New insights into the biological role of COMMD1
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CHAPTER 6

Loss of hepatocyte COMMD1 results in increased levels of circulating low-density lipoprotein cholesterol

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In preparation
ABSTRACT

An elevated level of circulating low-density lipoprotein (LDL) is a key risk factor for various cardiovascular diseases, and levels of LDL are tightly controlled by the hepatic LDL receptor (LDLR). Although the general concept by which plasma LDL levels are kept in control is known, the exact mechanism regulating the LDLR function is still poorly understood. Here we identified COMMD1 as a new gene regulating cholesterol homeostasis. Absence of hepatic COMMD1 increases the plasma levels of the atherogenic LDL lipoprotein in mice and dogs. COMMD1 physically associates with the LDLR and facilitates the cellular uptake of LDL. Depletion of Commd1 markedly impairs the proprotein convertase subtilisin/kexin type 9-mediated LDLR proteolysis, suggesting that COMMD1 coordinates the intracellular sorting of the LDLR. Since COMMD1 was initially reported to mediate biliary copper excretion, our data point to COMMD1 as a general adaptor protein in the intracellular sorting of various cargos, including the LDLR.
INTRODUCTION

The low-density lipoprotein receptor (LDLR) in the liver plays a pivotal role in controlling the level of circulating low-density lipoprotein (LDL) [1, 2]. A high level of LDL is a major risk factor for the development of atherosclerosis, and most of the knowledge about the mechanism regulating LDL levels has been obtained from patients suffering from familial hypercholesterolemia (FH).

FH is a genetic disorder characterized by elevated plasma levels of LDL [3, 4]. It is frequently caused by mutations in LDLR leading to impaired clearance of LDL from the bloodstream. Most common are heterozygous, mild mutations occurring with an estimated frequency of 1/500 in the general population [2, 4, 5]. The prevalence of FH patients with homozygous LDLR mutations is much lower (1 in a million), and these patients generally have a more severe phenotype [5]. The variations in LDLR are categorized depending on their outcome. The outcomes include no LDLR synthesis or production of a receptor defective in either LDL-cholesterol (LDL-c) binding, receptor internalization or recycling, or failing to move the receptor from the endoplasmic reticulum (ER) to the Golgi apparatus [4, 5].

In normal conditions, upon maturation, LDLR is transported to the cell surface where it binds to LDL-c. The LDLR-LDL-c complex is internalized via clathrin-coated pits, and directed to the early and then to the late endosomal compartments, where acidic pH triggers dissociation of LDL-c from its receptor. LDL-c is subsequently targeted to the lysosomes for degradation; the LDL receptor is either recycled back to the membrane or degraded by the lysosomes [6].

The lysosomal degradation of LDLR is mediated by proprotein convertase subtilisin/kexin type 9 (PCSK9) [7-10]. Gain-of-function mutations in PCSK9 are associated with hypercholesterolemia [11] caused by increased LDLR proteolysis mediated by PCSK9 [7, 12]. Apart from PCSK9, the autosomal recessive hypercholesterolemia protein (ARH) was also shown to be involved in the control of LDLR signaling and trafficking. Patients with ARH mutations show many clinical similarities with FH patients. ARH binds to the cytosolic domain of LDLR and acts as an adaptor protein to regulate the endocytosis of LDLR, which is markedly impaired in ARH patients [13-15]. Although additional proteins, such as Idol and β-Arrestin2, have been identified in the LDLR pathway [16, 17], the exact mechanism by which the intracellular trafficking of the LDLR is regulated is still not completely understood.

In this study, we identified COMMD1 as a novel gene reducing the plasma LDL-c levels in mice and dogs. COMMD1 belongs to the COMMD family of proteins [18], and loss of COMMD1 has initially been associated with progressive hepatic copper accumulation in dogs, which we recently reproduced in mice [19-21]. Several studies indicate that COMMD1 regulates biliary copper excretion via the copper-transporting protein ATP7B [21-24]. Although the exact mechanism is still unclear, it has been postulated that COMMD1 might be involved in the retrograde trafficking or the proteasomal degradation of ATP7B [23, 25]. Here, we show that COMMD1 interacts with LDLR to mediate the cellular uptake of LDL, and that COMMD1 deficiency elevates the levels of plasma LDL-c in mice and dogs.
RESULTS

HEPATIC COMM1 KNOCKOUT MICE AND COMM1-DEFICIENT DOGS ARE HYPERCHOLESTEROLEMIC

In order to better understand the biological role of Comm1 in the liver, we identified the characteristics of the liver-specific Comm1 knockout mice (Comm1\(^{ΔHe}\)) [21] with regard to their hepatic and plasma cholesterol and triglyceride concentrations. We investigated both chow-fed (Supplementary Table 1) and high-fat high-cholesterol (HFC, cholesterol 0.2%)-fed (Supplementary Table 2) groups of mice (n=6-8). As shown previously [21], body weight and hepatic copper levels were not altered between Comm1\(^{ΔHe}\) mice and littermate Comm1\(^{loxP/loxP}\) mice, which from now on are referred to as wild-type (WT) mice. Hepatic cholesterol and triglyceride (TG) concentrations were also unaffected. Remarkably, however, the plasma cholesterol levels in chow-fed Comm1\(^{ΔHe}\) mice were approximately 35% higher and in HFC-fed Comm1\(^{ΔHe}\) mice approximately 39% higher than those of WT mice (Fig. 1a). The plasma triglyceride levels remained unchanged (Fig. 1b). A significant increase in plasma cholesterol was also seen in Comm1\(^{ΔHe}\) mice at ten weeks of age (Supplementary Fig. 1a). Of note, we observed no differences in cholesterol levels between WT and transgenic mice expressing the Cre recombinase in hepatocytes (Alb-Cre), which excludes the possibility that exotic expression of Cre recombinase in the liver affects cholesterol metabolism (Supplementary Fig. 1b).

Next, we evaluated whether COMM1 deficiency in dogs [19] also affects the level of circulating cholesterol. We measured the plasma total cholesterol levels in COMM1\(^{+/−}\) dogs and in COMM1\(^{+/−}\) littermates (aged approximately 3-3.5 yrs) [20] (Fig. 1c). COMM1\(^{+/−}\) dogs showed a 50% increase in plasma total cholesterol compared to control littermates, but their plasma TG levels remained unaffected (Fig. 1d).

Figure 1. Hepatic Comm1 knockout mice and Comm1\(^{+/−}\) dogs are hypercholesterolemic. (a) Plasma total cholesterol and (b) triglyceride (TG) levels of hepatic Comm1 knockout mice fed chow or a high-fat high-cholesterol (HFC) diet for 20 weeks. (c) Plasma total cholesterol and (d) TG levels of dogs heterozygous (+/−) or homozygous (−/−) for a loss-of-function mutation in COMM1. (e) Plasma total cholesterol in two groups of Labrador retrievers: copper toxicosis-unaffected (unaff.) and affected (aff.). Group averages are plotted with SEM error. Significance in (a) and (b) was tested against a wild-type control group on each diet. Significance in (c) and (d) was tested against COMM1\(^{+/−}\) control group. Significance in (e) was tested against the unaffected group. *P<0.05, **P<0.01, ***P<0.001.
Despite the fact that high copper is linked to reduced levels of circulating cholesterol, possibly due to a decrease in Vldl synthesis and secretion [26], we evaluated the effect of progressive copper accumulation in the liver on plasma cholesterol levels in dogs. As a model we studied Labrador retrievers affected with copper toxicosis (Supplementary Fig. 2b). Similar to Bedlington terriers (Supplementary Fig. 2a), severe hepatic copper accumulation has been described in Labrador retrievers and although the mutation underlying copper toxicosis in Labrador retrievers is unknown, COMMD1 has been excluded as the causal gene [27-29]. Plasma total cholesterol in affected Labrador retrievers was similar to unaffected Labrador retrievers (aged ~4.5 yrs.) (Fig. 1e), excluding the possible causality between hepatic copper accumulation and elevated circulating cholesterol.

**CIRCULATING LDL-C LEVELS ARE MEDIATED BY COMMD1**

We then determined the lipoprotein profiles in pooled plasma samples of WT and Commd1**ΔHep** mice (n=6-8) fed either a chow (Fig. 2a) or HFC diet (Fig. 2b) for 20 weeks. Lipoproteins from plasma were separated by means of fast-performance liquid chromatography (FPLC). In Commd1**ΔHep** mice we found increased levels of Ldl-c (Fig. 2a,b), a lipoprotein that is normally present in mouse plasma in very low concentrations, whereas the high-density lipoprotein cholesterol (Hdl-c) is the most prevalent circulating cholesterol in mice. Detailed quantification of the different cholesterol forms demonstrated that the increase in the total cholesterol levels was mainly due to increase in Ldl-c (Supplementary Fig. 3a,b). To confirm that the isolated fractions correspond to Ldl and Hdl, we performed an immunoblot analysis to detect the apolipoproteins ApoB100 and ApoA1 (Fig. 2c,d), the apolipoproteins associated with Ldl and Hdl, respectively, and we also determined the triglyceride content in each fraction (Supplementary Fig. 3c,d).

The distribution of cholesterol across the lipoprotein fractions of COMMD1**−/−** and COMMD1**+/−** dogs (Fig. 3a) as well as of the unaffected and affected Labrador Retrievers (Fig. 3b) was also determined. Cholesterol was mainly present in VLDL and LDL particles in COMMD1**+/−** dogs, whereas cholesterol in their littermates (COMMD1**+/+** dogs) and Labrador retrievers (unaffected and affected dogs) was predominately detected in the HDL fraction. Similar to mice, due to the lack of cholesteryl ester transfer protein (CETP) activity, the most prevalent circulating cholesterol in dogs is HDL [30]. Together, these data indicate that hepatic Commd1 is essential to control plasma Ldl-c levels in mammals.

**VLDL-TG PRODUCTION IS UNAFFECTED BY ABLATION OF COMMD1 IN HEPATOCYTES**

To evaluate whether the elevated plasma Ldl-c levels in Commd1**ΔHep** mice result from an increased very low-density lipoprotein (Vldl) production and secretion, we assessed the changes in plasma Vldl-TG after intraperitoneal injection of Poloxamer-407. The production of Vldl-TG was not altered between Commd1**ΔHep** and WT mice (Fig. 4a, b).
Figure 2. Lipoprotein composition is altered in hepatic Commd1-deficient mice. Pooled plasma samples of each experimental group of mice fed either (a) chow or (b) HFC diet were separated using FPLC gel filtration. 50 fractions were collected from each separation. Total cholesterol content was determined and lipoprotein profile was plotted. (c) Fractions #13-26 containing cholesterol were collected through FPLC from each experimental group, loaded on an SDS polyacrylamide gel and blotted against ApoA1 and ApoB100 lipoproteins. The chow group and (d) HFC group are shown.

Figure 3. COMMD1 deficiency in dogs results in aberrant distribution of cholesterol among the different lipoproteins. Pooled plasma samples of each experimental group of dogs were separated using FPLC gel filtration. 50 fractions were collected from each separation. Total cholesterol content was determined and lipoprotein profile was plotted. (a) FPLC lipoprotein profile of dogs heterozygous (+/-) or homozygous (-/-) for a loss-of-function mutation in COMMD1. (b) FPLC cholesterol profile of copper toxicosis in unaffected (unaff.) and affected (aff.) Labrador retrievers.
Since Ldl-c is mainly cleared from the bloodstream via Ldlr-mediated endocytosis in the liver, we investigated whether hepatic Commd1 deficiency affects the Ldlr levels. No clear differences in hepatic Ldlr mRNA or protein levels were observed. In addition, no changes were found in Arh or Lrp (a member of the LDLR family) levels (Fig. 4c). Furthermore, hepatic mRNA expression of genes involved in cholesterol uptake, synthesis or efflux were unaltered (Fig. 4d). Together, these results exclude the possibility that the increased plasma Ldl-c levels in hepatic Commd1-deficient mice are caused by differences in Vldl synthesis and secretion, or by an aberrant expression of genes/proteins involved in LDL homeostasis.

Figure 4. VLDL-TG production and hepatic expression of Ldlr and other lipid-related genes are not affected by the loss of hepatic Commd1. (a) After intraperitoneal injection of poloxamer 407, blood was taken via orbita puncture at time points 0, 30, 60, 120 and 240 min. TG concentration was determined and plotted against time. (b) VLDL-TG production rate was calculated based on the TG concentration curve and corrected for the total time of experiment (4 h) and body weight of mice. (c) Livers of chow-fed mice were homogenized and 30 μg of protein was subjected to immunoblot analysis. Levels of Ldlr, Lrp-1, Arh and Tubulin were determined. Three representative samples from WT and Commd1ΔHep mice are shown. (d) Hepatic mRNA levels were analyzed in chow fed WT and Commd1ΔHep mice and presented relative to the WT control group. Group average values are presented with SEM. *P<0.05.

Plasma levels of LDL-c are determined by an accurate intracellular trafficking of the LDLR, and since COMMD1 mediates the trafficking of various transmembrane proteins, we determined the relative subcellular distribution of Commd1 in relation to Ldlr, Arh
and endocytic markers by performing a continuous sucrose gradient fractionation. The presence of Commd1, Ldlr, Arh and the endocytic proteins in fractions of WT mouse liver was determined by immunoblot analysis. Ldlr appeared in the middle and high-density fractions, whereas Commd1 was present in the low and middle density fractions (Fig. 5a). Commd1 and Ldlr were both detected in the same middle-density fractions. Furthermore, Commd1 co-sedimented with Arh, and the early endosomal marker, Eea-1. As demonstrated previously [14], only limited co-sedimentation with Ldlr and Arh was seen. A high percentage of Commd1 was also detected in the same fractions as the Rab11, a marker for the recycling endosomes [31].

Figure 5. COMMD1 is associated with LDLR. (a) Liver of WT chow fed mouse was homogenized and loaded on a continuous 10-40% sucrose gradient. Fractions were separated by ultracentrifugation and immunoblotted against Commd1, together with different endosomal and LDLR trafficking markers: Ldlr, Arh, Eea-1, Rab11. The figure represents the results of four independent experiments. * Commd1 and Ldlr were both detected in the same middle-density fractions (b) HEK293T cells were transfected with constructs expressing Flag-LDLR with either COMMD1-GST or GST alone. Interaction with COMMD1 was detected via pull-down using glutathione sepharose beads. (c) HEK293T cells were transfected with Flag-LDLR vector and interaction with endogenous COMMD1 was detected using immunoprecipitation with rabbit anti-Flag antibody. (d) HEK293T cells were transfected with Flag-LDLR vector together with either GST alone, COMMD1-GST, 1-118-GST (GST-tagged COMMD domain) or 119-190-GST (GST-tagged N-terminal region of COMMD1). Interaction with LDLR was detected by pull-down using glutathione sepharose beads. (e) HEK293T cells were transfected with Ha-COMMD1 construct together with GST alone, GST-LDLRct (GST-tagged cytosolic domain of LDLR) or GST-LDLRct Y807A (GST-tagged mutated cytosolic domain of LDLR). Interaction with COMMD1 was detected through pull-down with glutathione sepharose beads.
COMMD1 IS PHYSICALLY ASSOCIATED WITH THE LDLR

Since Commd1 co-sediments with the Ldlr, we determined whether COMMD1 associates with the LDLR. HEK293T cells were transiently transfected with constructs expressing LDLR-Flag together with either COMMD1 fused with glutathione S-transferase (GST) or GST alone (Fig. 5b). Cell lysates were incubated with glutathione (GSH) sepharose beads, and the presence of co-precipitated LDLR was detected by immunoblot analysis. A clear association between COMMD1 and LDLR was observed. This interaction was confirmed by using cell lysates of LDLR-Flag-transfected HEK293T cells, where endogenous COMMD1 was evidently present in the LDLR-Flag precipitates (Fig. 5c). To identify which region of COMMD1 is necessary for the formation of the COMMD1-LDLR protein complex, we transfected HEK293T cells with vectors co-expressing LDLR-Flag either with full length COMMD1, its N-terminal region outside of the COMMD domain (1-118-GST), or its C-terminal COMM domain (119-190-GST) (Fig. 5d). The interaction was only observed with the full-length COMMD1 protein or with the fragment encoding the COMM domain.

Intracellular transport of LDLR depends on various signals in its cytoplasmic tail, including the NPXY motif. Mutation of tyrosine (Y807) in the NPXY motif affects the binding of ARH, β-arrestin and clathrin, all of which are involved in LDLR trafficking. To determine whether the NPXY motif is also important for LDLR-COMMD1 association, we performed a GST-pull down assay with cell lysates expressing either the wild-type LDLR cytoplasmic tail fused with GST (GST-LDLRct) or mutant LDLRct Y807A (Fig. 5e). Substitution of tyrosine with alanine (Y807A) markedly abrogated the binding between LDLRct and COMMD1. Together, these results suggest that COMMD1 associates with the LDLR and that its binding with the LDLR depends on the NPXY motif in the cytoplasmic tail of the LDLR. In line with previous studies (reviewed by Fedoseienko et al. [32]), the above data suggest that COMMD1 is associated with endocytic compartments.

COMMD1 DEFICIENCY IN MOUSE EMBRYONIC FIBROBLASTS MODERATES LDL UPTAKE

Next, we investigated the role of Commd1 in the cellular uptake of LDL using Commd1 knockout (KO) mouse embryonic fibroblasts (MEFs) [24] (Fig. 6a). Fluorescently-labeled LDL (Dil LDL) was added to the medium, and cells were incubated at 4°C for 1 h in order to increase the binding of Dil LDL to the LDL receptor. Next, we induced endocytosis by placing the cells at 37°C for 5 min. Despite the increased mRNA (Fig. 6b) and protein expression of Ldlr (Fig. 6a) in Commd1 KO MEFs, we observed an approximately 40% decrease in LDL uptake in Commd1 KO MEFs compared to WT cells (Fig. 6c). To assess whether Commd1 deficiency specifically affects LDL uptake, we also measured the uptake of labeled transferrin (Tf) by the Tf receptor (TfR). The intracellular trafficking of TfR shows many similarities to LDLR regulation mechanistically; both receptor cargos are internalized...
by clathrin-mediated endocytosis [33]. No differences in transferrin uptake were observed between Commd1 KO and WT cells (Fig. 6c), suggesting that Commd1 mediates specifically the uptake of LDL in MEFs.

Figure 6. LDL uptake and LDLR degradation by PCSK9 is impaired in COMMD1-deficient MEFs. (a) Ldlr and Commd1 levels in WT and Commd1-knockout (KO) MEFs. (b) Gene expression of Ldlr in KO MEFs relative to WT cells. (c) In vitro uptake assay. Dil-labeled LDL [5 μg/ml] or Alexa-633-labeled Transferrin [5 μg/ml] was added to the serum-depleted medium and incubated with MEFs at 4°C for 1h and subsequently at 37°C for 5 min. Dil-labeled LDL uptake was measured by FACS analysis. The percentage of positive KO cells was plotted against values from WT cells. Data represent results of four independent uptake experiments. (d) MEFs were incubated with human recombinant PCSK9 [5 μg/ml] in a serum depleted medium for 0, 4, 8 h. Cells were lysed and subjected to immunoblot analysis. The presented blots are representative of four independent experiments. (e) Quantification of Ldlr protein optical density corrected for optical density of β-actin in PCSK9 assay. Values plotted are calculated as a percentage of density at T = 0. All group averages are presented with SEM. *P<0.05, **P<0.01, ***P<0.001.

**PCSK9-MEDIATED LDLR DEGRADATION IS IMPAIRED BY COMMD1 DEFICIENCY**

Degradation of the LDLR induced by extracellular PCSK9 depends on LDLR internalization and its subsequent routing to the lysosomes. To gain more insight into the mechanism of how COMMD1 mediates the LDLR function, we assessed the consequences of Commd1 deficiency on PCSK9-mediated LDLR proteolysis [7, 8]. For this, we incubated WT and Commd1 KO MEFs with recombinant PCSK9 for 4 h and 8 h and determined the total Ldlr levels by immunoblot analysis. Recombinant PCSK9 clearly induced the degradation of Ldlr in WT cells. However, upon depletion of Commd1, the PCSK9-mediated
proteolysis was markedly decreased (Fig. 6 d,e). These data suggest that COMMD1 participates in the intracellular sorting of LDLR.

**DISCUSSION**

An elevated level of circulating LDL cholesterol is a high risk factor for the development of cardiovascular diseases, including atherosclerosis. Despite broad knowledge about the regulation of cholesterol homeostasis, there are still many patients with high plasma cholesterol levels with unknown etiology, suggesting the presence of additional, unknown modulators of cholesterol homeostasis. Here we identified COMMD1 as a novel gene regulating the levels of circulating LDL cholesterol.

Hepatic Commd1 knockout mice and Commd1-deficient dogs are both hypercholesterolemic. Although in both species the main circulating lipoprotein is HDL, it was either Ldl, or VLDL/LDL that was primarily increased in these animal models, respectively. Our data suggest that COMMD1 modulates the plasma LDL levels via the LDLR; this is supported by several observations. First, we excluded the possibility that elevated plasma LDL-c level was caused by increased VLDL synthesis and secretion. Second, COMMD1 physically associates with the LDLR, which is consistent with a subset of Commd1 being co-sedimented with Ldlr. Third, ablation of Commd1 in MEFs reduces the uptake of labeled LDL, and finally, absence of Commd1 impairs PCSK9-mediated proteolysis of the Ldlr. Thus, it appears that COMMD1 mediates the intracellular trafficking of the LDLR, although the mechanism remains elusive.

These results are in line with previous findings on the biological function of COMMD1, which show that it modulates the biliary copper excretion via the copper transporting protein ATP7B [19, 21, 23, 34, 35]. COMMD1 physically associates with ATP7B. Under basal conditions, ATP7B is localized in the trans-Golgi network (TGN), but it redistributes to cytoplasmic vesicles when cells are exposed to high copper levels, and cycles back to the TGN when copper returns to normal physiological levels. Miyayama and colleagues [25] demonstrated that the loss of COMMD1 affects the retrograde transport of ATP7B back to the TGN in mouse hepatoma cells. However, another study suggested that COMMD1 promotes the proteolysis of misfolded ATP7B protein [23], but the latter observation could not be confirmed in vivo [21]. COMMD1 also coordinates the subcellular localization of several other transmembrane proteins, such as the epithelial sodium channel ENaC, Na-K-2Cl cotransporter NKCC1, and the cystic fibrosis transmembrane conductance regulator CFTR [36-40]. COMMD1 promotes the expression of CFTR and NKCC1 at the plasma membrane, but it reduces the level of δENaC at the cell surface, and targets δENaC to recycling endosomes. In all pathways a correlation between COMMD1 and the level of ubiquitination of its clients, suggesting that COMMD1 might be involved in a ubiquitin-dependent protein sorting.
In line with our results, COMMD1 colocalizes with various cellular compartments of the sorting machinery, such as early, late and recycling endosomes, and lysosomes [39-41]. Moreover, COMMD1 interacts with phosphorylated phosphatidylinositols, in particular with the phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) [41]. PtdIns(4,5)P$_2$ is a membrane-anchoring molecule for vesicular transport, which interacts with clathrin, ARH, and Dab2. All proteins bind to LDLR, and facilitate the internalization of LDLR [42-44]. Therefore, it is tempting to speculate that COMMD1 is an adaptor protein and participates in a dynamic network of proteins to sort the LDLR to the correct vesicular cellular compartment.

Together, our study has identified COMMD1 as a novel player in LDL cholesterol homeostasis. Our results and existing data on the biological role of Commd1 in vesicular trafficking indicate that COMMD1 facilitates the intracellular transport of LDLR. This discovery can lead to a better understanding of the molecular mechanism by which the LDLR is regulated, and possibly help with the development of new therapies to lower circulating cholesterol and reduce atherosclerosis. Furthermore, the regulatory function of COMMD1 in intracellular trafficking of transmembrane proteins appears to hold true for several pathways. Our findings are therefore relevant to discovering the general mechanistic details of how COMMD1 sorts its clients.

**METHODS**

**ANIMALS**

Hepatocyte-specific Commd1 knockout mice (Commd1$^{\DeltaHep}$) [21] were backcrossed for more than 8 generations in a C57BL/6J background. Ldl receptor-deficient mice (Ldlr$^{-/-}$) were purchased from The Jackson Laboratory (Bar Harbor, USA, stock number 002207). All mice were individually housed males, fed ad libitum with either standard rodent chow diet (RMH-B, AB Diets, the Netherlands) or, starting at 8-9 weeks of age, a high-fat, high-cholesterol (HFC) diet (45% calories from butter fat) containing 0.2% cholesterol (SAFE Diets), n=6-8. HFC feeding lasted for 20 weeks. Mice were sacrificed following a 4-hour morning fasting period. Tissues for mRNA and protein expression analysis were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was drawn by means of heart puncture, and plasma was isolated by centrifugation at 3000 rpm for 10 min. at 4°C. All animal studies were approved by either the Institutional Animal Care and Use Committee of the University of Groningen (Groningen, the Netherlands) or the Care and Use Committee of the University of Texas Southwestern Medical Center (Texas, USA). All canine samples originated from an earlier study [29].

**PLASMA CLEARANCE OF $^{[125I]}$LDL**

Mouse LDL was prepared and radiolabeled with sodium $^{125}$I. Clearance of $^{125}$I-labeled apoB (apoB48 plus apoB100) from plasma of WT and Commd1$^{\DeltaHep}$ mice was measured at the indicated time. All procedures were performed as described before [45].
IN VIVO VLDL-TG PRODUCTION

The experiment was performed on 10-13 week old chow-fed mice. After a 4 h morning fast, animals were intraperitoneally injected with poloxamer 407 (BASF, Ludwigshaven, Germany) solution in saline (1 g/kg body weight). Blood was drawn by retro-orbital puncture at the following time points: 0, 30, 60, 120, 240 min. Collected samples were used for TG determination and calculation of VLDL-TG production rate.

HEPATIC LIPID EXTRACTION

Liver homogenates prepared as 15% (w/v) solutions in PBS were subjected to lipid extraction according to the Bligh & Dyer method [46]. Obtained samples were used for further determination of cholesterol and TG content.

CHOLESTEROL AND TRIGLYCERIDE ANALYSIS IN PLASMA AND LIVER HOMOGENATES

Total cholesterol (TC) levels were determined using colorimetric assay (11489232, Roche Molecular Biochemicals, Almere, the Netherlands) with cholesterol standard FS (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) as a reference. Triglyceride (TG) levels were determined using Trig/GB kit (1187771, Roche Molecular Biochemicals) with Roche Precimat Glycerol standard (16658800) as a reference.

ANTIBODIES

In the experimental procedures described, the following antibodies were used: rabbit polyclonal antibody against COMMD1 (11938-1-AP, Proteintech Group, USA), rabbit polyclonal antibody against LDLR (PAB8804, Abnova GmbH, Heidelberg, Germany), rabbit polyclonal antibody against GST (Z-5) (sc-459, Santa Cruz Biotechnology, Inc., Heidelberg, Germany), goat anti-rabbit IgG (H + L)-HRP Conjugate (170-6515, Bio-Rad Laboratories BV, Veenendaal, the Netherlands), goat anti-mouse IgG (H + L)-HRP Conjugate (170-6516, Bio-Rad Laboratories BV), mouse anti-β-Actin (A5441, Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands), rabbit anti-Tubulin (AB4047, Abcam, Cambridge, UK), rabbit anti-Rab11 (700184, Invitrogen, Leek, the Netherlands), rabbit antiEEA1 (AB2900, Abcam). Rabbit anti-ApoB100 and rabbit anti-ApoA1 antibodies were a gift from Prof. A.K. Groen. Rabbit polyclonal antibody against ARH was a gift from Prof. H.H. Hobbs. Density analysis of Western blot images was performed using Image Lab 3.0.1 Software (Bio-Rad Laboratories).

EXPRESSION CONSTRUCTS

The following vectors were used in the experiments described: peBB-COMMD1-Flag [18, 47], peBB-GST, peBB-COMMD1-GST, peBB-1118-GST, peBB-119190-GST [48],
pcDNA3.1-Flag-LDLR, and pcDNA3.1-PCSK9. Full-length Flag-tagged LDLR receptor was obtained from Dr. N. Freedman [17] and subcloned into pDNA3.1. pcDNA3.1-PCSK9 was kindly provided by Dr. P. Costet [49].

GENE EXPRESSION ANALYSIS

Pieces of murine liver of approximately 100 mg were homogenized in 1 ml QIAzol Lysis Reagent (Qiagen). Total RNA was isolated by chloroform extraction. Isopropanol-precipitated and ethanol-washed RNA pellets were dissolved in RNase/DNase free water. 1 µg of RNA was used to prepare cDNA with the Quantitect Reverse Transcription Kit (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. 20 ng cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories BV) and 7900HT Fast Real-Time PCR Systems (Applied Biosystems). The following temperature program was used: 50°C/2 min., 95°C/10 min., 40 cycles with 95°C/15 sec and 60°C/1 min. Expression data was analyzed using SDS 2.3 software (Applied Biosystems) and the standard curve method of calculation. Mouse cyclophilin A was used as an internal control gene. Primers used for the expression studies are listed in Table S3.

FAST-PERFORMANCE LIQUID CHROMATOGRAPHY (FPLC)

Plasma samples within each murine or canine experimental group were pooled together and fractionated using the fast performance liquid chromatography (FPLC) method. All 50 collected fractions were analyzed to determine TC and TG content. Fractions containing LDL and HDL were further analyzed by means of immunoblot using anti-ApoA1 and anti-ApoB100 antibodies.

The total cholesterol distribution among the main lipoprotein classes of the individual plasma samples was measured using FPLC analysis as described previously but with some minor modifications [50]. In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and an UV-975 UV/VIS detector (Jasco, Tokyo, Japan). An extra PU 2080i-plus pump (Jasco) was used for in-line cholesterol RTU enzymatic reagent (Biomerieux, Marcy l’Etoile, France) addition at a flow rate of 0.1 mL/min. EDTA plasma was diluted 1:1 with Tris buffered saline and 30 µL sample/buffer mixture was loaded on a Superose 6 HR 10/30 column (GE Health care, Life sciences division, Diegem, Belgium) for lipoprotein separation at a flow rate of 0.31 mL/min. Chromatographic profiles of commercially available plasma lipid standards (SKZL, Nijmegen, the Netherlands) served as a reference.

CELL LINES AND CELL CULTURE

Human embryonic kidney 293T cells (HEK293T) (obtained from ATCC, Manassas, VA, USA) and mouse embryonic fibroblasts (MEFs) [24] were cultured using Dulbecco’s
Modified Eagles Medium – DMEM with Glutamax (GIBCO), supplemented with 10% FBS (Invitrogen) and antibiotics.

**SUCROSE GRADIENTS**

Sucrose gradient separation of fractions obtained from fresh liver tissue was performed as described before by Jones et al. [14] with the following modifications: chow-fed mice were fasted for 4 hours before sacrificing, liver homogenates were spun for 16 h using a Beckman Coulter ultracentrifuge equipped with a swinging bucket rotor SW55 Ti at 40,000 rpm, and fractions of 285 µl were collected from the top of the tube. 1/10 of each fraction was mixed with SDS sample buffer and used for further immunoblot analysis.

**IMMUNOPRECIPITATION ANALYSIS**

The immunoprecipitation experiments were performed as described before [51].

**DIL LDL UPTAKE ASSAY**

Culture medium was replaced with empty DMEM culture medium supplemented with Dil LDL [5 µg/ml] (Molecular Probes, Invitrogen). Cells were kept for 1 h at 4°C, then for 5 min. at 37°C. Then they were immediately placed on ice, washed with cold PBS and scraped. As a control for the specificity of the investigated uptake pathway, cells were incubated with Alexa633-Transferrin [5 µg/ml]. Cells were centrifuged at 300 g for 5 min. at 4°C and resuspended in 50 µl of FACS buffer (PBS with 2% FCS and 5 mM EDTA) supplemented with additional 5% FBS. Cell pellets were vortexed, 2 ml of FACS buffer was added, samples were centrifuged as before and resuspended in 200 µl of the FACS buffer. Cells were kept on ice at all times and subjected to immediate FACS analysis. The number of positive cells was counted and recorded as a percentage of the whole population. Data of four independent experiments were presented relative to uptake results of the control WT population (set as 100%).

**PCSK9 ASSAY**

Cells were grown in 12-well plates and incubated for 0, 4 h and 8 h with 5 µg/ml recombinant PCSK9 (#PC9-H5223 ACRO Biosystems, Greater London, UK) in an empty medium. Cells were lysed in sample buffer and analyzed by Western Blot.

**STATISTICAL ANALYSIS**

All results are expressed as a mean ± SEM. Statistical analysis was performed using Prism 5.00 for Windows (GraphPad Software, CA, USA) and the unpaired Student’s t test. Results of P<0.05 were considered statistically significant: *P<0.05, **P<0.001, ***P<0.0001.
ACKNOWLEDGEMENTS

We would like to thank Niels Kloosterhuis and Henk van der Molen for technical assistance with mouse experiments, Prof. Uwe Tietge for providing us with P407, Tineke Jager for help with plasma lipid analysis, Karin Klappe for help with setting-up the sucrose gradient experiments. We thank A.K. Groen for rabbit anti-ApoB100 and anti-ApoA1 antibodies and H.H. Hobbs for rabbit polyclonal antibody against ARH. We thank Jackie Senior for critically reading the manuscript. The work was partly funded by the Groningen University Institute for Drug Exploration (GUIDE) and TransCard FP7-603091–2.
COMMD1 and LDLR trafficking

REFERENCES


COMMD1 and LDLR trafficking


SUPPLEMENTARY FIGURES

Figure S1. Total plasma cholesterol levels in WT, Commd1^{ΔHep}, and Alb-Cre mice. Plasma total cholesterol levels of (a) 10-week old chow-fed wild-type and hepatic Commd1 knockout mice and (b) 20-week long HFC-fed Alb-Cre control mice. Group averages are plotted with SEM error. Significance in (a) and (b) was tested against the WT mice. **P<0.01

Figure S2. Hepatic copper concentrations. Hepatic copper levels in (a) dogs heterozygous (+/-) or homozygous (-/-) for loss-of-function mutation of COMMD1 and (b) copper toxicosis in unaffected (unaff.) and affected (aff.) Labrador retrievers. Group averages are plotted with SEM error. Significance in (b) was tested against the unaffected dogs. *P<0.05

Figure S3. Cholesterol distribution among the lipoprotein particles. (a) and (b) Pooled plasma samples from (a) chow-fed and (b) 20-week long HFC-fed WT and Commd1^{ΔHep} mice were size-fractionated by FPLC. Total cholesterol (TC) and cholesterol content of each fraction was determined. (c) Triglyceride (TG) levels in FPLC-fractionated plasma samples from chow-fed and (d) 20-week long HFC-fed WT and Commd1^{ΔHep} mice. Group averages are plotted with SEM error. Significance in (a) and (b) was tested against the WT control group. *P<0.05, **P<0.01
### SUPPLEMENTARY TABLES

#### Table S1. Parameters of WT and *Commd1*^A^Hep mice fed chow diet. Values ±SEM.

<table>
<thead>
<tr>
<th>Age</th>
<th>28 weeks</th>
<th>28 weeks</th>
<th>Significance</th>
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<tr>
<td>Genotype</td>
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<td>n</td>
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<td>7</td>
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<td>BW [g]</td>
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<td>Liver TC [μmol/g liver]</td>
<td>2.41 ± 0.43</td>
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<td>84.63 ± 8.93</td>
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#### Table S2. Parameters of WT and *Commd1*^A^Hep mice fed HFC diet. Values ±SEM.

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<tr>
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<td>BW [g]</td>
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<td>Liver Copper [μg/g dry liver]</td>
<td>17.52 ± 2.11</td>
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#### Table S3. qRT-PCR primer sequences.

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