SUPPLEMENTAL MATERIAL

Ablation of plasma prekallikrein decreases LDL cholesterol by stabilizing LDL receptor and protects against atherosclerosis

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Expanded Materials and Methods

Supplemental Table

Supplemental Figures

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Plasmids

We constructed the plasmids by standard molecular cloning techniques. The coding sequence (without stop codon) of *Klkb1*(NM_008455.3), *Ldlr* (NM_010700.3), *PCSK9* (NM_174936.4), *KPNB1*(NM_001276453.2), *CSE1L* (NM_001316.4) was amplified by polymerase chain reaction (PCR) and recombined into expression vector pCMV-14-3×Flag. pCMV-LDLR-MYC encodes mouse LDLR followed by 5×MYC epitope. pCMV-LDLR-ECD-HIS encodes mouse LDLR 1-788 animal acids followed by 6×HIS epitope. pCMV-FXII-MYC-HIS encodes mouse FXII followed by 5×MYC and 6×HIS epitope. pLVX-Tet-on-PK encodes mouse full-length PK. The PK truncated variants were generated with Quick-Change mutagenesis.

Doxycycline (Dox)-induced expression of PK in Huh7 cells

Lentiviral vector particles of Tet-On inducible PK-Blast were produced in HEK293T cells, and packaged viruses were used to infect Huh-7 cells for 16 h. Cells were exposed to 30 μg/ml blasticidin for 72 h to generate Huh7/Tet-on-PK cells. Huh7/Tet-on-PK cells were set up (3×10^5 cells per well) in medium A. Twenty-four hours later, 2 μg/ml Dox was added. Cells were harvested for immunoblotting analysis after 24 hours.

Immunoblotting

Total proteins in treated cells or tissues were extracted with ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM $MgCl_2$, 1.5% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with protease inhibitor cocktail, 1 mM phenylmethyl sulfonyl fluoride, 10 μM MG132, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 25 μg/ml ALLN and 1 mM dithiothreitol. The concentrations of protein lysates were quantified by BCA protein assay kit (Thermo Fisher Scientific). Samples were mixed with 4 × loading buffer (150 mM Tris-HCl, pH 6.8, 12% SDS, 30% glycerol, 6% 2-mercaptoethanol and 0.02% bromophenol blue) and boiled at 95 °C for 10 min. Forty micrograms of proteins were separated by SDS-PAGE and transferred to PVDF membranes. Blots were blocked with 5% non-fat milk in TBST buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 0.075% Tween-20) at room temperature for 1 h, and then incubated with indicated primary antibodies in TBST buffer containing 5% BSA at 4 °C for 12-16 h. After washing in TBST buffer, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (0.1

μg/ml) in TBST buffer supplemented with 5% non-fat milk at room temperature for 1-2 h followed by at least 3 washes with TBST. Pierce ECL Plus western blotting substrate (Thermo Fisher Scientific) was used to visualized the proteins. Primary antibodies used for immunoblotting were as follows: monoclonal antibody (clone M2) against Flag tag (Sigma, F1804, 0.5 μg/ml); mouse monoclonal antibodies (clone 9E10) against MYC tag (0.5 μg/ml) were prepared from hybridomas (ATCC); rabbit polyclonal antibodies against LDLR (0.5 μg/ml) were prepared in our laboratory; goat polyclonal antibody against PK (R&D, AF2498, 0.5 μg/ml); mouse monoclonal antibody (Clone 23) against CHC (BD Transduction Laboratories, 610500, 0.2 μg/ml); mouse monoclonal antibody (clone AC-15) against β-actin (Sigma, A1978, 0.1 μg/ml); rabbit polyclonal antibodies against GAPDH (Proteintech, 10494, 0.1 μg/ml). Horseradish peroxidase-conjugated goat anti-mouse (115-035-003, 0.1 μg/ml) and anti-rabbit (111-035-144, 0.1 μg/ml) secondary antibodies were from Jackson Immuno Research Laboratories. Mouse anti-goat IgG-HRP (sc-2354, 0.1 μg/ml) was from Santa Cruz Biotechnology. The rat monoclonal antibody (clone 2H5, 1 μg/ml) against PK were prepared from DIA-AN (Wuhan, China), and polypeptides GCARKDQPGVYTKVSEYMDWILEKTQSSDV were used as the antigen.

Immunoprecipitation

Cells were lysed in 1 ml lysis buffer on ice for 20 min. After vortexing for 15 s, lysates were subjected to centrifugation at 4 °C for 10 min at 13,200 rpm. Ninety microliters of supernatants were mixed with 30 μl 4 × loading buffer (150 mM Tris-HCl, pH 6.8, 12% SDS, 30% glycerol, 6% 2-mercaptoethanol and 0.02% bromophenol blue) as input sample. Eight-hundred microliters of supernatants were incubated with 30 μl anti-MYC beads or anti-Flag beads at 4 °C for 2-4 h. After washing in lysis buffer for 5 times at 4 °C, beads were boiled with 120 μl 1 × loading buffer at 95 °C for 10 min, and then centrifugated at 13,200 rpm for 5 min. The supernatants were used as pellet samples. Twenty microliters of samples were analyzed by immunoblotting.

Purification of recombinant proteins

HEK293T cells were set up (8×10^6 cells per 15-cm dish) in medium A and transfected with 20 μg DNA (plasmid expressing Flag-tagged PK or Flag-tagged PCSK9 or HIS-tagged LDLR-ECD_{1-788}). Twenty-four hours later, the culture medium was replaced with DMEM and incubated for 24 h. Cultured medium was collected and centrifugated at 2,000 g for 10 min. Supernatants (containing PK or PCSK9) were slowly drained through the anti-Flag M2 beads packed column at 4 °C. After extensive washes with PBS, the bound proteins were eluted with Flag peptide.

Supernatants containing LDLR-HIS were concentrated with Centricon filters (50 KDa-cut-off, Millipore) and the solution was replaced with PBS containing 1 mM $CaCl_2$. HIS-tagged proteins were purified by nickel-nitrilotriacetic acid agarose according to the manufacturer's instructions (Qiagen). The solution buffer of proteins was replaced with PBS containing 1 mM $CaCl_2$ and concentrated using 10 kDa-cutoff ultrafiltration filter. The final protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) and protein purity was monitored with Coomassie Brilliant Blue R-250 staining (Bio-Rad). The purified proteins were stored at -80 °C in small aliquots.

Degradation of LDLR by PK and PCSK9

Huh-7 cells were set up (5×10^5 cells per well) in medium A. Twenty-four hours later, cells were washed with PBS and incubated with cholesterol-depleted medium for 16 h. Cells were washed twice with PBS and incubated with DMEM supplemented with 10 μg/ml leupeptin and indicated amount of proteins for 8 h. Cultured medium was collected and centrifugated 2,000 g for 5 min. Supernatants were mixed with 4 × loading buffer and boiled at 95 °C for 10 min. Cells were harvested in RIPA lysis buffer. The medium and whole-cell lysate proteins were analysis by immunoblotting.

RNA interference

Duplexes of siRNA were synthesized by RIBOBIO (Guangzhou, China). The sequence of siRNA targeting human *IDOL* was 5'-GACTTTAGCCCAATTAATA -3'. Two sequences of siRNA targeting human *KLKB1* were 5'-GTGTAAGTGTTTCTTAAGA -3' and 5'-CCCAGAAGACTGTAAGGAA-3', respectively. The sequence of control siRNA was 5'-TTCTCCGAACGTGTCACGTTT-3'. Huh7 cells were set up (3×10^5 cells per well) in medium A and transfected with siRNA using lipofectamine RNAiMAX (Invitrogen) as described by the manufacturer. Cells were harvested 48 h later.

Preparation of adeno-associated virus (AAV)

Three types of DNA vectors (AAV-U6-shRNA vector, delta F6 helper plasmid, Rev cap 2/9 vector) were transfected into HEK293T cells to generate viral particles. After 60 h of transfection, HEK293T cells were collected and suspended in lysis buffer (150 mM NaCl, 20 mM Tris, pH 8.0), followed by three cycles of freezing-thawing to release virus. The nucleic acid fragments were digested with benzonase (Sigma, E8263). The viral particles in lysate were separated with density gradient centrifugation (17%-25%-40%-60% gradient iodixanol solutions) at

53,000 rpm for 160 min at 14 °C. The viral fraction was harvested from the 40% layer, and the solution buffer was replaced to PBS with filter column (MWCO 100 kDa, Millipore). The viral titer was determined by quantitative real-time PCR (qPCR).

LDL uptake assay

Human LDL was isolated from normal human plasma by sequential density gradient ultracentrifugation in our laboratory. To prepare fluorescent 1,1'-dioactadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled LDL (Invitrogen), 12 μl of DiI (6 mg/ ml in dimethyl sulfoxide) was added into 1 ml of 0.5 mg/ml LDL in 150 mM NaCl (pH 7.2). After vortexing, the DiI-LDL mixture was incubated at 37 °C for 3 h and filtered using the 0.45 μm filter.

SiRNA-treated Huh7 cells on glass coverslips were incubated in cholesterol-depleted medium at 37 °C for 12 h. Cells were washed twice with PBS and refed with pre-warmed DMEM containing 10 μg/ml DiI-LDL and incubated at 37 °C for 1 h. Cells were washed three times with ice-cold PBS and then fixed with 4% paraformaldehyde in PBS for 25 min in the dark. The cell fluorescent signal was examined by Leica SP8 confocal microscopy.

Blood collection

Animals were anaesthetized with isoflurane, and the blood samples were collected by retro-orbital bleeding or taken from heart. Blood was centrifuged at 2,000 g for 30 min at 4 °C to obtain the plasma or serum.

Fast protein liquid chromatography (FPLC)

Equal volume samples were loaded to Superdex 75 10/300 GL (GE Healthcare) column and separated by FPLC (AKTA, GE Healthcare). Fractions were eluted with PBS containing 5 mM EDTA at a constant rate of 0.3 ml/min, and collected with 300 μl per tube. Forty microliters of fractions were used to determine the lipids levels in each fraction. The cholesterol or triglyceride distribution curves of lipoproteins were generated by GraphPad Prism 7 software.

Blood and liver chemistry

The lipids in liver were extracted with 1.2 ml of chloroform and methanol mixed at the ratio of 2:1. The organic phase was dried on 50 °C incubator in fume hood, and lipids were dissolved in ethanol. The equal volume of plasma/serum samples or liver extracts were mixed with 200 μl of working buffer prepared with total cholesterol and triglyceride kit (Kehua Bioengineering) in the 96-well plate. The mixtures were

incubated at 37 °C for 5 min, then the absorbance was measured at 550 nm using a microplate reader (Molecular Devices, spectra MAX 190). The concentration of lipids was determined by calculating the absorbance value of the standard and the sample. The AST and ALT in plasma were determined with reagent kits (C009-2-1 and C010-2-1, NJJCBIO) according to manufacturer's instructions.

Activated partial thromboplastin time (aPTT) analysis

Whole blood collected by right ventricular puncture from anaesthetized animals with plastic syringe contenting anticoagulant sodium citrate (0.109 M). And the volume ratio of blood with anticoagulant was 9:1. The whole blood specimen was spun at 2,000 g for 20 min at 4 °C to obtain the platelet-poor plasma. The activated partial thromboplastin time was measured using the aPTT reagent kit (Shanghai, SUNBIO, 2010) according to the manufacturer's instructions.

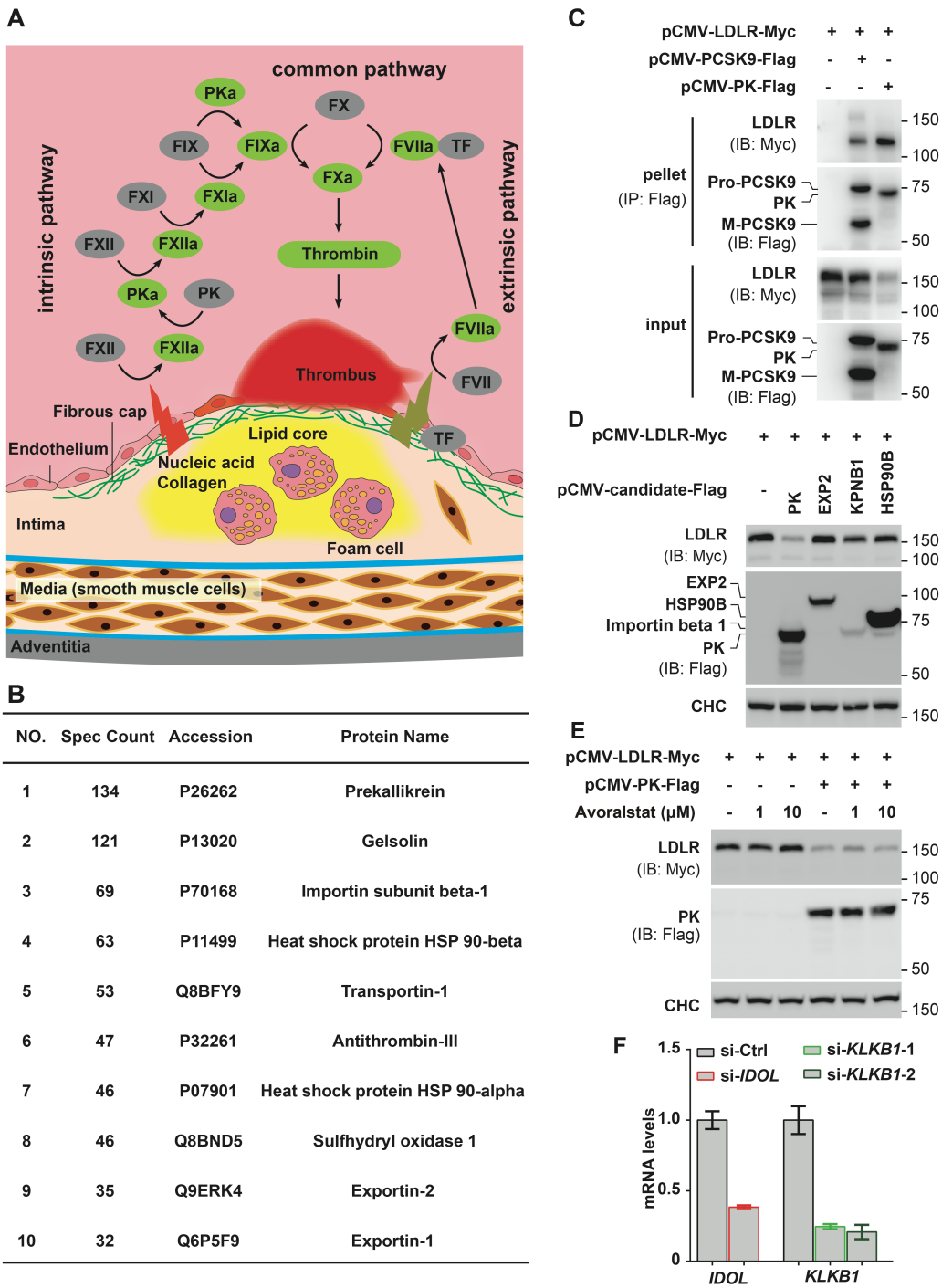
Quantitative real-time PCR

Total RNA was extracted from cells or liver tissue with Trizol Reagent (Invitrogen). Two microliters of total RNA were mixed with 0.1 μg of random hexamers primer to synthesis cDNA using M-MLV Reverse Transcriptase (Promega) in 25 μl of reaction volume. qPCR was administered by the Hieff qPCR SYBR Green Master Mix (Yeasen Biotech) and analyzed on a Bio-Rad CFX96 apparatus (Bio-Rad).

	Less than median group（n=98）	More than median group（n=100）	P value
Demographic characteristics			
Age (years)	20 [20-21]	20.5 [20-21]	0.428
Female sex (%)	57 (58.2%)	55 (55%)	0.653
Height (cm)	167 [162-174]	169 [163-175]	0.532
Weight (kg)	58 [51.45-66.15]	61 [53-72]	0.061
Blood routine examination			
Platelet count (10^9/l)	255.95±50.10	267.25±52.11	0.122
Red blood cell count (10^{12}/l)	4.88±0.53	4.91±0.60	0.775
White blood cell count (10^9/l)	6.55±1.40	6.62±1.59	0.715
Hepatic and renal function			
Alanine aminotransferase (U/l)	15.1 [12.36-18.93]	16.5 [12.19-25.08]	0.141
Aspartate aminotransferase (U/l)	18.35 [15.77-20.75]	17.19 [15.81-21.00]	0.747
Creatinine (μmol/l)	77.85±12.78	78.81±13.13	0.605
Urea (μmol/l)	4.33 [3.61-5.02]	4.39 [3.88-5.10]	0.289
Blood glucose and lipid index			
Total cholesterol (mmol/l)	3.77±0.56	4.08±0.61	0.001
HDL (mmol/l)	1.46 [1.24-1.61]	1.41 [1.16-1.60]	0.368
LDL (mmol/l)	2.02±0.52	2.32±0.52	0.001
TG (mmol/l)	0.71 [0.54-0.96]	0.91 [0.69-1.32]	0.001
Fasting blood-glucose (mmol/l)	5.03 [4.81-5.24]	5.12 [4.84-5.37]	0.363
Blood pressure			
Systolic pressure (mmHg)	110 [100-120]	110 [100-120]	0.076
Diastolic pressure (mmHg)	60 [60-70]	60 [60-70]	0.510

Supplemental Table I. Basic demographic information of 198 study participants.

SPSS24.0 was used for data analysis, and the Kolmogorov-Smirnov method or Shapiro-Wilk normality test was used to test the normality of the measurement data. The normally distributed measurement data are expressed as the mean ± standard deviation, and the two independent samples t test was used for comparisons between the two groups. The non-normally distributed measurement data are expressed as the median [interquartile range], and the Wilcoxon rank-sum test was used for comparisons between groups. Counting data are expressed as the number of cases (percentage). Chi-square test or Fisher's exact test was applied. PK median was 53.51 (μg/ml); HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; TG: triglyceride.



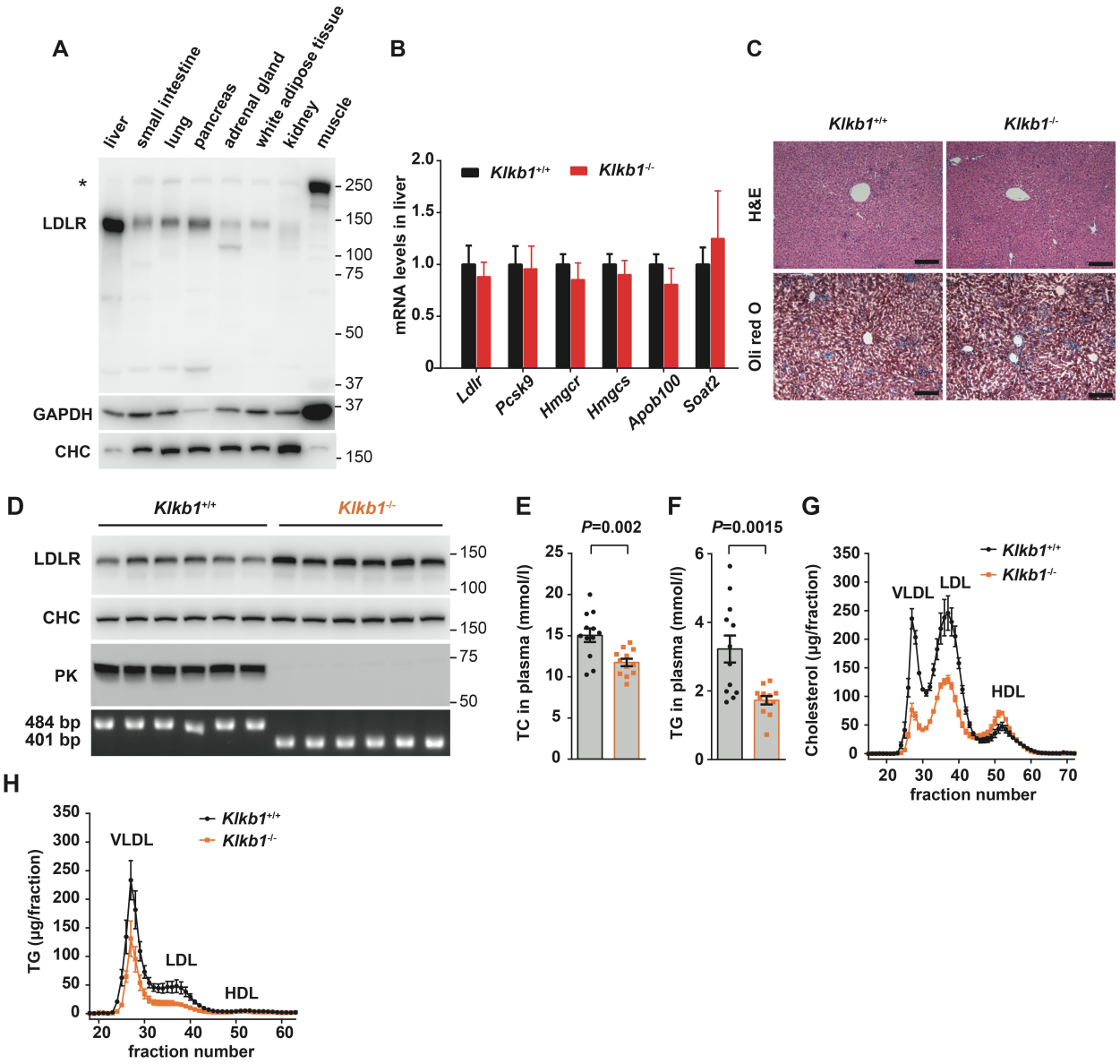
B

NO.	Spec Count	Accession	Protein Name
1	134	P26262	Prekallikrein
2	121	P13020	Gelsolin
3	69	P70168	Importin subunit beta-1
4	63	P11499	Heat shock protein HSP 90-beta
5	53	Q8BFY9	Transportin-1
6	47	P32261	Antithrombin-III
7	46	P07901	Heat shock protein HSP 90-alpha
8	46	Q8BND5	Sulfhydryl oxidase 1
9	35	Q9ERK4	Exportin-2
10	32	Q6P5F9	Exportin-1

Supplemental Figure I. Identification of PK as a binding partner for LDLR.

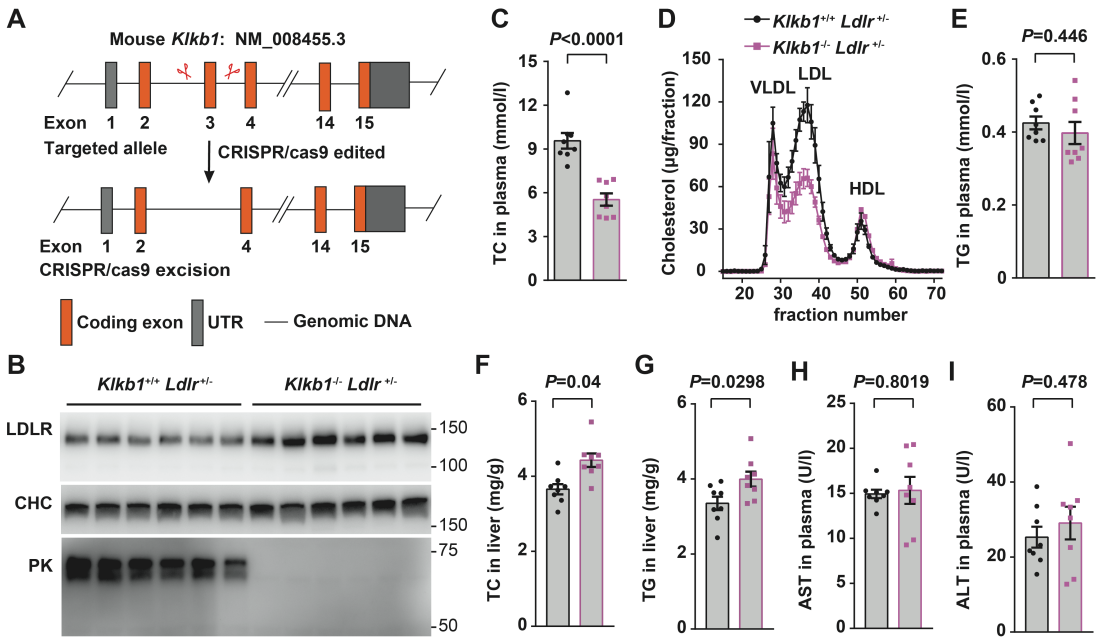
(A) Cartoon showing the role of PK in coagulation. The coagulation cascade is divided into three parts: intrinsic, extrinsic and common pathways. In the intrinsic pathway, nucleic acids and other negatively charged surface activate FXII. The activated FXII (FXIIa) cleaves PK to generate PKa. PKa in turn digests FXII to FXIIa and amplifies the cascade. FXIIa then cleaves FXI to produce FXIa, and FXIa next converts FIX to FIXa. PKa can also directly cleave FIX to generate FIXa. FIXa then cleaves FX to FXa. In the extrinsic pathway, the exposed tissue factor (TF) induces the conversion of FVII to FVIIa, which then forms a complex with TF to cleave FX to FXa. In the common

pathway, FXa cleaves prothrombin to thrombin that finally generates a fibrin and platelet cross-linked clot. a, activated. **(B)** Mass spectrometry analysis of LDLR binding proteins. The LDLR-Flag recombinant protein was purified from HEK293T cells and incubated with the anti-Flag coupled agarose. Mouse plasma deleted of lipoproteins were then added. After extensive washes, the bound proteins were eluted with Flag peptides and analyzed using mass spectrometry. Top ten candidates are listed. **(C)** Co-immunoprecipitation of LDLR with PK and PCSK9. Huh7 cells were transfected with the indicated plasmids. After 48 h, cells were harvested and LDLR was immunoprecipitated with the anti-Flag coupled agarose followed by probing for the indicated antibodies. M-PCSK9, the mature form of PCSK9; Pro-PCSK9, the precursor form of PCSK9. **(D)** Effects of different proteins on the LDLR level. Huh7 cells were transfected with the indicated plasmids for 48 h. Cells were harvested for immunoblotting. EXP2, exportin-2; KPNB1, gene name for importin subunit beta-1; HSP90B, heat shock protein 90-beta. CHC, clathrin heavy chain. **(E)** The enzymatic activity is not required for PK to regulate the LDLR degradation. Huh-7 cells were transfected with the indicated plasmids. After 24 h, cells were incubated with the PKa inhibitor Avoralstat at the indicated concentrations for 24 h and harvested for immunoblotting. **(F)** Knockdown efficiency of *IDOL* and *KLKB1*. Huh7 cells were transfected with control (Ctrl) siRNA or siRNAs targeting *KLKB1* (si*KLKB1*-1 and si*KLKB1*-2) or *IDOL* for 48 h. Cells were harvested for quantitative real-time PCR (qPCR) analysis**.** Data are normalized to control cells and presented as mean ± s.e.m. Related to Figure **1E**.



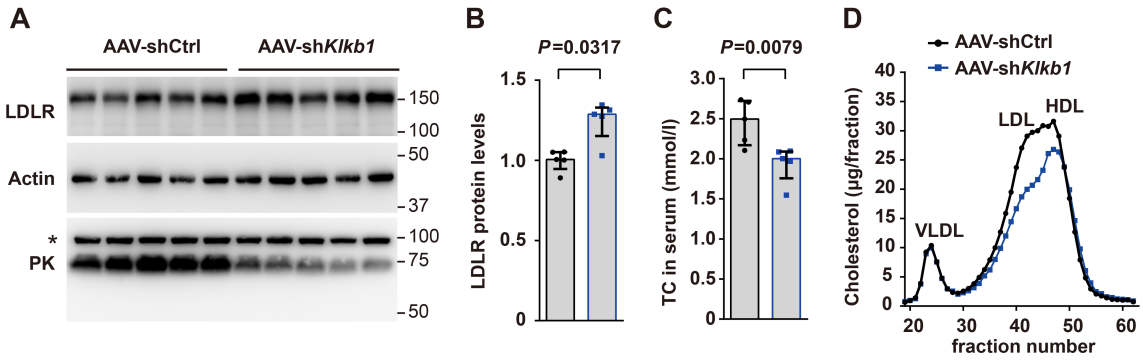
Supplemental Figure II. Characterization of *Klkb1*$^{-/-}$ hamsters.

(A-C) Five-month-old male hamsters on a HCD were analyzed. **(A)** Immunoblotting analysis of LDLR expression in different tissues of wild-type hamsters. Asterisk indicates the non-specific band. **(B)** The mRNA levels of different genes in the liver of *Klkb1*$^{+/+}$ and *Klkb1*$^{-/-}$ hamsters analyzed by qPCR. **(C)** Haematoxylin and eosin (H&E) and Oil red O staining of liver sections from *Klkb1*$^{+/+}$ and *Klkb1*$^{-/-}$ hamsters. Scale bars, 200 µm. (**D-H**) Five-month-old female hamsters (n=12 per genotype) were fed a HCD for 4 weeks. (**D**) Immunoblotting analysis of LDLR in the liver and PK in the plasma. Bottom showing the genotyping results. (**E**) Total cholesterol (TC) in plasma. **(F)** Triglyceride (TG) in plasma. **(G)** Cholesterol content in very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) fractioned by fast protein liquid chromatography (FPLC). **(H)** TG content in different lipoproteins fractioned by FPLC. Data in (**B, E, F**) are presented as mean ± s.e.m. Statistical significances were determined using unpaired two-tailed Student's t-test (**E, F**).



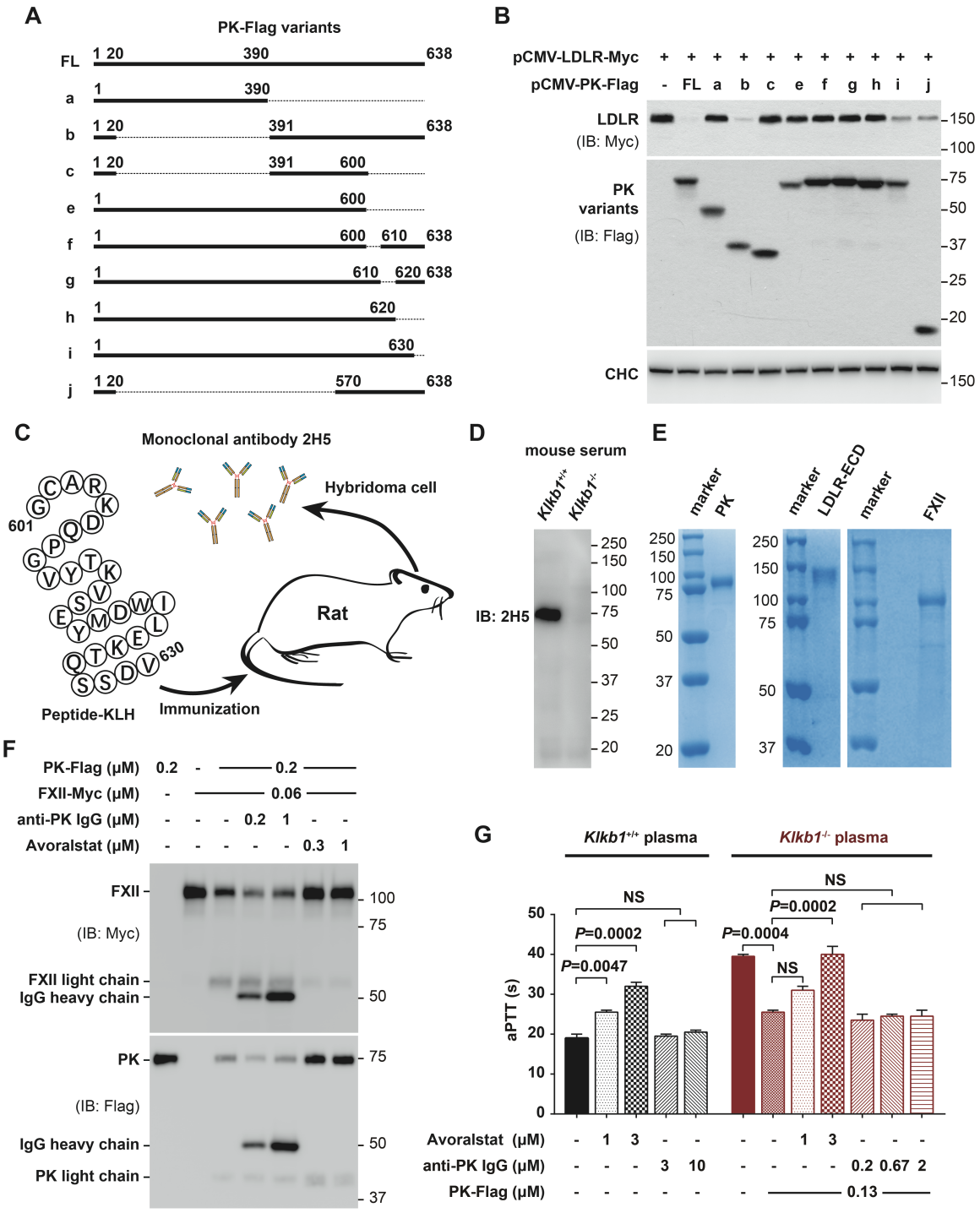
Supplemental Figure III. *Klkb1* deficiency increases hepatic LDLR protein levels and lowers circulating cholesterol in *Ldlr* $^{+/-}$ mice.

(A) Strategy of generation of *Klkb1* whole-body knockout mice using the CRISPR/Cas9 system. **(B-J)** Six-month-old *Klkb1*$^{+/+}$*Ldlr*$^{+/-}$ and *Klkb1*$^{-/-}$*Lldl*$^{+/-}$ male mice (n=8 per group) were fed a HCD for 4 weeks. **(B)** Immunoblotting analysis of LDLR, CHC and PK in the liver. **(C)** TC in plasma. **(D)** Cholesterol content in different lipoproteins fractioned by FPLC. **(E)** TG in plasma. **(F)** TC in liver. **(G)** TG in liver. **(H)** Aspartate aminotransferase (AST) levels in plasma. (**I**) Alanine aminotransferase (ALT) levels in plasma. Data in (**C, E-I**) are presented as mean ± s.e.m, with statistical significances determined using unpaired two-tailed Student's t test.



Supplemental Figure IV. Knockdown of *Klkb1* using AAV-mediated shRNA system lowers blood cholesterol in rats.

Eight-week-old male Sprague-Dawley rats on a chow diet were intravenously injected with 3×10^{12} viral genomes (vg) of AAV2/9-sh-control or sh-*Klkb1* (n=5 per group) and sacrificed 2 weeks later. **(A)** Immunoblotting analysis of LDLR and actin in the liver and PK in the plasma. Asterisk indicates non-specific bands. **(B)** Quantification of LDLR protein levels in **(A)**. **(C)** TC in serum. **(D)** Cholesterol contents in different lipoproteins fractioned by FPLC. Data in (**B, C**) are presented as median and interquartile range, with statistical significances determined using Wilcoxon rank-sum test.



Supplemental Figure V. Generation and evaluation of the rat anti-PK monoclonal antibody.

(A) Schematic of various PK truncations. FL, full-length. **(B)** The effects of PK variants on LDLR levels. Huh7 cells were transfected with the indicated plasmids for 48 h and harvested for immunoblotting. **(C)** Schematic depicting the generation of rat anti-PK monoclonal antibody (2H5). **(D)** Immunoblot showing the specificity of 2H5 using serum from WT and *Klkb1*$^{-/-}$ mice. **(E)** Coomassie staining of the recombinant PK and LDLR-ECD proteins. Full-length PK with a Flag tag, LDLR-ECD (1-788) with a HIS

tag and FXII with a MYC-HIS tag were transiently expressed in HEK293T cells, respectively. Cell medium was harvested and proteins were purified using the anti-Flag agarose or nickel beads. After boiling, samples were analyzed by SDS-PAGE gel and stained with Coomassie brilliant blue R-250. **(F)** 2H5 does not inhibit PK activation on FXII. Purified PK protein was pre-incubated with 2H5 or Avoralstat as indicated on ice for 30 min. Purified FXII was then added as indicated and incubated at 37 °C for 30 min. After boiling, samples were analyzed by immunoblotting under reducing conditions. **(G)** 2H5 does not affect aPTT. In the left part (black bars), plasma from 9-month-old male wild-type hamsters (n=2 per group) were pre-incubated with Avoralstat and 2H5 as indicated on ice for 30 min, and then subjected to the aPTT assay. In the right part (red bars), plasma from 9-month-old male *Klkb1*$^{-/-}$ hamsters (n=2 per group) were incubated with recombinant PK protein, Avoralstat and 2H5 as indicated on ice for 30 min, and then subjected to the aPTT assay. Data are presented as mean ± s.e.m. Statistical significances were determined using one-way ANOVA followed by Dunnett's multiple comparisons test.