin, 25 μg/mL ALLN), and then passed 15 times through a 22-gauge needle followed by centrifugation at 13 200 rpm for 10 minutes at 4°C. Supernatants were pooled and precleared with 60 μL protein A/G agarose at 4°C for 2 hours, followed by centrifugation at 2000*g* for 10 minutes at 4°C. Supernatants were incubated with 200 μL anti-Flag M2 beads at 4°C for 8 hours. Beads were washed extensively in lysis buffer, followed by 2 rounds of washes in pH 8.0 buffer (20 mmol/L Tris, 100 mmol/L NaCl, 0.5 mmol/L $CaCl_2$, pH 8.0) and pH 5.0 buffer (56 mmol/L sodium acetate, 100 mmol/L NaCl, 0.5 mmol/L $CaCl_2$, pH 5.0).

Mice plasma was filtered through a 100-kDa–cutoff ultrafiltration filter (Millipore, MWCO, 100,000) to deplete lipoproteins and then precleared with anti-Flag M2 beads (Sigma, A2220). Plasma was incubated with LDLR-coupled beads or control IgG beads at 4°C overnight. After extensive washes, the bound proteins were eluted with Flag peptides (0.5 mg/mL in PBS). The proteins were analyzed by mass spectrometry.

In Vitro Binding Assay of PK and LDLR Extracellular Domain

Flag-tagged PK (5 μg) was mixed with or without 5 μg anti-PK antibody (2H5) in 50 μL binding buffer (PBS, pH 7.4, 5% glycerol, 0.1% Tween-20, 1 mmol/L $CaCl_2$) on ice for 30 minutes. Then the solution of PK or PK/2H5 mixture was supplemented with 8 μg LDLR extracellular domain, and LDLR extracellular domain was added to 50 μL binding buffer as a control. The proteins solutions were placed on ice for 1 hour followed by rotation with 30 μL anti-Flag M2 beads in 800 μL binding buffer at 4°C for 2 hours. The beads were washed with 1 mL binding buffer 5 times at 4°C. The bound proteins were eluted with 100 μL PBS containing 1 mg/mL Flag peptides. The samples were analyzed by immunoblot with anti-LDLR antibody and anti-Flag antibody.

Atherosclerosis Plaque Analysis

Eight-week-old *Klkb1*$^{+/+}$*Apoe*$^{-/-}$ and *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ mice were fed an HCD (Research Diet, D12109C) for 8 weeks, and blood was collected. Animals were euthanized by cervical dislocation and perfused with ≈20 mL saline through a left ventricle puncture. The liver samples were quickly frozen with liquid nitrogen and stored at −80°C. The heart and whole aorta were isolated and placed in 4% paraformaldehyde at 4°C for at least 24 hours. After removal of adjoining tissues, the aorta (from the aorta root through the bifurcation of the iliac arteries) was opened longitudinally, stained with Sudan IV for 15 minutes, and then washed with 70% ethanol for 3 minutes. The aortic tree was pinned onto a black rubber plate in PBS and imaged under an Olympus SZX16 stereoscopic microscope. The heart was dehydrated by 2 steps in 15% and 30% sucrose at 4°C sequentially and then embedded using optimal cutting temperature compound. A series of 8-μm frozen sections of aortic root were prepared, and the sections at the same site in each heart were processed for Oil Red O staining. The atherosclerotic lesions were quantified with ImageJ software.

Statistical Analysis

The Kolmogorov-Smirnov method or Shapiro-Wilk normality test was used to test the normality of the measurement data. GraphPad Prism 7 and SPSS24.0 were used for data analysis. The normally distributed data are presented as mean±SEM unless stated otherwise, and statistical differences were analyzed with unpaired 2-tailed Student *t* test for 2-group comparisons or 1-way ANOVA and 2-way ANOVA for multiple-group comparisons as indicated in the figure legends. The nonnormally distributed measurement data are presented as median (interquartile range), and the Wilcoxon rank-sum test was used for comparison between groups. Partial correlations were analyzed by the SPSS statistical software to determine the relationship between plasma lipids (total cholesterol [TC], triglyceride, LDL-C, high-density lipoprotein cholesterol) and PK protein controlling for age and sex.

RESULTS

PK Promotes LDLR Degradation

To identify new proteins in the circulation system that bind LDLR and regulate the LDLR level, we purified the Flag-tagged recombinant LDLR protein from HEK293T cells and used it as bait to capture proteins binding to LDLR from the plasma depleted of lipoproteins that may have masking effects. Mass spectrometry identified PK, encoded by the *Klkb1* gene, as a top candidate (Figure S1B). Antithrombin III and heat shock protein 90α and 90β were also identified. Previous studies showed that antithrombin III could inhibit the activity of PKa by forming a covalent bond at the active site,[21,22] whereas heat shock protein 90 may interact with PK and activate the PK–high-molecular-weight kininogen complex independently of FXII.[23] We next confirmed the interaction between PK and LDLR by coimmunoprecipitation using PCSK9, which is known to bind LDLR, as the positive control (Figure 1A and Figure S1C). An interesting finding is that overexpression of PK could dramatically decrease LDLR protein levels (Figure S1D). Other candidates such as exportin-2, importin subunit β-1, and heat shock protein 90β had little effect on LDLR level (Figure S1D). To corroborate the effects of PK on LDLR stability, we generated Huh7 cells stably expressing doxycycline-inducible PK (Huh7/Tet-on-PK). Doxycycline-induced expression of PK decreased LDLR protein levels regardless of statin treatment (Figure 1B), suggesting that PK may promote LDLR degradation. Indeed, addition of the lysosome inhibitor NH_4Cl, but not the proteasome inhibitor MG132, reverted LDLR expression in the cells exposed to doxycycline (Figure 1C). Similar to PCSK9,[24] PK is expressed mainly in the liver and secreted into blood.[25] To examine whether the secreted PK protein could reduce cellular LDLR level, we purified PCSK9 and PK proteins and added them into the medium side by side. PK dose-dependently decreased LDLR level (Figure 1D). The PKa inhibitor avoralstat did not block LDLR

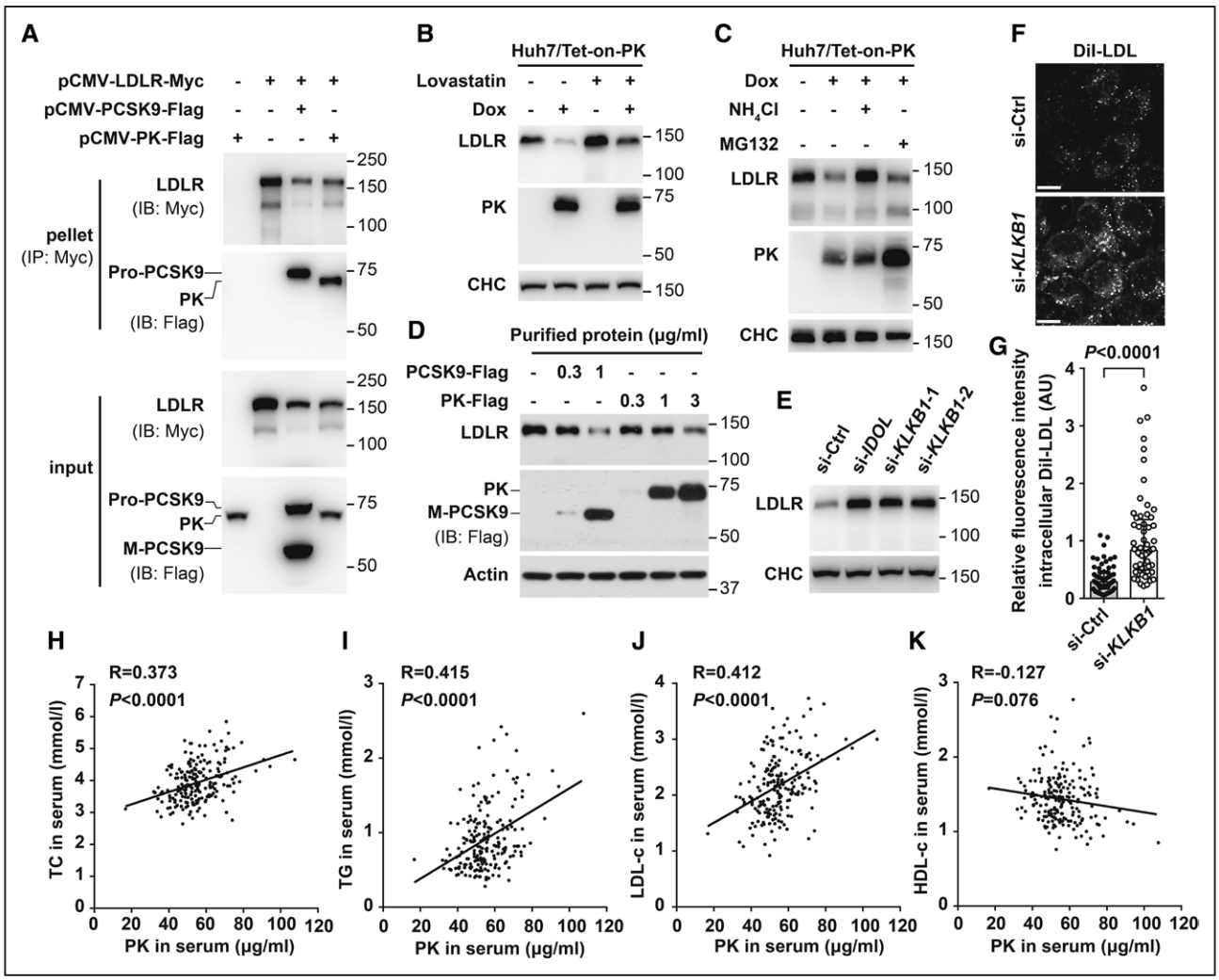

Figure 1. PK promotes LDLR degradation and reduces LDL uptake.
A, Coimmunoprecipitation of prekallikrein (PK) and low-density lipoprotein (LDL) receptor (LDLR). Huh7 cells were transfected with the indicated plasmids. After 48 hours, cells were harvested, and LDLR was immunoprecipitated with the anti-MYC–coupled agarose followed by probing for the indicated antibodies. **B**, Overexpression of PK decreases the LDLR protein level. Huh7/Tet-on-PK cells were treated with 2 μg/mL doxycycline (Dox) or 1 μg/mL lovastatin as indicated in the cholesterol-depletion medium containing 5% lipoprotein-deficient serum for 24 hours. Cells were harvested for immunoblotting. **C**, PK-induced LDLR degradation is blocked by the lysosome inhibitor NH_4Cl. Huh7/Tet-on-PK cells were treated with 2 μg/mL Dox, 5 mmol/L NH_4Cl, or 5 mmol/L MG132 as indicated for 18 hours. Cells were harvested for immunoblotting. **D**, PK induces LDLR degradation. Huh7 cells were depleted of cholesterol for 12 hours and incubated with purified Flag-tagged PCSK9 or PK at the indicated amounts for 8 hours. Cells were harvested for immunoblotting against LDLR, actin, and medium for immunoblotting against Flag. **E**, Knockdown of *KLKB1* increases the LDLR protein level. Huh7 cells were transfected with control (Ctrl) siRNA or siRNAs targeting *KLKB1* or *IDOL* for 48 hours. Cells were harvested for immunoblotting. **F** and **G**, Knockdown of *KLKB1* improves LDL uptake. Huh7 cells were transfected with the indicated siRNAs for 36 hours. Cells were then depleted of cholesterol for 12 hours followed by incubating with 10 μg/mL Dil-LDL for 1 hour. Scale bars, 10 μm. Representative images are shown in (**F**), and quantification of the relative fluorescence intensity of internalized Dil-LDL is shown in (**G**). Data are presented as mean and interquartile range (n=60 cells). Statistical significances were determined with the Wilcoxon rank-sum test. **H** through **K**, Partial correction analysis of serum PK protein concentration with (**H**) total cholesterol (TC), (**I**) triglyceride (TG), (**J**) LDL cholesterol (LDL-C), and (**K**) high-density lipoprotein cholesterol (HDL-C) in human subjects (n=198). Adjusted analyses include age and sex. *R* and *P* values were based on the correlation. CHC indicates clathrin heavy chain; CMV, Cytomegalovirus; M-PCSK9, mature proprotein convertase subtilisin/kexin 9; and Pro-PCSK9, precursor form of proprotein convertase subtilisin/kexin 9.

degradation induced by PK (Figure S1E), indicating that enzymatic activity is not required for PK to degrade LDLR. We further knocked down *KLKB1* using siRNA (Figure S1F) and detected increased levels of LDLR protein (Figure 1E). Inducible degrader of the LDLR as the E3 ubiquitin ligase known to mediate LDLR degradation[26–28] was the positive control. In line with LDLR elevation, the uptake of Dil-labeled LDL was dramatically increased in *KLKB1* knockdown cells (Figure 1F and 1G).

We further examined the correlation of serum PK concentrations with lipid levels in 198 randomly chosen young Chinese Han adults (Table S1). Serum levels of the PK protein were positively associated with those of LDL-C, TC, and triglyceride but not of high-density

lipoprotein cholesterol (Figure 1H–1K). Together, these results demonstrate that PK binds LDLR, induces LDLR degradation, and influences circulating cholesterol levels.

PK Deficiency Increases Hepatic LDLR and Decreases LDL-C in Rodents

Golden Syrian hamsters resemble humans more closely than mice in terms of lipid metabolism.[29] In particular, hamsters exhibit lipoprotein profiles similar to those of humans and are susceptible to HCD-induced hypercholesterolemia with high LDL-C levels.[30] LDLR was highly expressed in the hamster liver (Figure S2A). To investigate the effect of PK on lipid metabolism in vivo, we generated *Klkb1* whole-body knockout (*Klkb1*$^{-/-}$) hamsters using the CRISPR/Cas9 system (Figure 2A). No apparent differences in physical appearance, behavior, reproductive capacity, body weight (Figure 2B), and food intake (Figure 2C) were observed between WT and *Klkb1*$^{-/-}$ hamsters. However, the LDLR protein levels were significantly increased in the liver and other tissues of male *Klkb1*$^{-/-}$ hamsters, which, as expected, exhibited no detectable levels of PK in the plasma (Figure 2D). The mRNA levels of *Ldlr* and cholesterogenic genes were not changed in the *Klkb1*$^{-/-}$ liver (Figure S2B), confirming that PK promotes LDLR degradation at the posttranslational level (Figure 1). Compared with the WT controls, the plasma levels of TC and triglyceride were decreased by 49.2% and 43.6%, respectively, in *Klkb1*$^{-/-}$ hamsters (Figure 2E and 2F). Fast protein liquid chromatography analysis of plasma showed that circulating cholesterol and triglyceride were particularly reduced in very-low-density lipoprotein and LDL of *Klkb1*$^{-/-}$ hamsters (Figure 2G and 2H). The levels of TC and triglyceride in the liver (Figure 2I and 2J) and those of aspartate aminotransferase and alanine aminotransferase in the plasma were similar in both groups (Figure 2K and 2L). No obvious abnormalities or lipid accumulation was observed in the *Klkb1*$^{-/-}$ liver (Figure S2C), indicating that *Klkb1* deficiency does not cause liver damage. Female *Klkb1*$^{-/-}$ hamsters also demonstrated marked increases in hepatic LDLR protein levels (Figure S2D) and significant decreases in plasma TC and triglyceride levels (Figure S2E and S2F). The cholesterol and triglyceride contents were decreased dramatically in very-low-density lipoprotein and LDL fractions in *Klkb1*$^{-/-}$ female hamsters (Figure S2G and S2H). Collectively, these results indicate that ablation of PK increases hepatic LDLR and thus the clearance of plasma LDL.

It is known that mice exhibit extremely low LDL-C, most likely as a result of high levels of LDLR, which is different from humans who have high LDL-C.[31,32] We therefore depleted *Klkb1* on the *Ldlr*$^{+/-}$ background to study the effects of PK on cholesterol metabolism in mice (Figure S3A). Consistent with the results in hamsters, the hepatic LDLR protein level was dramatically increased in *Klkb1*$^{-/-}$*Ldlr*$^{+/-}$ mice compared with *Klkb1*$^{+/+}$*Ldlr*$^{+/-}$ mice (Figure S3B). Circulating TC was decreased by 42% (Figure S3C), primarily in the LDL fractions (Figure S3D). Plasma triglyceride was slightly, but not significantly, decreased (Figure S3E). *Klkb1*$^{-/-}$*Ldlr*$^{+/-}$ mice had significantly increased TC and triglyceride levels in the liver (Figure S3F and S3G). These results suggest that more circulating lipids are internalized into the liver in the absence of PK. No liver damage was observed, as revealed by the aspartate aminotransferase and alanine aminotransferase assays (Figure S3H and S3I).

We further used adeno-associated virus–mediated shRNA to knock down PK expression in rat liver. Compared with those injected with control shRNA, rats receiving adeno-associated virus–sh*Klkb1* showed largely reduced PK protein in the plasma and significantly increased LDLR protein in the liver (Figure S4A and S4B). As a result, the serum TC was decreased by 19.8% (Figure S4C). Knockdown of *Klkb1* decreased cholesterol contents in LDL fractions (Figure S4D).

PK and PCSK9 Function Additively to Induce LDLR Degradation

We next investigated whether PK functioned additively with PCSK9 to degrade LDLR. As Figure 3A shows, although either PCSK9 or PK alone could decrease LDLR protein levels, a much stronger effect was observed when both were applied. We also injected evolocumab (Amgen; a human monoclonal antibody with higher affinity for human, cynomolgus monkey, and hamster PCSK9 but lower affinity for mouse PCSK9) into WT and *Klkb1*$^{-/-}$ hamsters. Those lacking *Klkb1* had higher hepatic LDLR levels than WT controls (Figure 3B). Evolocumab increased LDLR levels (Figure 3B) and decreased plasma lipid levels in both WT and *Klkb1*$^{-/-}$ hamsters (Figure 3C and 3D), despite the fact that the decrease of triglyceride in *Klkb1*$^{-/-}$ hamsters did not reach significance (Figure 3D). *Klkb1*$^{-/-}$ hamsters receiving evolocumab injections had the lowest very-low-density lipoprotein and LDL cholesterol content (Figure 3E). Together, these results suggest that PCSK9 and PK have additive effects on LDLR degradation and lipid lowering.

Anti-PK Neutralizing Antibody Increases Hepatic LDLR and Decreases Blood Cholesterol Without Affecting the Contact System

We next aimed to map the LDLR-binding sites of PK using a series of PK truncations (Figure 4A and Figure S5A). The full-length PK and truncations b (Δ21–390) and d (Δ21–390 and Δ631–638) were coprecipitated by LDLR and demonstrated a strong potency in lowering LDLR protein expression (Figure 4B), indicating that these variants contain the motifs responsible for LDLR interaction and degradation. In contrast, the PK trunca-

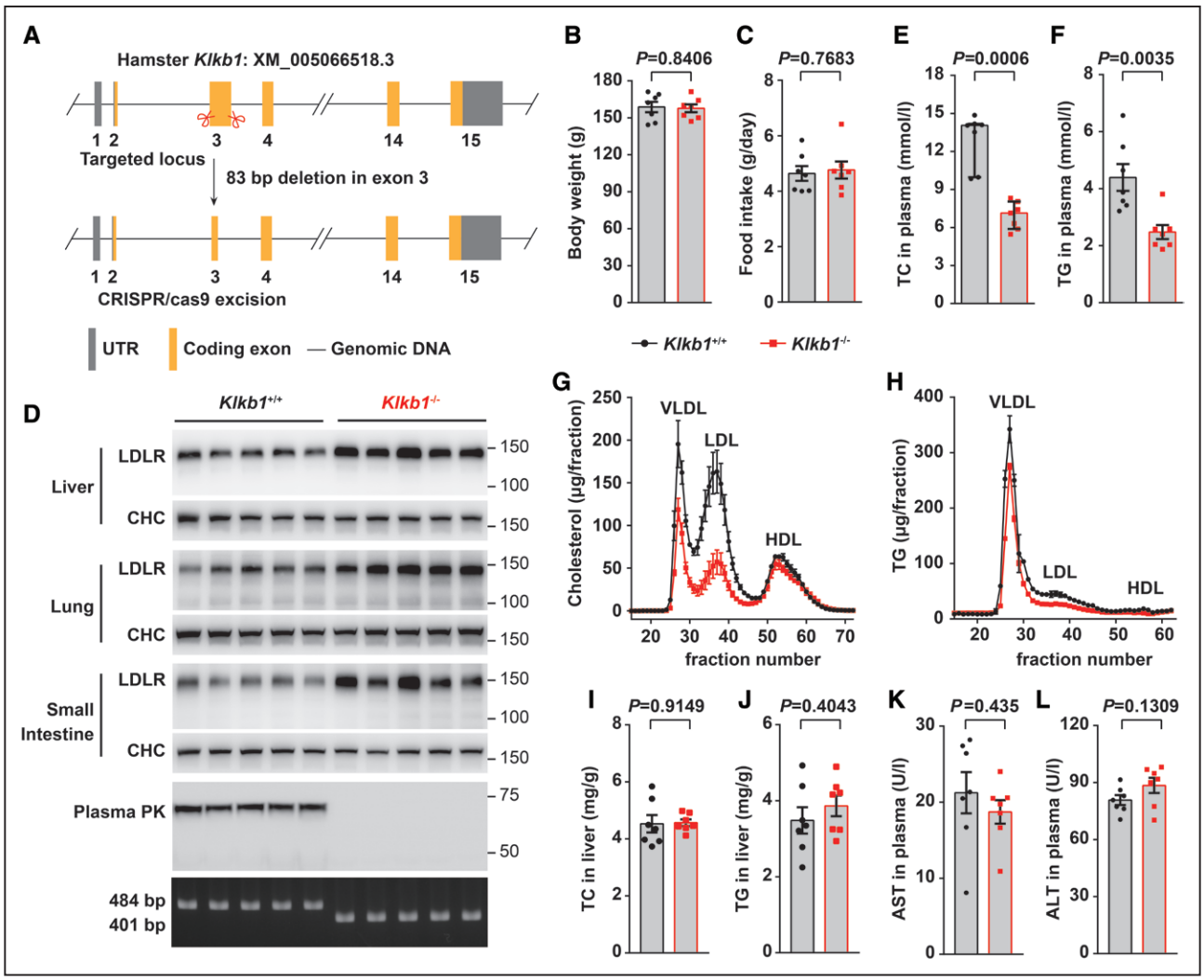

Figure 2. *Klkb1* deficiency increases hepatic LDLR protein level and lowers plasma cholesterol in hamsters.
A, Strategy of generation of *Klkb1* whole-body knockout (*Klkb1*$^{-/-}$) hamsters using the CRISPR/Cas9 system. **B** through **L**, Five-month-old male hamsters (n=7 per genotype) were fed a high-cholesterol diet (HCD) for 3 weeks. **B**, Body weight. **C**, Food intake. **D**, Immunoblotting analysis of low-density lipoprotein (LDL) receptor (LDLR) expression in different tissues and prekallikrein (PK) expression in plasma. **Bottom**, genotyping results. **E**, Total cholesterol (TC) in plasma. **F**, Triglyceride (TG) in plasma. **G**, Cholesterol content in very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) fractioned by fast protein liquid chromatography (FPLC). **H**, Triglyceride (TG) content in different lipoproteins fractioned by FPLC. **I**, TC in liver. **J**, TG in liver. **K**, Aspartate aminotransferase (AST) levels in plasma. **L**, Alanine aminotransferase (ALT) levels in plasma. Data are presented as mean±SEM in **B**, **C**, **F**, and **I** through **L**, with statistical significances determined with unpaired 2-tailed Student *t* test. Data are presented as median and interquartile range in **E**, with statistical significances determined with the Wilcoxon rank-sum test. CHC indicates clathrin heavy chain; and UTR, untranslated region.

tions a (Δ391–638) and c (Δ21–390 and Δ601–638) failed to bind LDLR or induce its degradation (Figure 4B). Deletion of 601 to 638 in PK (truncation e) abolished its activity of inducing LDLR degradation, whereas PK truncation i (Δ631–638) could still cause LDLR degradation (Figure S5B). Collectively, these results suggest that the region (a.a. 601–630) of PK is required for LDLR interaction and PK-induced LDLR degradation.

We then generated a rat monoclonal anti-PK antibody (called 2H5-IgG here) using the PK fragment (a.a. 601–630) as an antigen (Figure S5C). 2H5-IgG could specifically detect the PK protein in the serum of WT but not *Klkb1*$^{-/-}$ mice (Figure S5D). We purified LDLR extracellular domain and PK from the medium of HEK293T cells (Figure S5E) and mixed them with or without 2H5-IgG in vitro. The interaction between the LDLR extracellular domain and PK was effectively blocked by 2H5-IgG (Figure 4C). We also investigated whether 2H5-IgG could decrease plasma cholesterol levels. *Ldlr*$^{+/-}$ mice on an HCD received 3 intraperitoneal injections of 2H5-IgG or control IgG from day 7 and were euthanized 1 week later (Figure 4D). 2H5-IgG dramatically increased hepatic LDLR levels (Figure 4E) and reduced plasma TC levels (Figure 4G) in both very-low-density lipoprotein and LDL fractions (Figure 4F). The plasma triglyceride level was not changed by 2H5-IgG (Figure 4H). The TC and triglyceride levels in the liver showed a trend for yet not a statistically signifi-

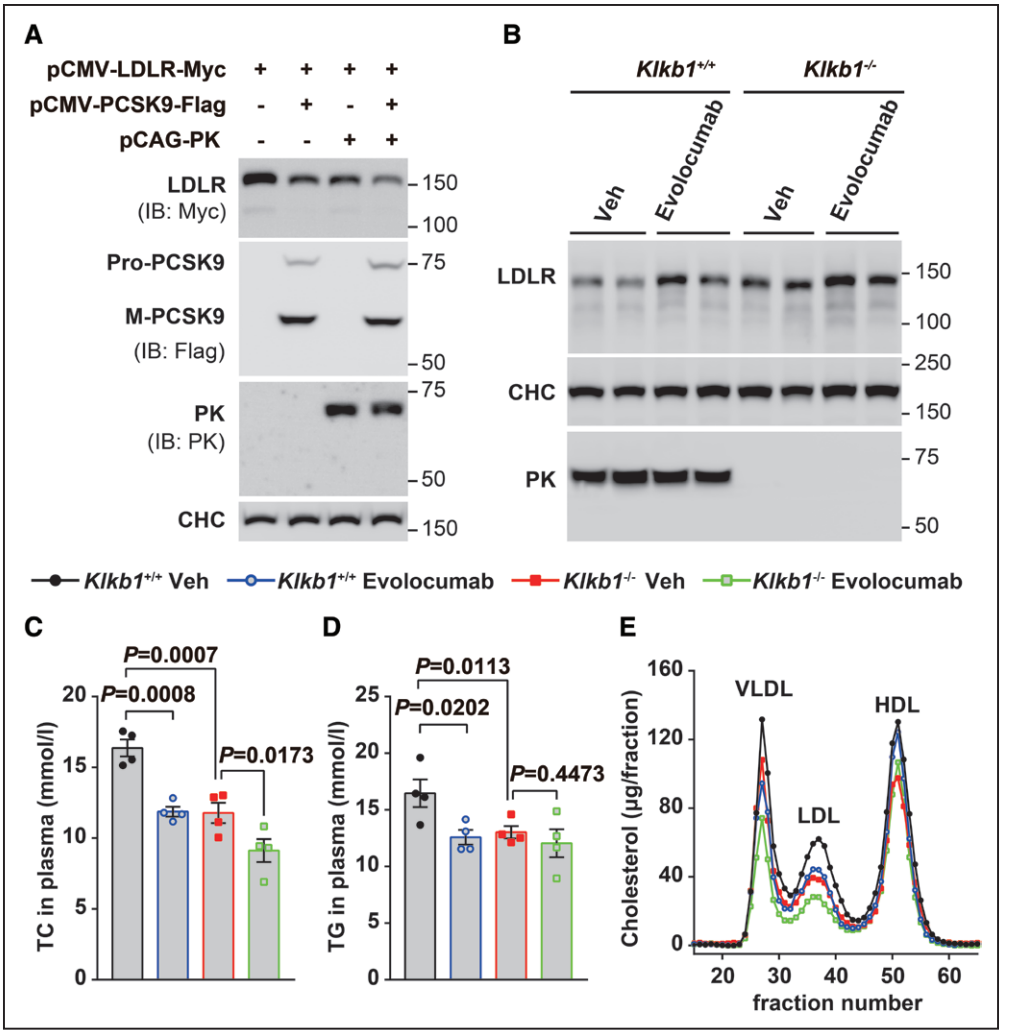

Figure 3. PK acts additively with PCSK9 on inducing LDLR degradation.
A, Huh7 cells were transfected with the indicated plasmids. After 48 hours, cells were harvested for immunoblotting. **B** through **E**, Seven-month-old male hamsters (n=4 per group) on a high-cholesterol diet were subcutaneously injected with 30 mg/kg evolocumab or equal amounts of vehicle (Veh) once a week for 2 weeks. **B**, Immunoblotting analysis of low-density lipoprotein (LDL) receptor (LDLR) expression in liver and prekallikrein (PK) expression in serum. **C**, Total cholesterol (TC) in plasma. **D**, Triglyceride (TG) in plasma. **E**, Cholesterol content in very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) fractioned by fast protein liquid chromatography. Data are presented as mean±SEM. Statistical significances were determined with 2-way ANOVA followed by the Fisher least significance difference test (**C** and **D**). CHC indicates clathrin heavy chain; CMV, Cytomegalovirus; M-PCSK9, mature proprotein convertase subtilisin/kexin 9; and Pro-PCSK9, precursor form of proprotein convertase subtilisin/kexin 9.

cant increase after 2H5 injections (Figure 4I and 4J). 2H5-IgG induced no liver toxicity as revealed by the aspartate aminotransferase and alanine aminotransferase assays (Figure 4K and 4L). Together, these data demonstrate that the anti-PK neutralizing antibody, by blocking the interaction between PK and LDLR, can stabilize hepatic LDLR protein, enhance LDL clearance by the liver, and lower circulating cholesterol levels.

We next tested whether 2H5-IgG could inhibit PKa activation of FXII. PK and FXII expression plasmids were transiently expressed in HEK293T cells, and the secreted proteins were purified from the medium (Figure S5E). The single chain revealed by SDS-PAGE under the reducing conditions corresponded to full-length PK and FXII, respectively (Figure S5E). It is known that PK is directly activated by purified FXII,[33] and the generated PKa in turn facilitates the production of FXIIa.[34] Consistently, incubation of purified PK protein with FXII induced the formation of cleaved PKa and FXIIa at the expense of the full-length ones (Figure S5F, lane 3). The PKa inhibitor avoralstat dramatically reduced FXIIa formation and increased the full-length forms of FXII and PK (Figure S5F, comparing lanes 6 and 7 with lane 3). The changes of PK light chain (lanes 3–5) were less dramatic because it was unstable. In contrast, 2H5-IgG had no effects on the mutual activation of PK and FXII (Figure S5F, comparing lanes 4 and 5 with lane 3) because neither the full-length nor the cleaved forms were obviously changed. These data demonstrate that 2H5 does not inhibit PKa activity.

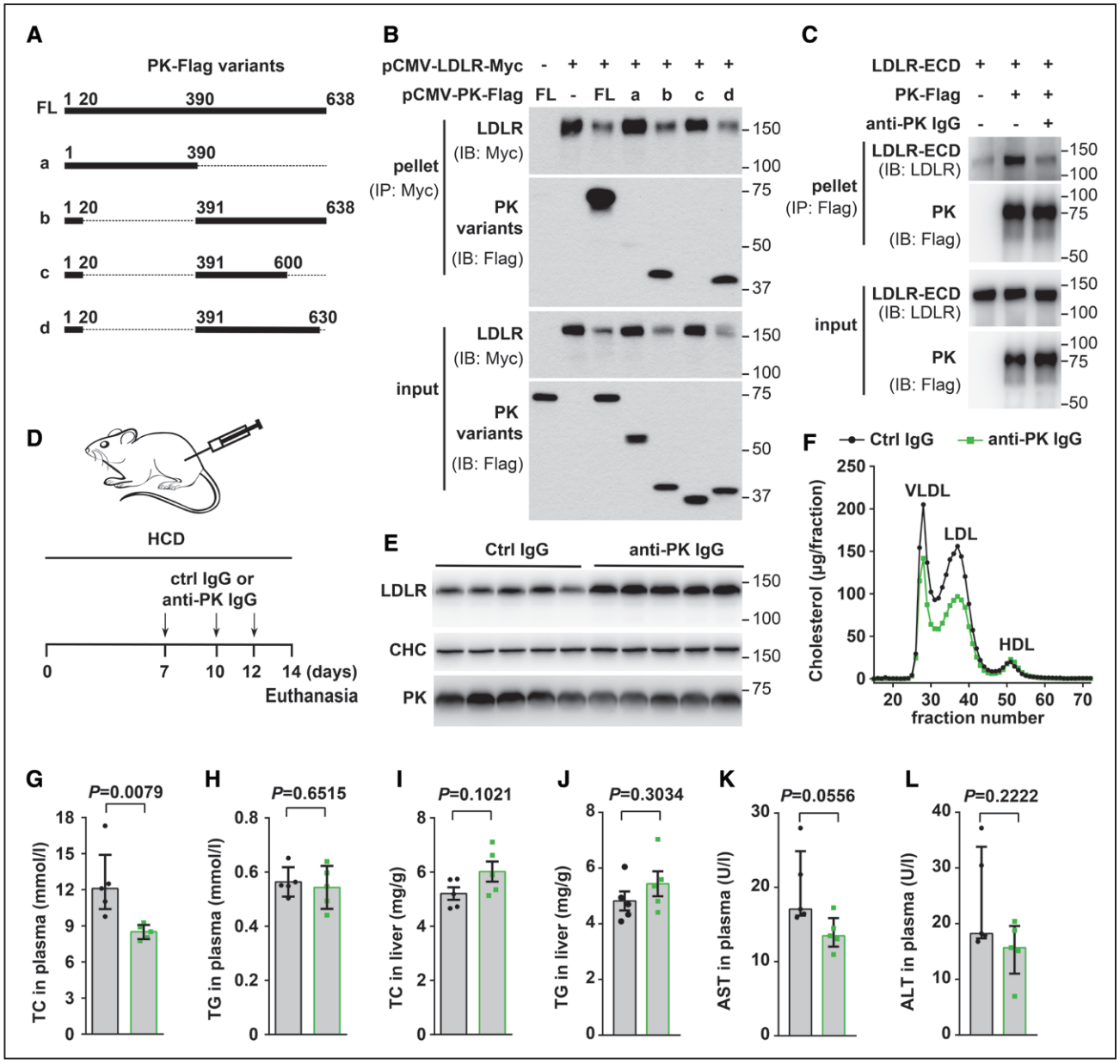

Figure 4. The anti-PK antibody blocks the PK-LDLR interaction, increases hepatic LDLR protein level, and lowers plasma cholesterol level.

A, Schematic of various prekallikrein (PK) truncations. **B**, The a.a. 601 to 630 of PK mediates its interaction with low-density lipoprotein (LDL) receptor (LDLR). Huh7 cells were transfected with the indicated plasmids. After 48 hours, cells were harvested, and LDLR was immunoprecipitated with the anti-MYC–coupled agarose followed by probing for the indicated antibodies. **C**, The anti-PK antibody blocks the interaction between PK and LDLR extracellular domain (ECD). Purified Flag-tagged PK protein (5 μg) was mixed with or without 5 μg anti-PK antibody on ice for 30 minutes and then incubated with 8 μg purified LDLR-ECD protein on ice for 1 additional hour. PK was immunoprecipitated with the anti-Flag–coupled agarose, and the eluents were probed for the indicated antibodies. **D** through **L**, Fourteen-week-old *Ldlr*$^{-/-}$ mice were fed a high-cholesterol diet (HCD) for 2 weeks and received a total of 3 intraperitoneal injections of 30 mg/kg control (Ctrl) or anti-PK immunoglobulin G (IgG; n=5 per genotype) at the indicated time points. **D**, Injection scheme. **E**, Immunoblotting analysis of LDLR expression in liver and PK expression in serum. **F**, Cholesterol content in very-low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) fractioned by fast protein liquid chromatography. **G**, Total cholesterol (TC) in plasma. **H**, Triglyceride (TG) in plasma. **I**, TC in liver. **J**, TG in liver. **K**, Aspartate aminotransferase (AST) levels in plasma. **L**, Alanine aminotransferase (ALT) levels in plasma. Data are presented as median and interquartile range in **G**, **K**, and **L**, with statistical significances determined with the Wilcoxon rank-sum test. Data are presented as mean±SEM in **H** through **J**, with statistical significances determined with unpaired 2-tailed Student *t* test. FL indicates full length; and CMV, Cytomegalovirus.

We also evaluated the effect of the anti-PK antibody on the contact system. The activated partial thromboplastin time (aPTT) is a measure of the intrinsic and common coagulation pathways. An increase in aPTT was detected in WT hamster plasma pretreated with avoralstat but not 2H5-IgG (Figure S5G, black bars). We next added the purified mouse PK protein into *Klkb1*$^{-/-}$ hamster plasma that showed elevated aPTT. The PK protein significantly

reduced aPTT, whereas avoralstat reverted aPTT to the level without treatment (Figure S5G, red bars). 2H5-IgG failed to block the lowering effect of PK on aPTT. These results demonstrate that 2H5 does not affect PKa protease activity on coagulation.

PK Deficiency Protects Against Atherosclerosis

To investigate whether depletion of *Klkb1* prevents the progression of atherosclerosis, we crossed *Klkb1*-deficient mice with *Apoe*-deficient mice to generate double knockouts (*Klkb1*$^{-/-}$*Apoe*$^{-/-}$). Mice were fed an HCD for 8 weeks. Compared with *Klkb1*$^{+/+}$*Apoe*$^{-/-}$ mice, *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ mice displayed higher hepatic LDLR protein, lower serum cholesterol, and similar serum triglyceride levels (Figure 5A–5C). The TC and triglyceride levels in the *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ livers were increased by ≈12% and 20%, respectively (Figure 5D and 5E). In line with reduced levels of circulating cholesterol, *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ mice had a decrease of ≈50% in atherosclerotic plaques along the whole aorta and ≈40% in lesion areas in the aortic root (Figure 5F–5I), suggesting that they were more resistant to atherosclerosis progression.

DISCUSSION

In this study, we found that the blood coagulation factor PK can induce LDLR degradation and prevent atherosclerosis progression. Genetic ablation or antibody neutralization of PK increases LDLR and decreases blood cholesterol level in multiple animal models. The effect of PK ablation is additive to the PCSK9 monoclonal antibody that has been approved to treat hyperlipidemia.[35] More important, reducing PK dramatically decreases atherosclerotic plaque formation. Given that atherosclerosis is expedited by high LDL levels and plaque rupture–induced thrombosis and that PK is involved in LDLR degradation, the contact system–initiated intrinsic pathway, and the kallikrein-kinin system, PK inhibition represents an effective and novel approach for treating ASCVD.

PK deficiency in humans is exceedingly rare, with only ≈60 patients being described in the United States and almost none reported in Chinese populations thus far.[36] However, in several cross-sectional studies, plasma PK has been shown to be associated with cardiovascular disease and its risk factors, including circulating lipid levels. Merlo et al[37] compared patients who survived myocardial infarction with healthy controls and found that a high level of plasma PK was a strong risk factor for myocardial infarction. Jaffa et al[38] analyzed patients with type 1 diabetes and showed that plasma PK level was significantly and positively associated with plasma TC and LDL-C in type 1 diabetes and that the group with the highest PK level had higher carotid intima-media thickness. Plasma PK level was also found to be positively correlated with circulating LDL-C in children.[39] Moreover, several *KLKB1* variants have been reported to be associated with coronary artery disease. Suhre et al[40] reported that rs4253252 of *KLKB1* had a weak association with coronary artery disease in the CARDIOGRAM study (Coronary Artery Disease Genome-Wide Replication and Meta-Analysis). Gittleman et al[41] also showed that the G allele of rs3733402 (*KLKB1*) significantly decreased the odds of having an angiographic coronary disease by 24%. Together, these results in different human subjects with various backgrounds are consistent with ours in young Chinese Han adults (Figure 1H–1K). Taking a step further, we dissected the mechanisms by which PK regulates serum lipid levels. We demonstrated that PK binds LDLR and induces its lysosomal degradation, therefore affecting circulating cholesterol levels and atherosclerosis progression.

PK also plays a vital role in kallikrein-kinin system. PKa digests high-molecular-weight kininogen to make bradykinin and cleaved kininogen, both of which are proinflammatory. Bradykinin activates the bradykinin receptors to increase the expression of adhesion receptors, whereas cleaved kininogen can induce the monocytes to release cytokines such as tumor necrosis factor-α and interleukin-1β.[42] In *Klkb1*-deficient mice, the generation of bradykinin was dramatically reduced, accompanied by a decrease in bradykinin receptor. The *Klkb1*-deficient mice also showed increased levels of Mas receptor and plasma prostacyclin, which increased the vasculoprotective factors sirtuin-1 and Kruppel-like factor 4 and decreased vascular tissue factor.[43] Thus, in addition to elevating LDLR and its effect on the contact activation system, ablation of PK alters the kallikrein-kinin system and improves vessel function, all of which contribute to atherosclerosis prevention.

Our results show that PK deficiency dramatically reduces atherosclerotic plaques in mice lacking apo E (Figure 5). These effects are indeed more pronounced than in some earlier studies on PCSK9 and may be attributable to diet differences. Specifically, Ason et al[44] showed that deletion of PCSK9 did not influence circulating cholesterol and the burden of atherosclerosis in *Apoe*-deficient mice under a chow diet for 8 and 24 weeks. Another study by Denis et al[45] reported that ablation of PCSK9 in *Apoe*-deficient mice on a chow diet for 6 months caused a trend of a decrease in plasma LDL-C and upregulation of liver LDLR. PCSK9 deficiency was also found to protect atherosclerosis development and to decrease plaque cholesterol ester. Notably, our *Apoe*$^{-/-}$ and *Klkb1*$^{-/-}$*Apoe*$^{-/-}$ mice were fed an HCD (40% kcal from fat, 1.25% cholesterol, and 0.5% sodium cholate) for 8 weeks. It is possible that different diets (chow diet versus HCD) may cause different results. For example, cholate can increase the apo B_{100}/apo B_{48} ratio through inhibiting apo B mRNA editing.[46] This results in increased production of apo B_{100}–containing lipoproteins that have higher affinity for LDLR than for apo B_{48}.

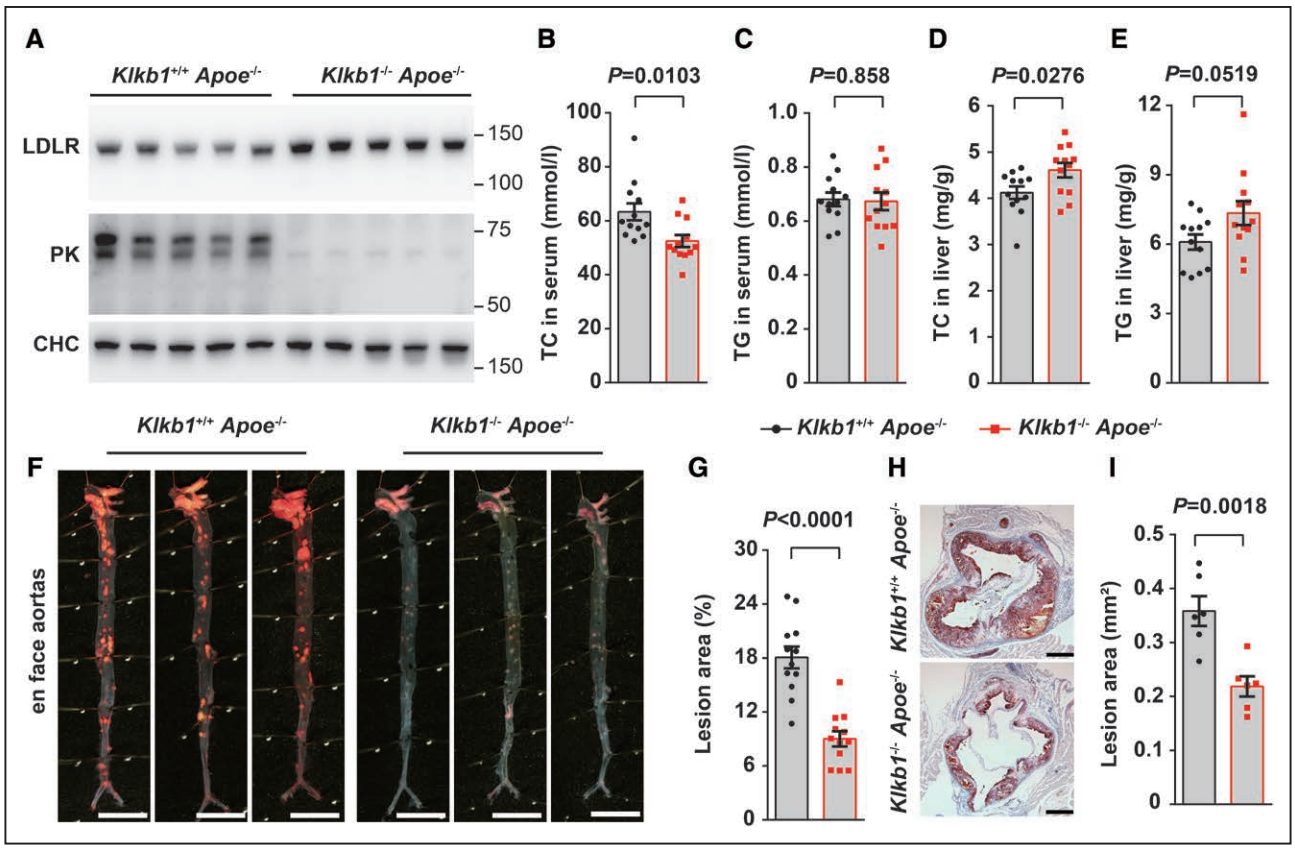

Figure 5. *Klkb1* deficiency protects against atherosclerosis.
Eight-week-old male *Klkb1*$^{+/+}$*Apoe*$^{-/-}$ *and Klkb1*$^{-/-}$*Apoe*$^{-/-}$ mice (n=12 per genotype) were fed a high-cholesterol diet for 8 weeks. **A**, Immunoblotting analysis of low-density lipoprotein receptor (LDLR) and prekallikrein (PK) expression in liver. **B**, Total cholesterol (TC) in serum. **C**, Triglyceride (TG) in serum. **D**, TC in liver. **E**, TG in liver. **F**, En face lipid staining of aortas. Scale bars, 5 mm. **G**, Quantification of atherosclerotic lesion areas (n=12 mice per genotype). **H**, Hematoxylin- and lipid-stained aortic root sections. Scale bars, 200 μm. **I**, Quantification of lesion areas in aortic root sections (n=6 mice per genotype). All data are presented as mean±SEM. Statistical significances were determined with unpaired 2-tailed Student *t* test. CHC indicates clathrin heavy chain.

The safety of PK inhibitors has been evaluated in patients with hereditary angioedema, a disease that results from deficiency of C1-esterase inhibitor and thus dysregulation of the kallikrein-kinin system with uncontrolled PKa and overproduction of bradykinin, which lead to recurrent angioedema attacks. Inhibition of PKa is an effective approach to treat hereditary angioedema. Berotralstat is a small-molecule PKa inhibitor and can significantly reduce the frequency of hereditary angioedema attacks. The most frequent side effects of berotralstat include vomiting, diarrhea, headache, and back pain.[47] Lanadelumab is a human monoclonal antibody against PKa. It is well tolerated up to a dose of 3.0 mg/kg, with no treatment-emergent adverse events observed in healthy subjects.[48] IONIS-PPK-LRx is an antisense oligonucleotide targeted to liver PK mRNA. After injections of 400 mg IONIS-PPK-LRx, a 94% reduction of PK in plasma was achieved with no serious adverse events reported.[49] It is important to note that none of these PKa inhibitors increase the bleeding risk or interfere the hemostasis. In our study, deletion of PK lowered circulating cholesterol and drastically inhibited the progress of atherosclerosis without causing any obvious adverse effects in multiple rodent models. Moreover, our anti-PK neutralizing antibody against the C terminus of PK interferes with its binding to LDLR but not the enzymatic activity (Figure 4 and Figure S5E and S5G). We therefore believe that PK is an attractive target for ASCVD. The long-term safety of PK inhibition remains to be evaluated.

CONCLUSIONS

Our study demonstrates that PK is a potent LDLR degrader. Given that it is dispensable for hemostasis, targeting PK offers a new and potentially safe therapeutic approach to treat hyperlipidemia, ASCVD, and other thrombotic diseases.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Expanded Materials and Methods
Table S1
Figures S1–S5

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