ORIGINAL RESEARCH

Prodomain of Furin Promotes Phospholipid Transfer Protein Proteasomal Degradation in Hepatocytes

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***Background*—**Phospholipid transfer protein (PLTP) is one of the major modulators of lipoprotein metabolism and atherosclerosis development; however, little is known about the regulation of PLTP. The effect of hepatic prodomain of furin (profurin) expression on PLTP processing and function is investigated.

***Methods and Results*—**We used adenovirus expressing profurin in mouse liver to evaluate PLTP activity, mass, and plasma lipid levels. We coexpressed PLTP and profurin in human hepatoma cell line cells and studied their interaction. We found profurin expression significantly reduced plasma lipids, plasma PLTP activity, and mass in all tested mouse models, compared with controls. Moreover, the expression of profurin dramatically reduced liver PLTP activity and protein level. We further explored the mechanism using in vivo and ex vivo approaches. We found that profurin can interact with intracellular PLTP and promote its ubiquitination and proteasomal degradation, resulting in less PLTP secretion from the hepatocytes. Furin does not cleave PLTP; instead, it forms a complex with PLTP, likely through its prodomain.

***Conclusions*—**Our study reveals that hepatic PLTP protein is targeted for proteasomal degradation by profurin expression, which could be a novel posttranslational mechanism underlying PLTP regulation. **(*J Am Heart Assoc*. 2018;7:e008526. DOI: 10.1161/JAHA.118.008526.)**

Key Words: atherosclerosis • furin (PCSK3) • lipids and lipoprotein metabolism • phospholipid transfer protein • profurin • proteasome

Phospholipid transfer protein (PLTP) expression is upregulated in different pathological conditions associated with an increased risk of coronary heart disease (CHD), such as obesity,[1,2] insulin resistance,[3] and type 2 diabetes mellitus.[4] Fifteen years ago, we reported that serum PLTP activity was increased in patients with CHD.[5] Despite many unresolved questions, we have since suggested that PLTP might be a therapeutic target for CHD. In the past decade, most human studies showed a positive association between plasma PLTP activity and atherosclerosis.[6–9] Using a PLTP gene score, constructed by a combination of 2 PLTP tagging single-nucleotide polymorphisms, Vergeer et al reported that PLTP gene variation, which confers lower hepatic PLTP transcription and plasma PLTP activity, leads to decreased risk of cardiovascular events among 5 cohorts comprising a total of 4658 cases and 11 459 controls.[10] In the Framingham Heart Study, which comprised a total of 2679 participants with 187 first events being ascertained during 10.4 years of follow-up, Robins et al found that higher plasma PLTP activity predicted a first cardiovascular event, defined as fatal or nonfatal CHD and stroke, among men.[11] Moreover, PLTP activity is also positively correlated with left ventricular systolic dysfunction.[12,13] Recently, we found that, after controlling for a variety of baseline variables, plasma PLTP activity levels were a strong and independent predictor of all-cause mortality in 5 years, and higher PLTP activity had higher mortality.[14] Contradictorily, PLTP mass was lower in a small group of patients with CHD compared with controls,[15]

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Accompanying Figures S1 through S4 are available at http://jaha.ahajournals.org/content/7/9/e008526/DC1/embed/inline-supplementary-material-1.pdf

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Clinical Perspective

What Is New?

- Liver prodomain of furin expression dramatically suppresses plasma phospholipid transfer protein activity, resulting in reduction of atherosclerosis in mouse models.
- Hepatocyte prodomain of furin expression reduces apolipoprotein B–containing secretion through promoting phospholipid transfer protein proteasomal degradation.

What Are the Clinical Implications?

- Phospholipid transfer protein liver-specific inhibitor, such as prodomain of furin, could be a novel therapeutic approach in the effort to moderate plasma very-low-density lipoprotein/low-density lipoprotein levels.

although it seems clear that the plasma PLTP protein concentration does not represent the preferred marker of PLTP-associated risk.[16,17] In addition, reported effects of PLTP on peripheral artery disease are both limited and inconsistent.[18,19]

In mouse models, it has been demonstrated that global PLTP deficiency reduces atherosclerotic lesion size,[20] whereas its overexpression shows the opposite effect.[21] Global PLTP deficiency in mice is also associated with a reduced thrombotic response[22] and a reduced abdominal aortic aneurysm.[23] In rabbits, overexpression of PLTP increases atherosclerotic lesions after a high-fat diet feeding, compared with controls.[24] In general, PLTP is a risk factor of atherosclerosis in animal models.

Proprotein convertase subtilisin/kexins (PCSKs) belong to a family of calcium-dependent serine endopeptidases. This family is composed of 9 members, including PCSK 1 to 9. PCSK3, also known as furin, is synthesized as prefurin. The "pre" signal peptide is removed in the endoplasmic reticulum (ER), and the full-length protein (amino acids 25–794; 110 kDa) is further processed by autocleavage in the secretory pathway to generate the N-terminal prodomain (prodomain of furin [profurin]; amino acids 25–107; 14 kDa) and mature, enzymatically active furin-cat (amino acids 108–794; 96 kDa), containing a C-terminal membrane binding domain[25] (Figure S1). Furin-cat is a type I transmembrane protein cycling between the Golgi, plasma membrane, and lysosome compartments, whereas profurin undergoes rapid intracellular degradation.[25] It is well known that proprotein convertases can mediate processing of various cellular proteins. Profurin is effective in inhibiting the processing of various cellular precursors, including nerve growth factor,[26] vascular endothelial growth factor-C,[27] and beta-amyloid-converting enzyme1.[28] Treatment with profurin markedly reduces C57BL/6 and low-density lipoprotein (LDL) receptor knockout mouse plasma lipid levels.[29,30] Notably, profurin expression inhibits PCSK3, PCSK5, and PCSK6.[26,29]

Although PLTP is one of the major modulators of lipoprotein metabolism and the development of atherosclerosis, little is known about the regulation of PLTP. In this study, we investigated an unexpected finding that plasma PLTP activity can be dramatically suppressed by profurin expression in the liver, and we explored the mechanism.

Methods

We agree to make the data and materials available on request. The corresponding author, Xian-Cheng Jiang, PhD, at Downstate Medical Center, State University of New York, will maintain availability of such data and materials.

Mice and Diets

LDL receptor knockout mice, apolipoprotein E (apoE) knockout mice, and wild-type (WT) mice (8 weeks old) on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME). Human PLTP transgenic mice were a gift from Dr R. de Crom (Erasmus Medical Center, the Netherlands). Animals were on a homogeneous C57BL/6 background (9 generation backcrosses). Experimental animals were housed in a temperature- and humidity-controlled room with a 12:12-hour light-dark cycle. We used both male and female mice (10 weeks old). Mice were fed a chow diet. Mouse adenovirus treatment included control adenovirus without a transgenic expression cassette (AdV-null) or adenovirus encoding human profurin (AdV-profurin; 2×10^{11} viral particles/mouse) via the tail vein (n=6 in each group). Experiments involving animals were conducted with the approval of State University of New York Downstate Medical Center Institutional Animal Care And Use Committee. The procedures followed were in accordance with institutional guidelines.

Antibodies and Materials

Monoclonal antibody for human PLTP was a gift from Dr John Albers (Department of Medicine, Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington, Seattle, WA). Antibodies against Flag, β-actin, ubiquitin, and albumin are from Sigma and Abcam. AdV-profurin and AdV-null were generated as previously described.[29] Complete protease inhibitor cocktail tablets were purchased from Roche (05892970001). Nitrobenzoxadiazole (NBD)-labeled phosphoethanolamine (N-360) was purchased from Molecular Probe (Life Technologies). MG132 (SML1135) was purchased from Sigma (Merck, Germany).

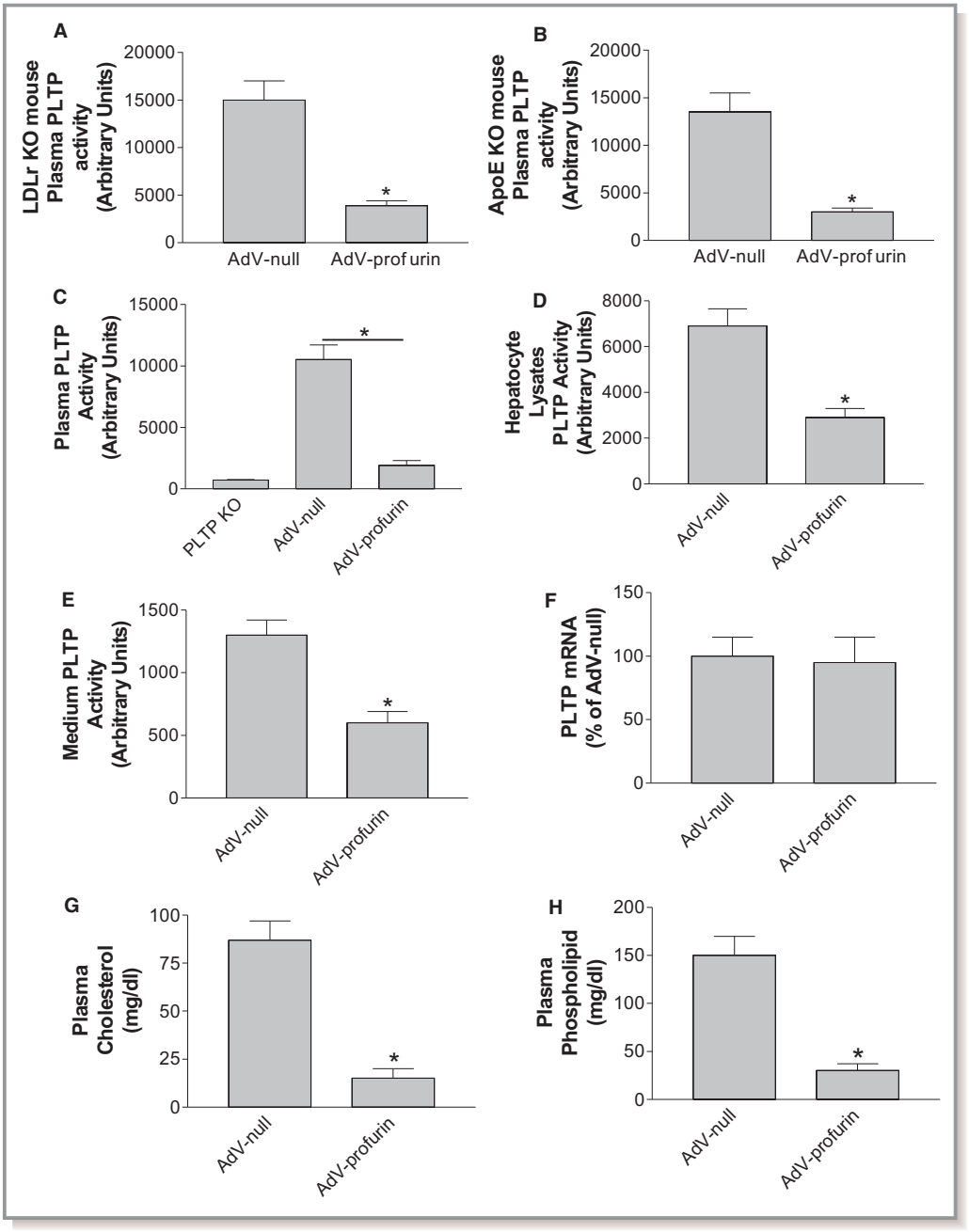

Figure 1. Adenovirus (AdV)–prodomain of furin (profurin) treatment reduces plasma and hepatocyte phospholipid transfer protein (PLTP) activity, as well as plasma lipid levels. Low-density lipoprotein receptor (LDLr) knockout (KO), apolipoprotein E (ApoE) KO, and wild-type (WT) mice were injected with AdV-null and AdV-profurin. Plasma was collected on day 3. A, LDLr KO mouse PLTP activity measurement. B, ApoE KO mouse PLTP activity measurement. C, WT mouse plasma PLTP activity measurement. D, PLTP activity in primary hepatocyte homogenate. E, PLTP activity in primary hepatocyte culture medium. F, Liver PLTP mRNA expression measured by real-time polymerase chain reaction. G, Plasma total cholesterol levels. H, Plasma total phospholipid levels. Values are mean±SEM (n=6). *P<0.001.

Lipid Assays

Plasma samples were collected on day 3 after adenovirus injection. Mice were fasted for 4 hours and bled from the retro-orbital plexus under isoflurane anesthesia using heparinized microcapillary tubes. Blood was centrifuged at 10 000g for 10 minutes at 4°C, and plasma was separated and used for the analysis and/or stored at −70°C. Total cholesterol and phospholipids were determined using commercially available kits (Wako Pure Chemical Industries Ltd, Richmond, VA).

PLTP Activity Assay

PLTP activity was determined following the method reported previously, with brief modification.[31] Before the assay, the donor and acceptor were prepared. The donor liposome labeled with NBD-phosphoethanolamine was in a stable and self-quenched status before use. The donor (3 μL) and acceptor (3 μL) were combined with plasma (3–5 μL) or concentrated cultured media (3–10 μL) in a final volume of 100 μL buffer (10 mmol/L Tris, 0.15 mol/L NaCl, 2 mmol/L EDTA, pH=7.4) at 37°C in a 96-well black microplate to allow the transfer of NBD-phosphoethanolamine mediated by PLTP. The fluorescence was detected every 10 minutes using a multifunctional microplate reader (Infinity F200; TECAN, Austria; excitation, 465 nm/emission, 535 nm). The transfer rate was expressed as pmol/μL per minute.

Primary Hepatocyte Isolation

Primary hepatocyte was isolated from WT and human PLTP transgenic mice on day 3 after AdV-null and AdV-profurin injection. The procedure was same as we reported before.[20]

Cell Culture and Transfection

The human hepatoma cell line (Huh7) was cultured in 5% CO_2 at 37°C in DMEM containing 100 U/mL penicillin and 100 μg/mL streptomycin supplemented with 10% (v/v) fetal bovine serum (Invitrogen). The cells were transfected with jetPEI (PolyPlus), according to the manufacturer's instructions. For 1 well of a 24-well plate, 1 μg of DNA (600 ng of PLTP-Flag or cholesteryl ester transfer protein-Flag plasmid, 400 ng of pcDNA with profurin or α-1 antitrypsin Portland variant plasmid) and 2 μL of jetPEI were used. The culture media were changed to serum-free DMEM at 24 hours after transfection. The medium was collected at 48 hours after transfection. The cells were lysed and collected after centrifugation at 12 000*g* for 10 minutes at 4°C. For MG132 treatment, Huh7 cells were treated with 1 μmol/L MG132 for 7 hours.

Protein Isolation, Electrophoresis, and Western Blot Analysis

Total protein extraction from mouse liver and cultured cells for Western blot analysis was described previously.[32] Proteins were isolated using cell lysis buffer with complete protease inhibitor cocktail tablets and PhoStop cocktail, which preserves phosphorylated sites in the presence of protease inhibitors. Densitometry analysis was conducted using Image-Pro Plus software, version 6.0 (Media Cybernetics Corp, Bethesda, MD).

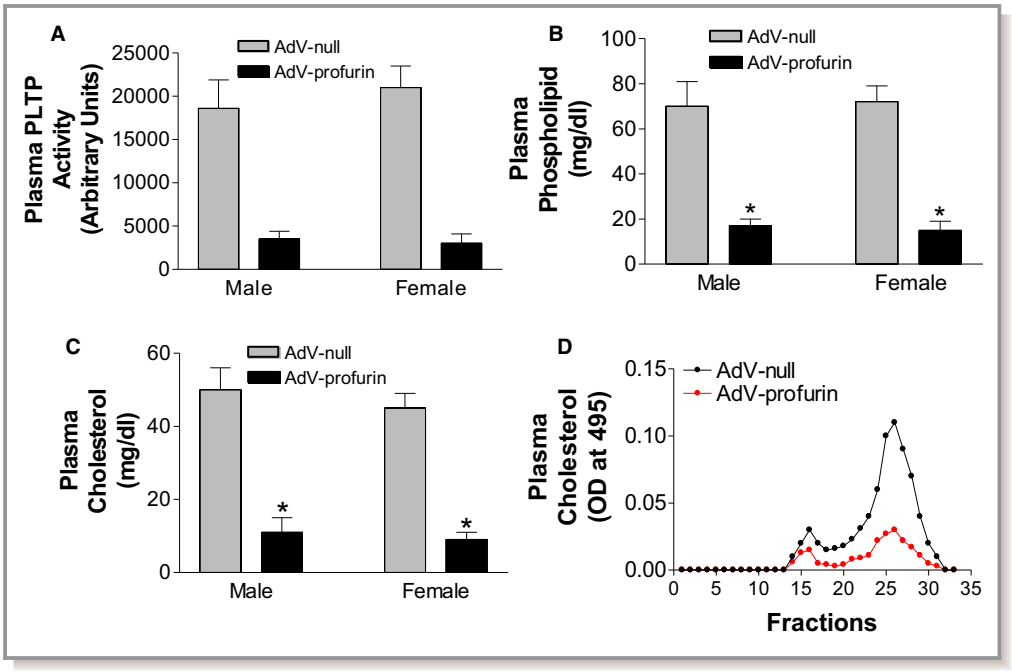

Figure 2. Adenovirus (AdV)–prodomain of furin (profurin) treatment reduces plasma phospholipid transfer protein (PLTP) activity and plasma lipid levels. Human PLTP transgenic mice (male and female) were injected with AdV-null and AdV-profurin. Plasma was collected on day 3. A, Plasma PLTP activity assay. B, Plasma total cholesterol levels. C, Plasma total phospholipid levels. D, Fast protein liquid chromatography, male mouse plasma cholesterol distribution. Values are mean±SEM (n=6). **P*<0.001.

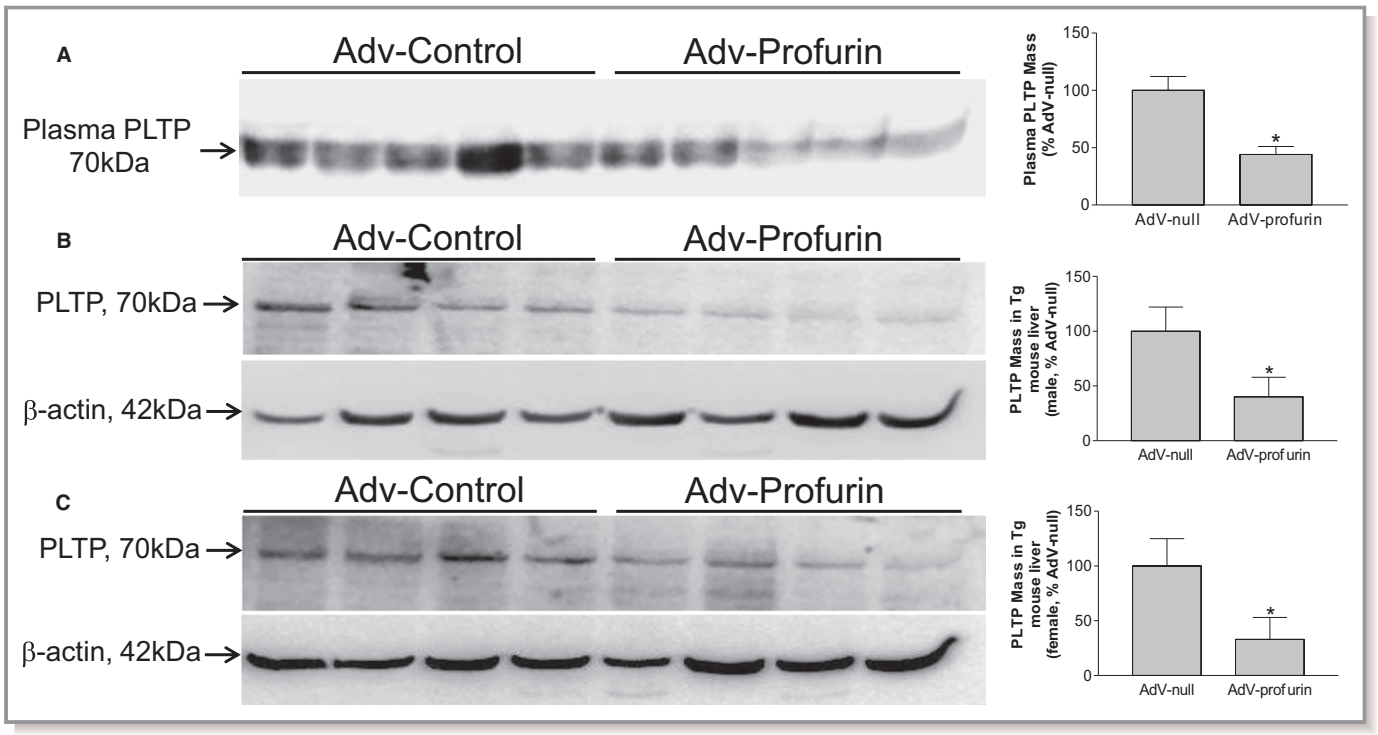

Figure 3. Adenovirus (AdV)–prodomain of furin (profurin) treatment reduces plasma and liver phospholipid transfer protein (PLTP). Human PLTP transgenic mice were injected with AdV-null and AdV-profurin. Plasma was collected on day 3. A, The effect of AdV-profurin on plasma PLTP (n=5). The liver homogenates were harvested for Western blot analysis from male and female mice (n=4). B and C, The effect of AdV-profurin on male and female PLTP transgenic mouse hepatic PLTP. Values were presented as mean±SEM. **P*<0.05.

PLTP mRNA Measurement

Total RNA from different tissues was extracted with RNeasy Mini kit reagents (Qiagen). RNA (2 μg) was reverse transcribed using a kit from Applied Biosystems, and PLTP mRNA levels were determined by real-time polymerase chain reaction, as we did previously.[33] The primers used for reverse transcription–polymerase chain reaction were as follows: PLTP forward, 5CATGCGGGATTCCTCACC3; and PLTP reverse, 5GAGGGGGCACTACAGGCTAT3.

Statistical Analysis

Results are expressed as mean±SEM. The statistical significance of the difference between 2 data means was determined with a 2-tailed exact Mann-Whitney test, and differences among multiple groups were assessed by 1-way ANOVA, followed by the Student-Newman–Keuls test. A difference for which *P* was <0.05 was considered statistically significant.

Results

Hepatic Profurin Expression Reduces Plasma PLTP Activity, Protein, and Plasma Lipids

In our previous study, we found that hepatic profurin expression significantly reduces plasma cholesterol, triglyceride, apolipoprotein B (apoB), and apolipoprotein A-I levels in LDL receptor knockout mice, resulting in reduction of atherosclerotic lesion formation.[30] The lipid-lowering effects of profurin are not completely understood. However, the phenotype of profurin expression is reminiscent of hepatic PLTP deficiency in mice.[34,35] Therefore, we hypothesized that profurin is involved in PLTP regulation. First, we measured plasma PLTP activity in LDL receptor knockout and apoE knockout mice and found that AdV-profurin treatment dramatically reduces plasma PLTP activity in these mice, compared with controls (Figure 1A and 1B). AdV-profurin treatment also significantly reduced plasma cholesterol levels, apoB levels, and atherosclerotic lesions in apoE knockout mice (Figure S2A through S2C). The results were similar to those obtained from LDL receptor knockout mice.[30]

Then, we repeated the previously described experiments in WT (male, C57BL/6) mice and measured plasma and liver PLTP activity in the mice. We found that AdV-profurin treatment also dramatically reduced plasma PLTP activity (Figure 1C). Consistently, profurin decreased PLTP activity of primary mouse hepatocytes in both lysates and culture media (Figure 1D and 1E). However, profurin expression had no effect on liver PLTP mRNA expression (Figure 1F), indicating a posttranslational effect on PLTP activity. In addition, AdV-profurin treatment substantially reduced plasma cholesterol and total phospholipid levels (Figure 1G and 1H), which are in

line with the lipid phenotype seen in hepatic PLTP-deficient mice.[36] Similar results were observed in female C57BL/6 mice (Figure S3).

We next used human PLTP transgenic mice to perform similar experiments for the following reasons: (1) to test the interaction of human profurin and human PLTP in vivo; (2) antibody excellent for human PLTP detection is on our hand; (3) to exclude the possibility that profurin reduces PLTP expression at a transcriptional level; and (4) to confirm the results observed in LDL receptor knockout, apoE knockout, and WT mice. In fact, AdV-profurin treatment dramatically reduced plasma PLTP activity (Figure 2A), plasma total cholesterol (Figure 2B), and total phospholipids (Figure 2C) in both male and female PLTP transgenic mice. We also measured lipoprotein distribution using fast protein liquid chromatography and found both non–high-density lipoprotein (HDL)-cholesterol and HDL-cholesterol were dramatically reduced (Figure 2D).

To further determine human PLTP protein level in the plasma of PLTP transgenic mice, we performed immunoblotting using a specific anti-human PLTP monoclonal antibody. Actually, the plasma PLTP protein level was significantly reduced after profurin treatment (Figure 3A). Moreover, PLTP protein level in liver homogenate from human PLTP transgenic male and female mice was measured. Accordingly, AdV-profurin treatment significantly decreased intracellular PLTP protein levels in the liver, compared with controls (Figure 3B and 3C).

Profurin Expression Promotes Intracellular PLTP Degradation

To investigate how expressed profurin decreases PLTP activity, we coexpressed profurin in increasing amounts with PLTP-Flag (with a C-terminal Flag tag that did not interfere with protein activity) in Huh7 cells, a human hepatoma cell line, and found that PLTP secretion was reduced in the culture media and lysate in a dose-dependent manner (Figure 4A). However, profurin had no effect on cholesteryl ester transfer protein, a protein in the same gene as PLTP (Figure 4B). We

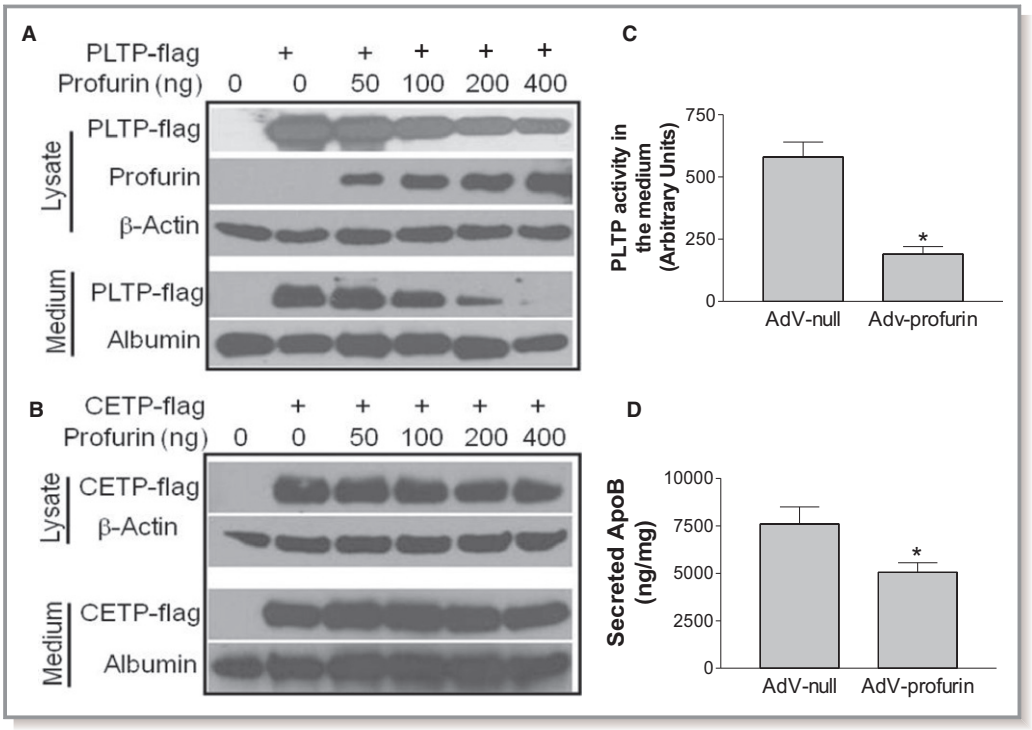

Figure 4. Effect of prodomain of furin (profurin) on cellular phospholipid transfer protein (PLTP) levels and apolipoprotein B (apoB) secretion. Human hepatoma cell line (Huh7) cells were cotransfected by PLTP-Flag (600 ng) or cholesteryl ester transfer protein (CETP)-Flag (600 ng) and different concentrations of profurin expression vectors (0, 50, 100, 200, and 400 ng) with pcDNA control vector (400, 350, 300, 200, and 0 ng). After 2-day transfection, medium was collected and cells were lysed. PLTP-Flag or CETP-Flag and profurin protein levels (anti–enhanced green fluorescent protein) were measured by Western blot analysis. A, The effect on PLTP. B, The effect on CETP. Blots are representative of 3 experiments. Huh7 cells were treated with adenovirus (AdV)-profurin, then PLTP activity and apoB in the cell culture medium were measured. C, PLTP activity. D, ApoB levels measured by ELISA (Mybiosource, ABG20796). Values are mean±SEM (n=5). *$P<0.05$.

next evaluated profurin expression on apoB secretion, a PLTP involved process[20,37,38]; we found that AdV-profurin treated Huh7 cells reduced cell PLTP activity and apoB secretion (Figure 4C and 4D).

We then hypothesized that profurin promotes the intracellular degradation of PLTP. Cultured cells expressing PLTP in the presence of MG132, a specific and potent proteasome inhibitor, resulted in significantly increased amount of PLTP protein (Figure 5A, lane 4 versus lane 2). However, cultured cells coexpressing profurin and PLTP showed less reduction of PLTP protein when they were treated with MG132 (Figure 5A, lane 5 versus lane 4), compared with absence of MG132 (Figure 5A, lane 3 versus lane 2), indicating at least proteasomal degradation is involved in this process.

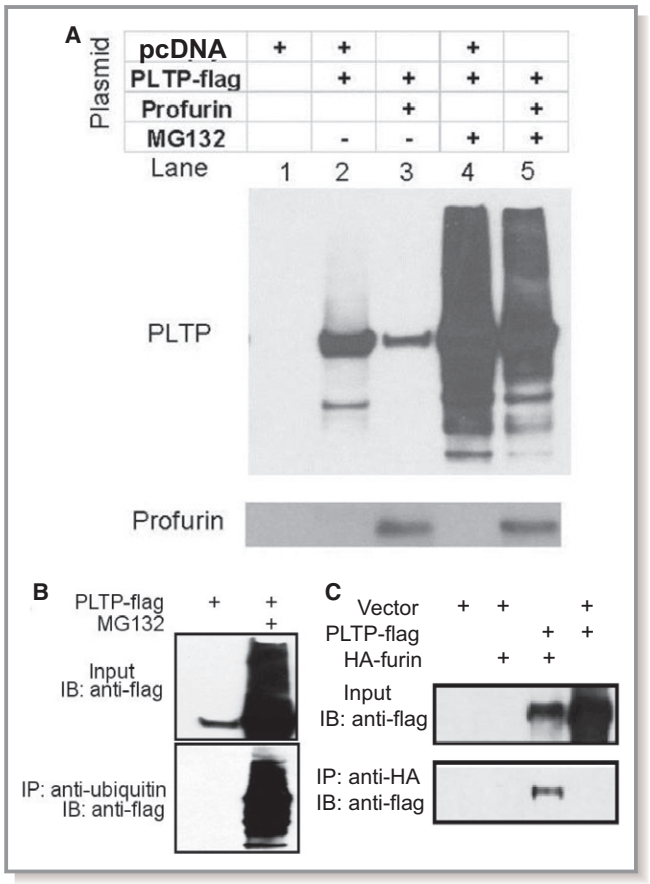

Figure 5. Effect of prodomain of furin (profurin) on cellular phospholipid transfer protein (PLTP) degradation. A, Effect of MG132 on PLTP-Flag protein level in human hepatoma cell line (Huh7) cell lysates cotransfected with or without profurin. B, PLTP is polyubiquitinated after MG132 treatment in Huh7 cells, which was validated by coimmunoprecipitation using an anti-ubiquitin antibody, and then Western blot analysis was performed with an anti-Flag antibody. C, Furin forms a complex with PLTP. HA-tag-furin/PLTP-Flag complex was coimmunoprecipitated using an anti-HA tag antibody, and then Western blot analysis was performed with anti-Flag antibody. Blots are representative of 3 experiments. IB indicates immunoblot.

Lanes 4 and 5 of Figure 5A demonstrate multiple forms of PLTP protein, consistent with the ubiquitination and possible degradation products. To test it, we transfected Huh7 cells with PLTP-Flag, which were further incubated with MG132 or vehicle. The anti-ubiquitin antibody was used to do the coimmunoprecipitation assay, and PLTP was shown to be highly ubiquitinated (Figure 5B).

To see whether profurin interacts with PLTP, we coexpressed PLTP-Flag and HA-tag-furin (with an N-terminal HA tag that did not interfere with activity) in Huh7 cells. We found that anti-HA-tag antibody can pull down a complex that contains PLTP (Figure 5C). HA was linked to the N-terminal of furin, which can be autocleaved into 2 parts (HA-profurin and furin-cat); thus, the interaction between furin and PLTP is most likely through profurin.

It is known that profurin expression inhibits furin (PCSK3), PCSK5, and PCSK6.[26,29] Besides the direct interaction of profurin on PLTP degradation, are these protein convertases involved in the reduction of PLTP by profurin? α-1 Antitrypsin Portland variant is a general protein convertase inhibitor, whereas prodomain of PCSK5 is an inhibitor for both PCSK3 and PCSK5. We coexpressed PLTP-Flag and plasmid containing either α-1 antitrypsin Portland variant cDNA or prodomain of PCSK5 in Huh7 cells and found both α-1 antitrypsin Portland variant and prodomain of PCSK5 have no effect on intracellular PLTP levels (Figure 6A and 6B). Thus, profurin specifically mediates PLTP intracellular degradation, which is not likely through its ability to inhibit the proprotein convertase activities.

Can expression of profurin affect ER stress, which mediates apoB degradation?[39] To answer this question, we measured the mRNA levels of binding immunoglobulin protein and X-box binding protein 1s, which are both ER stress-related proteins; after AdV-profurin treatment, we found that X-box binding protein 1s mRNA was significantly reduced, whereas binding immunoglobulin protein had no change (Figure S4). Thus, ER stress-associated apoB degradation seems not play a role here.

To further test the influence of profurin expression on intracellular PLTP, we also isolated primary hepatocytes from AdV-null and AdV-profurin treated PLTP transgenic mouse liver, and then treated with MG-132 or vehicle. We found that MG-132 treatment can prevent profurin-mediated PLTP degradation in hepatocytes (Figure 7A and 7B).

To test the observed phenotype is related with endogenous furin, we measured plasma PLTP activity in liver-specific furin-deficient mice.[40] Indeed, the deficient mice have significantly higher PLTP activity in the circulation (Figure 7C). It is possible that furin could decrease PLTP expression by cleaving it. However, our results showed it is unlikely to happen. We incubated recombinant human PLTP-Flag with different concentrations of recombinant HA-tag-furin for

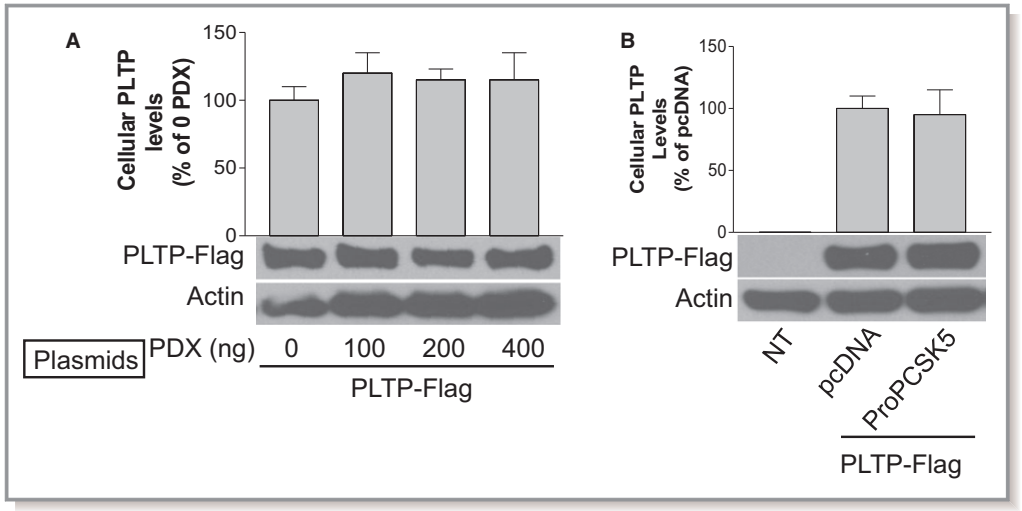

Figure 6. α-1 Antitrypsin Portland variant (α-1-PDX) and prodomain of PCSK5 (ProPCSK5) have no effect on cellular phospholipid transfer protein (PLTP) degradation. A, Human hepatoma cell line (Huh7) cells were cotransfected by PLTP-Flag (600 ng) and different concentrations of α-1-PDX expression vectors (0, 100, 200, and 400 ng) with pcDNA control vector (400, 300, 100, and 0 ng). PLTP-Flag levels in cell homogenate were detected by Western blot analysis. B, Huh7 cells were cotransfected by PLTP-Flag (600 ng) and ProPCSK5 (400 ng) expression vectors. PLTP-Flag levels in cell homogenate were detected by Western blot analysis. Blots are representative of 3 experiments. NT indicates no treatment.

4 hours. Then, Western blot analysis was performed with an anti-Flag antibody, and no PLTP cleavage was detected (Figure 7D).

Discussion

We reported previously that adenovirus-mediated hepatic profurin expression resulted in a significant reduction of atherosclerotic lesion development and plasma LDL-cholesterol in LDL receptor knockout mice, despite the fact that hepatic profurin expression markedly decreased HDL-cholesterol.[29] Furthermore, metabolic studies revealed lower secretion of apoB and triglycerides in very-low-density lipoprotein (VLDL) particles. More important, short-term hepatic profurin expression did not result in hepatic lipid accumulation.[30] However, the underlying mechanism by which profurin decreases apoB-containing lipoproteins is unknown.

In this study, we disclosed an interaction between profurin and PLTP. We found the following: (1) profurin significantly and dramatically reduces plasma PLTP activity in all tested mouse models, including LDL receptor knockout, apoE knockout, and WT mice, compared with controls; (2) profurin decreases PLTP at posttranslational level; (3) profurin promotes PLTP ubiquitination and ER/proteasome-associated degradation; and (4) furin can form a complex with PLTP, likely through profurin, and furin cannot mediate PLTP cleavage.

One of the surprising findings is that hepatic profurin expression reduces plasma PLTP activity (Figure 1A through 1C; Figure 2A), and this is because of reduction of PLTP mass in the circulation (Figure 3A). PLTP belongs to a family of lipid transfer/lipopolysaccharide-binding proteins, including lipopolysaccharide-binding protein, bactericidal/permeability-increasing protein, and cholesteryl ester transfer protein.[41] PLTP can be secreted by the liver.[35] Human PLTP transgenic mice, with a high level of liver expression, showed a 2.5- to 4.5-fold increase in PLTP activity in plasma, compared with controls.[42] The reason to use human PLTP transgenic mice in this study is that human PLTP monoclonal antibody is available. Indeed, we observed that human PLTP expression in primary hepatocytes from PLTP transgenic mice was suppressed by profurin treatment (Figure 3B and 3C). We believe that same situation also occurs in WT mice, when profurin is expressed in the liver. More important, the observed phenotype is related with endogenous furin, because liver-specific furin-deficient mice had significantly higher PLTP activity in the circulation (Figure 7C).

Why is regulation of PLTP important? We have found that PLTP deficiency causes a significant impairment in hepatic secretion of VLDL in mouse models.[20] Likewise, it has been reported that animals expressing human PLTP transgene exhibit hepatic VLDL overproduction.[43,44] The group of Lagrost and colleagues[24] found that human PLTP transgenic rabbits showed a significant increase of LDL, but not of HDL, in the circulation. Okazaki et al reported that, in concert with the increase in triglyceride synthesis, the increased PLTP activity permits triglyceride incorporation into large VLDLs.[45] Dashti and colleagues found that PLTP played a major role in the initiation of apoB-containing particle assembly in mouse

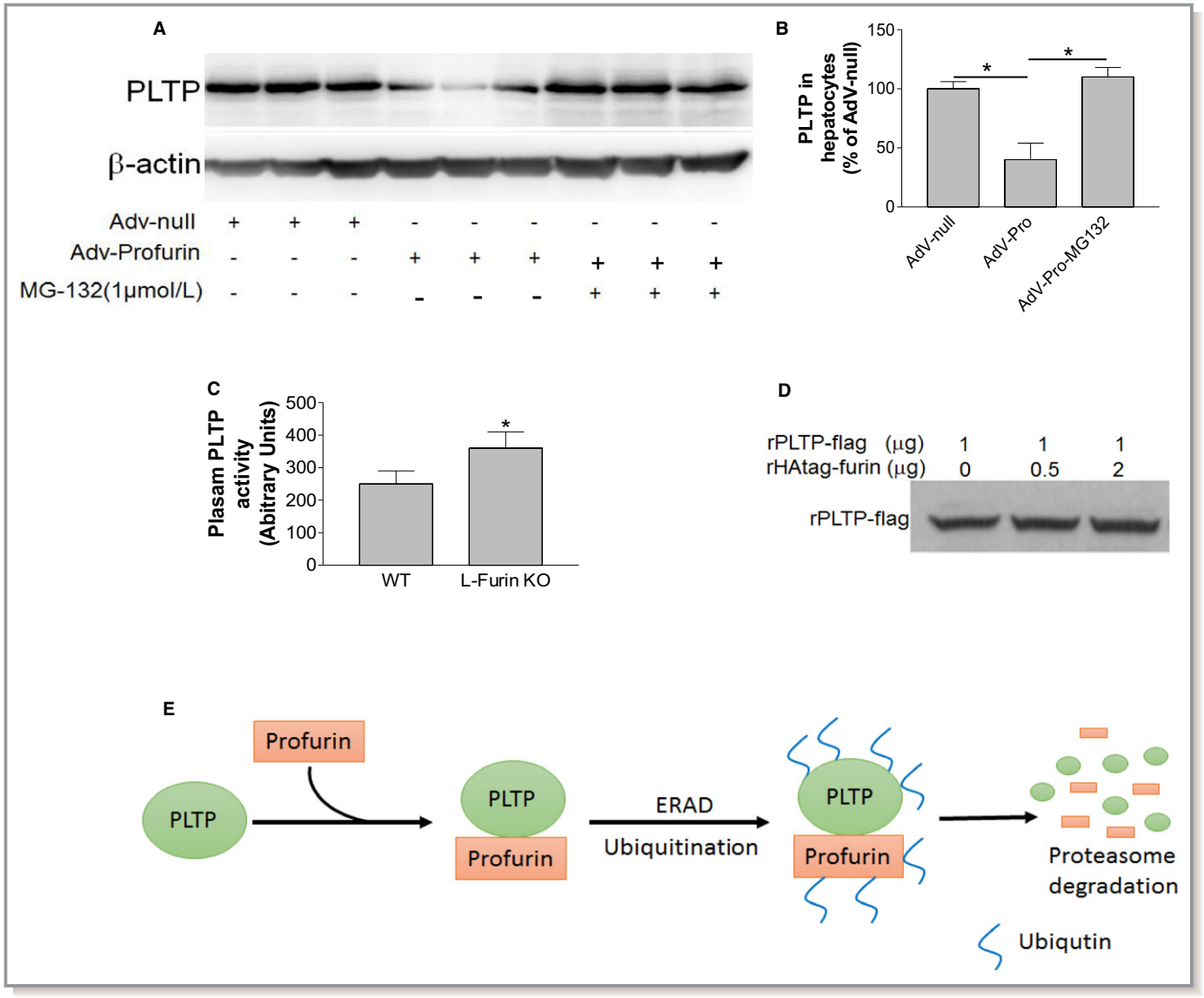

Figure 7. The influence of prodomain of furin (profurin) on phospholipid transfer protein (PLTP) in vivo. A, Primary hepatocytes from adenovirus (AdV)-null and AdV-profurin treated PLTP transgenic mice were isolated, and then treated with MG-132 or vehicle. PLTP in the cell homogenate was measured by Western blot analysis. B, Quantification. C, Plasma PLTP activity in liver-specific furin (L-Furin) knockout (KO) and wild-type (WT) mice (n=3). D, No effect of furin on PLTP cleavage. The recombinant human PLTP (rPLTP-Flag) was incubated with different concentrations of recombinant HA-tag-furin (rHAtag-Furin) for 4 hours. No cleavage was detected by Western blot analysis using anti-Flag antibody. E, The formation of profurin/PLTP complex initiates ubiquitination and then endoplasmic reticulum/proteasome-associated degradation (ERAD). Values were presented as mean±SEM. *P<0.01.

primary hepatocytes.[37] More important, human genome-wide association studies and many others have shown that human PLTP levels are positively associated with plasma triglyceride and apoB levels.[7,46] We reason that PLTP activity is involved in promoting VLDL lipidation, because PLTP activity and triglyceride enrichment are 2 factors regulating PLTP-mediated HDL enlargement,[47,48] a process similar to VLDL lipidation.[49]

Furin is one of the proprotein convertases responsible for the proteolytic cleavage of certain proteins for their biological functions. For instance, furin reduces endothelial lipase function through direct inactivating cleavage of the enzyme and through activating cleavage of angiopoietin-like protein 3, an endogenous inhibitor of endothelial lipase.[29] Furin could decrease PLTP expression by cleaving it. However, our results showed it is unlikely to happen (Figure 7D). Thus, furin/PLTP interaction does not result in proteolytic cleavage of PLTP by furin; rather, it results in targeting PLTP for intracellular degradation. Moreover, this degradation seems to be profurin specific, because inhibition of other protein convertases has no such activity (Figure 6A and 6B).

It is not unusual that proprotein convertases have functions other than their proteolytic activity. It is well known

that PCSK9-mediated LDL receptor degradation is independent of its enzymatic activity.[50] When PCSK9 is bound to the LDL receptor, mainly after extracellular interaction between LDL receptor and LDL particle, the LDL receptor is degraded and is no longer recycled back to the cell membrane surface to bind and ingest more LDL particles.[51] The difference between PCSK9-mediated LDL receptor degradation and profurin-mediated PLTP degradation is that the former occurs in the lysosome and the latter occurs in the proteasome. Further studies are required to answer the question of whether endogenous profurin is a significant player in regulating hepatic PLTP secretion and plasma lipid levels.

We summarized our finding in Figure 7E. We believe the following: (1) PLTP is involved in apoB particle lipidation and secretion in the liver[20,24,37,43,44]; without PLTP, VLDL production is greatly reduced.[20,37] (2) Profurin could regulate PLTP through promoting apoB particle intracellular degradation. The mechanism could be that profurin and PLTP form a complex that then initiates ubiquitination and degradation.

Our study reveals that hepatic PLTP protein is targeted for proteasomal degradation by profurin, which could be a novel posttranslational mechanism by which PLTP is regulated. Our early work demonstrated that liver PLTP is responsible for VLDL production. PLTP liver-specific inhibitor, such as profurin, could be a novel therapeutic approach in the effort to moderate plasma VLDL/LDL levels.

Author Contributions

Yu and Lei designed the study and performed most of experiments, and they contributed equally to this work; Jiang, Li, and Qin performed some experiments; Creemers provided liver-specific furin knockout mouse tissues; Jin and Jiang designed the study and wrote the article. All authors reviewed the results and approved the final version of the article.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Supplemental Figure Legends:

Figure S1. Maturation of Furin. Following signal peptide removal and translocation, the furin prodomain (profurin) acts as an intramolecular chaperone and autoinhibitor to facilitate folding and autocleavage of Furin in the secretory pathway, resulting in the active conformation (Furin-cat).

Figure S2. AdV-profurin treatment reduces apoE KO mouse plasma cholesterol levels, apoB levels, and atherosclerotic lesion area. (A) plasma cholesterol distribution, measured by FPLC. (B) plasma apoB levels measured by Western blot. (C) 5 month old apoE KO mouse atherosclerotic lesion measurement (*en face*). Values were presented as mean ± S.E., n=7, **P*<0.01.

Figure S3. AdV-profurin treatment reduces plasma PLTP activity, cholesterol and phospholipid in female WT mice. On day 3 after mice were injected with AdV-null and AdV-profurin, plasma PLTP activity (A), cholesterol (B) and phospholipid (C) were measured, respectively. Values were mean ± SD, N=6. **P*<0.001.

Figure S4. BiP and XBP1s mRNA measurement. On day 3 after mice were injected with AdV-null and AdV-profurin, liver BiP and XBP1s mRNAs were measured by Real-time PCR. Values were presented as mean ± S.E., n=4, **P*<0.01.

Figure S1.

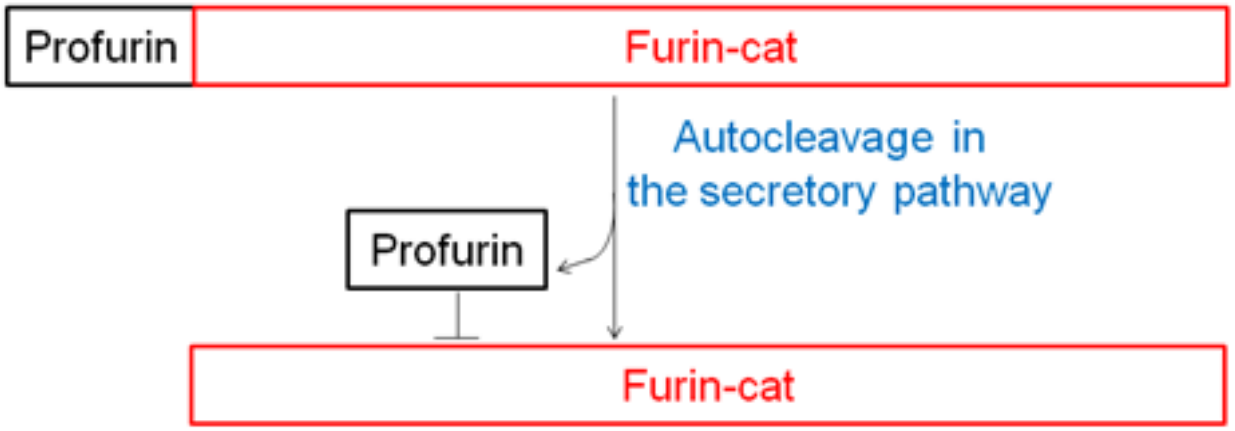

Profurin
Furin-cat
Autocleavage in
the secretory pathway
Profurin
Furin-cat

Figure S2

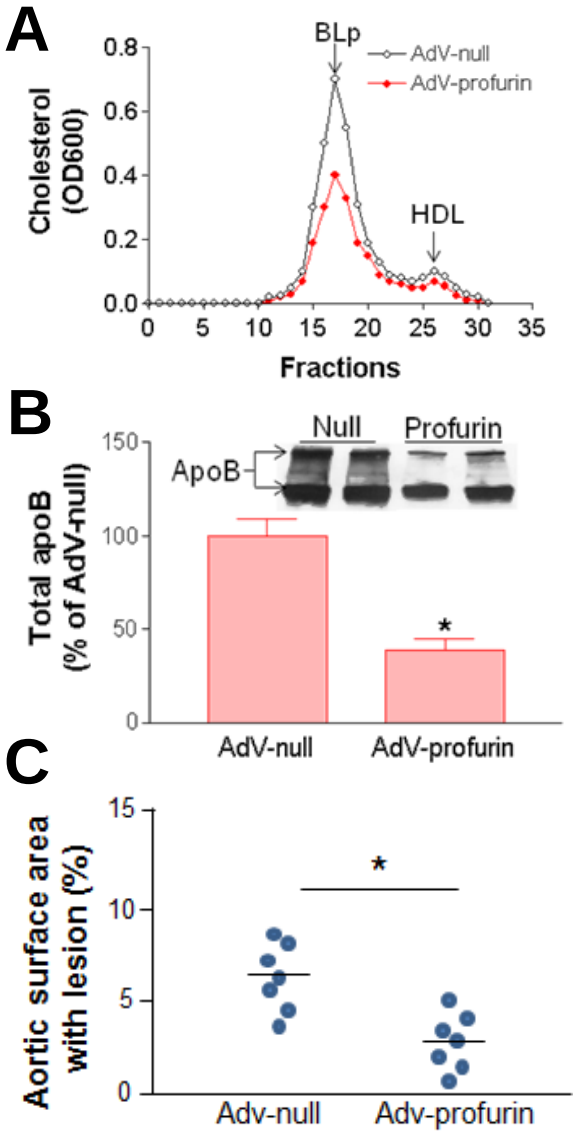

A
BLp
AdV-null
AdV-profurin
HDL
Cholesterol (OD600)
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
Fractions
B
Null
Profurin
ApoB
Total apoB (% of AdV-null)
150
100
50
0
*
AdV-null
AdV-profurin
C
Aortic surface area with lesion (%)
15
10
5
0
*
Adv-null
Adv-profurin

Figure S3.

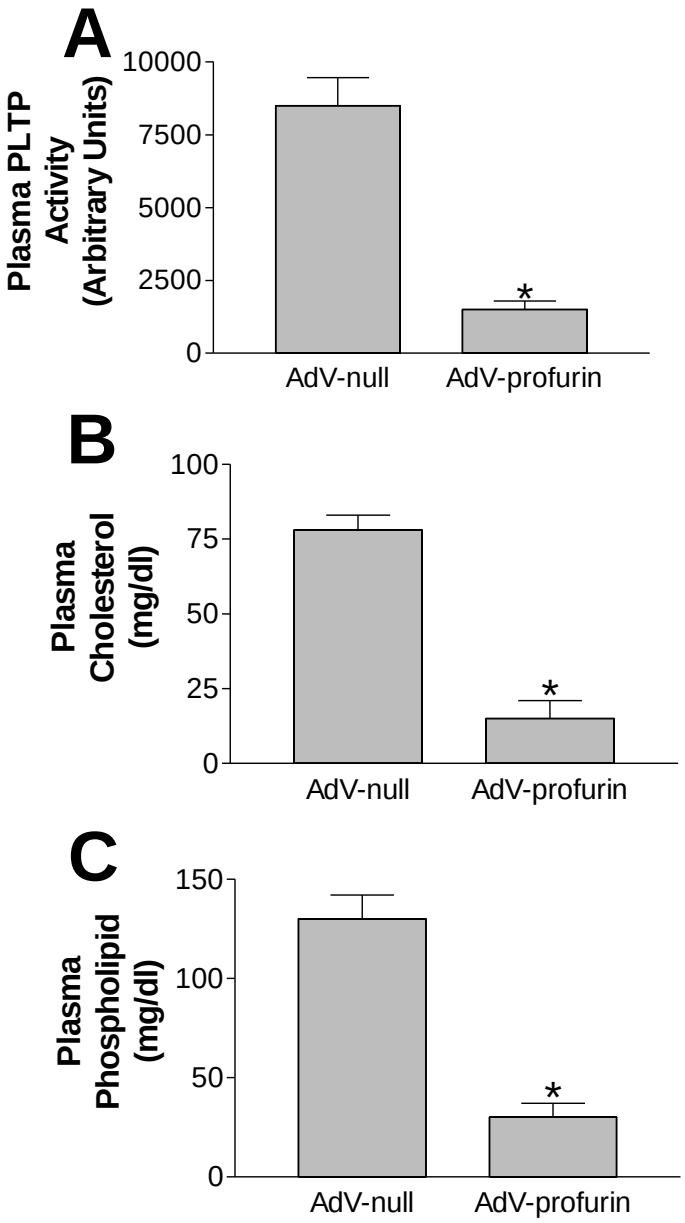
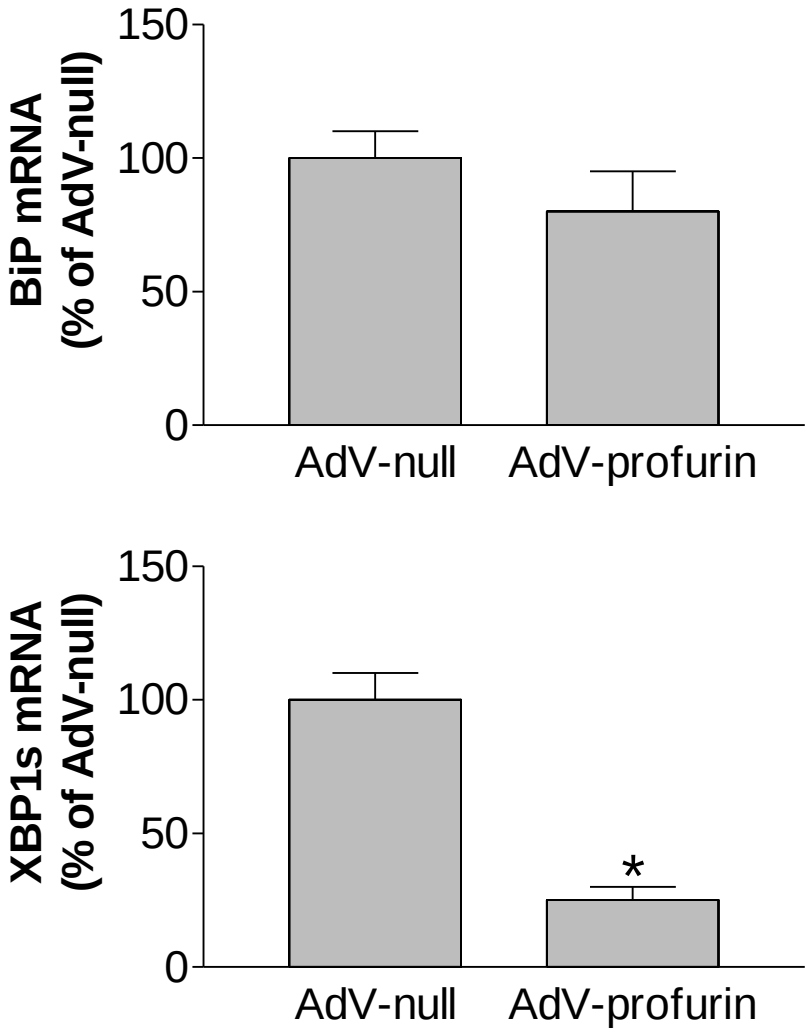

A
Plasma PLTP Activity (Arbitrary Units)
10000
7500
5000
2500
0
*
AdV-null
AdV-profurin
B
Plasma Cholesterol (mg/dl)
100
75
50
25
0
*
AdV-null
AdV-profurin
C
Plasma Phospholipid (mg/dl)
150
100
50
0
*
AdV-null
AdV-profurin

Figure S4.


BiP mRNA
(% of AdV-null)
150
100
50
0
AdV-null
AdV-profurin
XBP1s mRNA
(% of AdV-null)
150
100
50
0
*
AdV-null
AdV-profurin