CHAPTER 4

The COMMD family regulates plasma LDL Levels and attenuates atherosclerosis through stabilizing the CCC complex in endosomal LDLR trafficking

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Abstract

Rationale: Copper Metabolism MURR1 Domain-containing (COMMD) proteins are part of the COMMD-CCDC22-CCDC93 (CCC) complexes facilitating endosomal trafficking of cell surface receptors. Hepatic COMMD1 inactivation decreases CCDC22 and CCDC93 protein levels, impairs the recycling of the low-density lipoprotein receptor (LDLR), and increases plasma LDL cholesterol levels in mice. However, whether any of the other COMMD members functions similarly as COMMD1, and whether perturbation in the CCC complex promotes atherogenesis remains unclear.

Objective: To unravel the contribution of evolutionarily conserved COMMD proteins to plasma lipoprotein levels and atherogenesis.

Methods and Results: Using liver specific Commd1, Commd6 or Commd9 knockout mice we investigated the relation between the COMMD proteins in the regulation of plasma cholesterol levels. Combining biochemical and quantitative targeted proteomic approaches, we found that either hepatic COMMD1, COMMD6 or COMMD9 deficiency resulted in massive reduction in the protein levels of all ten COMMDs. This decrease in COMMD proteins levels coincided with destabilization of the core (CCDC22, CCDC93, C16orf62) of the CCC complex, reduced cell surface levels of LDLR and LRP1, followed by increased plasma LDL cholesterol levels. To assess the direct contribution of the CCC core in the regulation of plasma cholesterol levels, Ccdc22 was deleted in mouse livers via CRISPR/Cas9-mediated somatic gene editing. CCDC22 deficiency also destabilized the complete CCC complex, and resulted in elevated plasma LDL cholesterol levels. Finally, we found that hepatic disruption of the CCC complex exacerbates dyslipidemia and atherosclerosis in ApoE3*Leiden mice.

Conclusions: Collectively, these findings demonstrate a strong interrelationship between COMMD proteins and the core of the CCC complex in endosomal LDLR trafficking. Hepatic disruption of either of these CCC components causes hypercholesterolemia, and exacerbates atherosclerosis. Our results indicate that not only COMMD1, but all other COMMDs and CCC components may be potential targets for modulating plasma lipid levels in humans.

Keywords: hypercholesterolemia, low-density lipoprotein receptor (LDLR), LDLR-related protein 1 (LRP1), endosome, liver, WASH complex, CRISPR, atherosclerosis, low-density lipoprotein cholesterol, transgenic mice.
**Introduction**

Clearing circulating atherogenic low-density lipoprotein (LDL) cholesterol is highly dependent on the LDL receptor (LDLR) in hepatocytes (1, 2). LDL binds to LDLR at the cell surface, and the LDL-LDLR complex is endocytosed via clathrin-coated pits. On entering the endosomal network LDLR is subjected to one of two fates decisions, either LDLR is sorted into the lysosome for proteolysis or LDLR is reused and is recycled back to cell surface to take up the next cargo (3, 4). In the recent years, several proteins, such as proprotein convertase subtilisin/kexin type 9 (PCSK9) and inducible degrader of the LDLR (IDOL) (5-7), have been identified that avert the reuse of LDLR through directing LDLR to the lysosomes, but the mechanism preventing the lysosomal fate and directing LDLR back to the cell surface is still not fully elucidated.

We recently uncovered the Copper Metabolism MURR1 Domain 1 (COMMD1) as a novel factor in endosomal LDLR trafficking (8). Loss of hepatic COMMD1 impairs the endocytic LDLR recycling, which subsequently results in increased levels of plasma LDL cholesterol in dogs and mice (8). In addition to LDLR, COMMD1 also facilitates the endosomal trafficking of other receptors, such as the copper transporting protein ATP7A and ATP7B, to maintain copper homeostasis (9-11). COMMD1 is assembled into the CCC complex together with C16orf62 and the coiled-coil proteins CCDC22 and CCDC93 (9). The CCC complex localizes to endosomes through physical association with the Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) complex (9). The recruitment of the CCC and WASH complexes to the endosomes is dependent on the retromer subunit VPS35 (8, 9, 12). WASH activates the actin-related protein 2/3 (Arp2/3) complex to deposit branched actin filaments on endosomes, which is essential for the architecture of the endosomal and lysosomal network, and endosomal receptor trafficking (12-14). The exact function of the CCC complex within the endosomal sorting process is still unknown, but it is hypothesized to coordinate selective endosomal receptor trafficking.

COMMD1 belongs to the COMMD family, which consists of ten members, each characterized by a carboxyl-terminal COMM domain, which mediates physical interaction with other COMMD proteins (15). COMMD proteins are ubiquitously expressed and are conserved throughout evolution from lower organisms to higher vertebrates (15, 16). The COMMD proteins have a vital role in mouse embryonic development, as Commd1, Commd6, Commd9 or Commd10 knockout mice are embryonically lethal (17-20), and die at different stages of embryogenesis, suggesting non-redundant functions of COMMD proteins during
development. Although several studies have shown that the ten COMMD proteins exist in large macromolecular complexes containing the CCC core components CCDC22, CCDC93 and C16orf62 (9, 18, 21, 22), their role in the regulation of plasma cholesterol levels and atherogenesis has yet to be determined.

This study provides novel insights into the molecular organization of the endosomal sorting machinery in the coordination of intracellular trafficking of members of the LDLR family, such as LDLR and LDLR-related protein 1 (LRP1), and imply that all ten COMMD proteins and the CCC core components may be potential targets modulating plasma lipid levels in humans.

Results

Hepatic Commd6 ablation results in increased plasma LDL cholesterol levels

To understand the biological role of COMMD proteins in lipoprotein metabolism, we used COMMD6 as a prototype for the COMMD family, as COMMD6 primarily consists of the COMM domain (15), a region of the COMMD proteins that is crucial for multiple protein-protein interactions, including the interaction with LDLR and the CCC core component CCDC22 (8, 9). A Commd6 conditional knockout mouse model was generated by flanking exon 3 of Commd6 with loxP sites (Fig. S1A). Mouse genotypes were confirmed by Southern blot analysis and PCR (Fig. S1B, C). To assess the role of hepatic COMMD6 in lipoprotein metabolism, we deleted Commd6 in hepatocytes by crossbreeding Commd6F/F mice with transgenic mice expressing Cre-recombinase specifically in hepatocytes (Alb-Cre). Liver-specific Commd6 knockout mice (Commd6ΔHep) were born in the expected Mendelian frequency, and the absence of COMMD6 in hepatocytes was validated on mRNA and protein levels by quantitative RT-PCR and Western blot analysis, respectively (Fig. S1D, E). No overt differences were observed between hepatic Commd6 knockout animals and Commd6F/F littermate controls (hereafter referred to as WT), with regard to body weight, liver weight and liver histology (Fig. S2A, B, C). However, hepatic COMMD6 deficiency resulted a ~37% increase (P<0.01) in plasma total cholesterol (TC) levels on a chow diet and a ~40% increase (P<0.001) in plasma TC levels on a HFC compared to WT animals (Fig. 1A). Plasma triglyceride (TG), hepatic cholesterol and triglyceride concentrations were unaltered by hepatic Commd6 ablation (Fig. 1B, C, D). Using a specifically developed targeted proteomics approach we observed that the plasma levels of ApoB100 were significantly elevated (P<0.01) in Commd6ΔHep mice, both upon chow and HFC diet feeding (Fig. 1D, E, insets). Plasma ApoA1 levels were also increased (P<0.01) in Commd6ΔHep mice, but only after feeding chow diet and not upon HFC diet feeding (Fig.
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In line with the elevated plasma ApoB100 levels, the lipoprotein profile of WT and Commd6ΔHep animals fed a chow or HFC diet (Fig. 1E, F, Fig S2D, E) showed an increase in plasma LDL cholesterol in Commd6 knockout animals. Intriguingly, these data demonstrate that, similar to Commd1 (8), hepatic Commd6 ablation causes hypercholesterolemia.

COMMD6 colocalizes with the WASH complex and retromer

Previous in vitro data indicated that COMMD1 and the WASH complex act together to facilitate endosomal trafficking of LDLR (9). We investigated whether COMMD6 is also associated with the WASH complex on the endosome. Since there are no appropriate antibodies to determine the subcellular localization of COMMD6 by immunofluorescence staining, we used the CRISPR/Cas9 gene editing technology to fuse a V5-epitope tag to
endogenous **COMMD6** in HEK293T cells. These cells were targeted with a sgRNA recognizing the stop codon of **COMMD6** and a corresponding repair template to incorporate the V5-tag before the stop codon (Fig S3A, B, C, D, E). Immunofluorescence staining showed that COMMD6 strongly colocalizes with WASH1, FAM21 and COMMD1, almost to a similar degree as seen between COMMD1 and WASH1 and FAM21 (Fig. S4A, B, C and S5A). The recruitment of the WASH complex and COMMD1 to the endosomes rely on the retromer subunit VPS35 (8, 9, 12), and therefore we examined COMMD6 localization relative to VPS35. COMMD6 overlapped with VPS35 almost to the same extent as COMMD1 and VPS35 (Fig. S4A, B, C and S5A). Next, we determined the effect of COMMD6 deficiency on the total and surface levels of LDLR in primary hepatocytes. Ablation of COMMD6 impaired the total expression and the expression of LDLR at the plasma membrane (Fig. S5B). Similar effect on the total and plasma membrane expression of LRP1 was seen (Fig. S5B). The reduced total LDLR and LRP1 levels can likely be explained by increased lysosomal degradation due to impaired receptor recycling, similar as seen in WASH1 deficient mouse fibroblast cells (8, 23). These observations indicate that both COMMD6 and COMMD1 are localized in a WASH and retromer enriched subcompartment of the endosome, and suggest that COMMD6 may also participate in the CCC-WASH axis in endosomal sorting of receptors, including LDLR and LRP1.

**COMMD6 is indispensable for COMMD1, CCDC22 and CCDC93 protein expression**

To further evaluate the biological role of COMMD6 in CCC complex functioning, we stably downregulated **COMMD6** expression in cells of different origins. The protein and mRNA levels of COMMD6 were both markedly reduced in HEK293T and RAW264.7 cells expressing short hairpin RNA (shRNA) targeting **COMMD6** (Fig. 2A, B). Unexpectedly, silencing of **COMMD6** significantly decreased COMMD1 protein levels (Fig. 2A) without affecting **COMMD1** mRNA (Fig. 2B). However, silencing of **COMMD1** did not reduce COMMD6 protein levels (Fig. 2A). Taken the effect of liver specific ablation of **Commd6** on plasma lipid levels, we also silenced **COMMD6** in the hepatocellular carcinoma cell lines HepG2 and Huh7, and found similar effects as described above (Fig. 2C). To assess whether reduced COMMD1 levels in COMMD6 knockdown (KD) cells are caused by increased proteasomal degradation of COMMD1, we treated control and COMMD6 KD cells (HEK293T and RAW264.7) with the proteasomal inhibitor MG132. Blocking proteasome activity partially restored COMMD1 protein levels in COMMD6 KD cell lines (Fig. 2D), suggesting that COMMD1 instability is partially ubiquitin dependent. In line with previous studies (15, 24), we observed that COMMD1 and COMMD6 are physically associated with each other, as determined by co-immunoprecipitation assay (Fig. 2E).
The results of COMMD6 ablation on COMMD1 levels in different cell lines led us to investigate the effect of hepatic COMMD6 inactivation on the protein levels of COMMD1, and the CCC proteins regulate plasma lipid levels.
components CCDC22 and CCDC93 in mice. As observed in COMMD6 KD cells, depletion of Commd6 in mouse hepatocytes blunted COMMD1 protein levels, accompanied by reduced protein levels of the CCC core components CCDC22 and CCDC93 (Fig. 2F). The decreased COMMD1, CCDC22 and CCDC93 levels were not caused by aberrant gene expression (Fig. 2G). These data indicate that COMMD6 form a protein complex with COMMD1 that is indispensable for the protein stability of CCC complex in endosomal receptor recycling.

**Stability of COMMD proteins associated with the CCC complex depends on other COMMD proteins**

Since all COMMDs can participate in the CCC complex through an interaction with CCDC22 (16), we assessed whether COMMD6 deficiency also affects the protein levels of other COMMD family members. Deletion of hepatic Commd6 markedly decreased all detectable COMMD proteins (Fig. 3A), however not all to the same degree (Fig. 3A), as COMMD3 levels were only moderately decreased compared to other COMMD proteins (Fig. 3A). The adverse effect of Commd6 deletion on COMMD1 protein levels made us decide to also assess the consequence of hepatic COMMD1 deficiency on the protein levels of COMMDs in mice. The effect of hepatic Commd1 deletion on COMMD protein amounts was similar to Commd6 depletion (Fig. 3A, B).

Given the difficulty in detecting all COMMD proteins using specific antibodies, we measured the COMMD protein concentrations in liver homogenates with a specifically developed targeted proteomics assay. We used isotopically labeled standards combined with LC-MS analysis for accurate quantification of the proteins studied (25). In line with the Western blot results, hepatic Commd1 and Commd6 depletion in mice significantly reduced the expression all COMMD proteins (Fig. 3C, Table S1). Furthermore, the CCC core subunits were reduced by ~70% (P<0.05) in COMMD1 and COMMD6 deficient livers (Fig. 3C, Table S1), while the expression of the retromer subunits, LDLR and LRP1 were unaffected (Fig. 3C, Table S1). Intriguingly, the expression of the WASH component WASH1 was increased by ~50% (P<0.05) following loss of COMMD1 or COMMD6. Altogether, these results suggest that the integrity of CCC core complex (CCDC22, CCDC93, C16orf62), and the protein expression of all COMMDs depend on the expression of COMMD1 and COMMD6.

Although the amount of most COMMD proteins was reduced by ~70% in Commd1 and Commd6 KO livers compared to WT livers (P<0.05), COMMD3 protein levels were only decreased by ~50% (P<0.05). To understand which fraction of COMMD3 is adversely affected by COMMD1 deficiency we assessed the relative distribution of COMMD1 and
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COMMD3 within CCC-, WASH- and retromer-positive endocytic compartments in cells using sucrose gradient fractionation. In WT livers, COMMD1 and COMMD3 had a very similar distribution, and partially co-sedimented with vesicles containing the CCC (CCDC22 and CCDC93) and WASH (WASH1, FAM21) complexes. Furthermore, a percentage of COMMD1 and COMMD3 were present in the same fractions as LDLR (Fig. 4A). Hepatic COMMD1 inactivation not only blunted the expression of CCDC22 and CCDC93 (Fig. 4A), but also markedly abolished COMMD3 associated with the CCC and WASH complexes and LDLR, without affecting COMMD3 present in other fractions (Fig. 4A). These results suggest that the main disturbance of COMMD3 levels upon COMMD1 deficiency is on the fraction of COMMD3 that is associated with the CCC and WASH complexes, and LDLR.

To assess whether all COMMD proteins and CCC core subunits bind to COMMD1 in mouse
primary hepatocytes, we measured the amount of COMMD and CCC core molecules in COMMD1-immunoprecipitates using a targeted proteomics approach. We found that all COMMD and CCC core proteins interact with COMMD1 (Fig. 4B). These proteins were not detected in IgG negative control immunoprecipitates.

The association of COMMD1 with all COMMD proteins made us decide to investigate the role of other COMMDs on the protein stability of the COMMD family and CCC core subunits. We quantified the COMMD and CCC proteome also in Commd9ΔHep livers by proteomics analysis. Ablation of Commd9 reduced the expression of the CCC complex subunits CCDC22, CCDC93 and C16orf62 (P<0.05) to a similar extent as COMMD1 or COMMD6 inactivation (Fig. 3C, Table S1). However, COMMD9 appears to be less essential for COMMD stability, because COMMD1, 2, 3, 6, and 8 expression were only reduced by 40–50% (P<0.05) following Commd9 depletion (Fig. 3C). This partial reduction in COMMD1 and COMMD6 levels was confirmed by Western blot analysis (Fig. 4C, D).

As heterozygous loss of Commd1 or Commd6 does not alter plasma cholesterol levels (8, 10, 26), and since COMMD1 and COMMD6 levels were only reduced by ~40% in COMMD9 deficient livers (Fig. 3C, Table S1), we proceeded by investigating the effect of hepatic COMMD9 deficiency on plasma TC levels. Ablation of hepatic Commd9 increased plasma TC levels (P<0.001), ApoB100 levels (P<0.001), including plasma LDL cholesterol (Fig. 6A, B), to a similar degree as hepatic Commd1 (5) or Commd6 depletion (Fig. 1A), without effecting body weight, liver weight or liver histology (Fig. S6C, D).

Taken together, these results indicate that the protein expression of COMMDs relies on each other through forming multiprotein complexes and the degree of dependency differs between COMMD proteins. In contrast, the stability of the CCC core components (CCDC22, CCDC93, C16orf62) seems to be entirely dependent on the COMMD proteins. This specific decrease in the CCC core components in all three models of hepatic COMMD deficiency coincides with elevated plasma cholesterol levels.

**Hepatic ablation of the CCC component CCDC22 increases circulating cholesterol levels**

To directly examine whether this specific loss of the CCC core underlies the increased circulating plasma cholesterol in our mouse models, we targeted one of its key component Ccdc22 in hepatocytes of mouse livers using CRISPR/Cas9 gene editing technology. Three gRNAs were designed to simultaneously target exon 1 and exon 2 of Ccdc22 (Fig. S7A). These gRNAs (Ad-gRNA_Ccdc22) were directed to the liver of either WT or liver-specific
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Figure 4. Depletion of either COMMD1 or COMMD9 perturbs the formation of the CCC complex. (A) Western blotting of WT and Commd1 KO mouse liver homogenates fractionated by ultracentrifugation on 10–40% sucrose gradient. (B) Number of COMMD and CCC core components associated with COMMD1 in primary hepatocytes as determined by immunoprecipitation assay and subsequently targeted proteomics analysis (n=5). (C) COMMD1, COMMD6, CCDC22 and CCDC93 protein expression in livers of WT and Commd9KO were determined by immunoblotting. (D) Quantification of Western blot results depicted in (C). Group averages and SEM are shown. *p<0.05, **p<0.01, ***p<0.001 (compared to control groups).

Cas9-expressing C57BL/6J mice using an adenoviral gene delivery system. One week before virus administration, blood was collected to ensure the plasma cholesterol values of WT and
Cas9-expressing C57BL/6J mice were similar (Fig. S7B). Twenty-one days after virus injection, blood and tissues were collected for analysis (Fig. S7B). Hepatic expression of the three gRNAs reduced CCDC22 expression by ~70% and diminished the levels of CCDC93, C16orf62 and COMMD1 (Fig. 5A, B). Targeted proteomics analysis showed that the expression of all COMMD proteins was significantly reduced by targeting Ccdc22, with the exception of COMMD6 (Fig. S7C). CCDC22 inactivation did not alter the body weight, liver weight, or cause overt liver pathology (Fig. S7C, D), but it increased plasma TC levels by ~35% (P<0.01) compared to WT mice injected with Ad-gRNA_Ccdc22 (Fig. 5C). Lipoprotein fractionation by FPLC revealed that increased plasma cholesterol levels were due to increased plasma LDL cholesterol (Fig. 5D), supported by the increased plasma ApoB100 levels (Fig. 5E). In summary, these results indicate that proper formation of the core of the CCC complex in hepatocytes is required to control plasma LDL cholesterol levels.

**Hepatic CCC complex ablation exacerbates hyperlipidemia and accelerates atherosclerosis**

The reduced surface levels of both LDLR and LRP1 upon inactivation of the CCC complex (Fig. S5B) suggest that the CCC complex orchestrates trafficking of both receptors. As LDLR can compensate for the loss of LRP1, studying LRP1 functioning in the clearance of plasma ApoE and ApoB-containing particles in mice is only possible in a hepatic LDLR-deficient background (27). To assess the role of the CCC complex in LRP1 functioning, we overexpressed a gain-of-function variant of PCSK9-D377Y in the liver of WT and Commd1ΔHep mice by intravenously injecting an adeno-associated virus (AAV) expressing human PCSK9-D377Y (28). PCSK9-D377Y expression blunted the protein expression of LDLR, but not LRP1, in the liver of WT and Commd1ΔHep mice (Fig. 6A). As expected, LDLR deficiency led to a significant increase in plasma TC and TG (Fig. 6B) in WT mice. Animals that lack the protein expression of both COMMD1 and LDLR showed higher increase in plasma TC and TG levels as compared to mice only deficient for LDLR in the liver (Fig. 6B). The plasma concentrations of ApoB100 and ApoB48 were examined by Coomassie staining and showed that LDLR deficiency resulted in a marked increase in both apolipoproteins (Fig. 6C). In contrast, hepatic ablation of both LDLR and COMMD1 further increased ApoB48 levels as compared to control animals (Fig. 6C). This dramatic increase in ApoB48 in mice deficient for both COMMD1 and LDLR is supported by targeted proteomics analysis of the plasma samples of both groups in which we specifically determined the plasma concentration of ApoB100 and ApoB48 (Fig. 6D). This further rise in both plasma TC and ApoB48 levels was also reflected by the marked change in their plasma lipoprotein profile (Fig. 6E). Hepatic deficiency of both COMMD1 and LDLR led to a large increase of plasma lipoproteins in the chylomicron remnant/VLDL and LDL size range. A similar increase in plasma ApoB48 levels and change in lipoprotein profile has been
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seen upon hepatic depletion of both Ldr and Lrp1 (27), suggesting that the CCC complex also facilitates the functioning of LRP1.

Next, we investigated the contribution of CCC-mediated endosomal LDLR/LRP1 trafficking on the progression of atherosclerosis. We used ApoE3*Leiden (ApoE3*L) transgenic mice as a model for atherosclerosis (29) since these mice are hyperlipidemic, develop atherosclerosis and express LDLR in their livers (30), which we thought to be essential in evaluating the contribution of the CCC complex to dyslipidemia and atherogenesis. Liver-specific Commd1 knockout (Commd1<sup>ΔHep</sup>) mice were crossbred with ApoE3*L mice to ablate Commd1 in hepatocytes (ApoE3*L;Commd1<sup>ΔHep</sup>). Hepatic COMMD1 deficiency did not affect the total protein expression of LDLR and LRP1 in the liver of ApoE3*L (Fig. S8A) mice fed a HFC diet

Figure 5. Hepatic CRISPR/Cas9-mediated Ccdc22 editing increases plasma cholesterol levels. (A) Protein levels of CCDC22, CCDC93 and COMMD1 determined by immunoblotting. (B) Quantification of immunoblot results depicted in (A). (C) Total cholesterol levels in of WT and Cas9 mice on chow, 7 days before and 21 days after injection with Ad-gRNA-Ccdcc22 (n=5-6). (D) The average total cholesterol levels of FPLC fractionated plasma of WT and Cas9 mice 21 days after Ad-gRNA-Ccdc22 injection. (E) Plasma ApoB100 and ApoA1 levels of WT and hepatic CCDC22 deficient mice, indicated by fold change versus WT controls (n=4-6). Group averages and SEM are shown. *p<0.05, **p<0.01, ***p<0.001 (compared to control groups)
for 12 weeks but resulted in a dramatically rise in plasma TC and TG levels (~100% increase, P<0.001) (Fig. 7A). VLDL- and LDL-cholesterol levels were notably increased by inactivation of the CCC complex (Fig. 7B), supported by the increased plasma ApoB100 levels. This rise in plasma cholesterol levels coincided with ~50% larger atherosclerotic lesion area (P<0.01) in the aortic root of these animals (Fig. 7C, D). Atherosclerotic lesions in the aortic root were small and foam-cell rich (Fig. 7C, D). We then assessed atherosclerotic lesion area in the aortic arch by performing en face analysis using Oil Red O staining. We observed no atherosclerotic lesions in the aortic arch of both genotypes (Fig. S8B), indicating that atherogenesis did not yet develop in this part of the aorta. Taken together, hepatic perturbations in the CCC complex exacerbates hyperlipidemia - likely due to a defect in LDLR and LRP1-mediated lipoprotein clearance - and accelerates atherosclerosis in ApoE3*L mice.

Figure 6. CCC complex inactivation further increases dyslipidemia in hepatic LDLR deficient mice. (A) Hepatic LDLR and LRP1 protein levels of uninjected and AAV-PCSK9-D377Y-injected WT and Commd1ΔHep mice. (B) Plasma total cholesterol and triglyceride levels of WT and Commd1ΔHep mice before and 3 weeks after injection of AAV-PCSK9-D377Y (n=9-10). (C) Plasma of uninjected and AAV-PCSK9-D377Y-injected WT and Commd1ΔHep mice was separated by SDS gel electrophoresis and ApoB100 and ApoB48 were visualized by Coomassie staining. (D) Plasma ApoB100, ApoB48, and ApoA1 levels of WT and Commd1ΔHep mice 3 weeks after injection of AAV-PCSK9-D377Y (n=6). (E) Average total cholesterol in FPLC fractionated plasma of AAV-PCSK9-D377Y-injected WT and Commd1ΔHep mice (n=9-10). Group averages and SEM are shown. *p<0.05, **p<0.01, ***p<0.001 (compared to controls).
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Discussion

Removal of atherogenic LDL cholesterol from the circulation is highly dependent on hepatic LDLR, and progress has been made in our understanding of how LDL trafficking is mechanistically coordinated. Recently, the CCC complex - a multiprotein complex associated with vital elements of the endocytic sorting machinery (9) – was identified as a novel factor of endosomal LDLR trafficking ((8), Fig. S9). In this study, we revealed that the integrity of the CCC core components, namely CCDC22, CCDC93 and C16orf62, is not exclusively controlled by COMMD1 (8), but by numerous members of the COMMD family (Fig. S9). In addition, our data also suggest that the stability of the COMMDs in hepatocytes relies on the CCC core component CCDC22, indicating a strong interrelation between the COMMD family and the core of the CCC complex in endosomal trafficking of cell surface receptors, including LDLR.

Figure 7. Destabilization of the CCC complex exacerbates dyslipidemia and atherosclerosis. (A) Total cholesterol and triglyceride levels in plasma of ApoE3*L and ApoE3*L;Commd1ΔHep mice fed a high-fat high-cholesterol (HFC) diet for 12 weeks (n=8–11). (B) Average total cholesterol in FPLC fractionated plasma of mice fed a HFC diet for 12 weeks. (C) Representative images of toluidin blue staining of aortic root of APOE3*L and ApoE3*L;Commd1ΔHep mice after 12 weeks of HFC feeding. (D) Quantification of plaque size in ApoE3*L and ApoE3*L;Commd1ΔHep mice after 12 weeks of HFC feeding. Lesion area is the average of 10 cross-sections per animal (n=8-11). Group averages and SEM are shown. **p<0.01, ***p<0.001 (compared to controls).
and LRP1 (8).

It has been implicated that the COMMDs form heterogeneous CCC complexes that facilitate receptor-specific endosomal trafficking (9, 18, 21, 22), but their contribution in the formation of the CCC complex in endosomal LDLR trafficking was still unclear. To investigate this, we generated and characterized different liver specific Commd knockout mouse models (Commd1, Commd6 and Commd9), and we identified for the first time that the stability of the core of the CCC complex depends on numerous COMMDs, as CCDC22, CCDC93 and C16orf62 levels were massively reduced upon hepatic deletion of Commd1, Commd6 or Commd9. This loss of the CCC core subunits was correlated with elevated plasma LDL cholesterol levels in all our models (8), indicating that specific disruption of the CCC core leads to the hypercholesterolemic phenotype (8, 11). Indeed, using CRISPR/Cas9-mediated somatic gene targeting, we achieved extensive reduction in hepatocyte CCDC22 levels, and provided evidence for a direct role for the core of the CCC complex in the regulation of plasma cholesterol levels in mice. In addition, we found that perturbation of the CCC complex contributes to the progression of atherosclerosis in ApoE3*L mice, a mouse model with a human-like lipoprotein profile. Although the total protein levels of hepatic LDLR and LRP1 in WT and ApoE3*L mice are not affected upon CCC inactivation, this study and our previous work (8) showed that hepatic CCC deficiency reduces surface levels of LDLR and LRP1 – likely due to impaired endosomal receptor trafficking (8) – leading to increased plasma LDL cholesterol levels and accelerated atherosclerosis (Fig. S9). In humans, mutations in CCDC22 cause the severe developmental disorder X-linked intellectual disability (XLID), and we recently showed that the CCDC22 mutations are also correlated with hypercholesterolemia in these patients (8). In this study, we provide genetic evidence that CCDC22 insufficiency directly cause high plasma LDL cholesterol levels in XLID-patients, and establish that CCC complex-mediated LDLR trafficking is conserved between mice and humans. However, whether human genetic variants in COMMDs, CCDC93 and C16orf62 exist that are associated with plasma lipid levels and cardiovascular events needs to be validated.

This study shows also for the first time that the expression of the COMMD proteins in hepatocytes relies on each other, but intriguingly the degree of dependency differs between COMMD proteins. For example, COMMD5 and COMMD10 are reduced by ~70%, whereas COMMD3 is only diminished by 50% upon Commd1, Commd6 or Commd9 depletion. The exact reason for these differences is unclear, but our cell fractionation experiments showed that only the fraction of COMMD3 proteins associated with the CCC and WASH complexes are adversely affected following Commd1 deletion. These results suggest that the portion
of the COMMDs assembled into the CCC complexes is particularly disturbed by removal of another COMMD protein. The fraction of a COMMD protein that is not affected by the loss of another COMMD protein is likely present in other cellular compartments, where they likely mediate other biological activities, similarly to what has been reported for COMMD1, which can also regulate NF-κB and HIF-1 signaling (17, 19, 31).

The reduction of all COMMDs and the CCC core subunits (CCDC22, CCD93, and C16orf62) upon loss of any COMMD studied is very remarkable, and mystifies the biological relevance of hepatic expression of ten different COMMD proteins that are all assembled into a CCC complex (9, 18, 21, 22). Since, only COMMD5 and COMMD9 regulate Notch receptor recycling (18), and only a few COMMDs, including COMMD1, bind to the copper transporting protein ATP7B to mediate its trafficking (11), it has been postulated that the COMMDs define multiple different CCC complexes that act in conjunction with the WASH complex for selective endosomal trafficking of transmembrane proteins. We now show that in addition to COMMD1, also COMMD3 and COMMD6 are associated with the WASH complex (9). Based on these novel findings, it is reasonable to speculate that elevated plasma LDL cholesterol after downregulation of one COMMD protein is indirect and is the consequence of destabilizing of different CCC complexes, including the CCC complex that directly mediates endosomal LDLR sorting. Further studies are needed to elucidate the stoichiometry of these heterogeneous CCC complexes in receptor-specific endosomal trafficking.

We recently speculated that LDLR is retrieved by sorting nexin 17 (SNX17) from the lysosomes before LDLR is transferred to the CCC and WASH complexes for endosomal recycling (Fig. S9) (8). SNX17 also facilitates endosomal trafficking of LRP1 (32-34). LDLR and LRP1 contain a NPxY motif in their cytoplasmic tail, and both SNX17 and COMMD1 can bind to this NPxY motif (8, 34-36), suggesting that the CCC complex can facilitate the endosomal trafficking of various members of the LDLR-family that contain this motif, including LRP1 (37). Indeed, our \textit{in vitro} and \textit{in vivo} results indicate that the functioning of LDLR and LRP1 are both affected by inactivation of the CCC complex (Fig. S9B). We therefore speculate that upon entering the endosomes LDLR and LRP1 are coupled to SNX17 and are recycled by CCC and WASH complexes (8). McNally and co-workers recently identified a multiprotein complex, called retriever, which is composed of DSCR3, VPS29 and C16orf62 (33). In this study, the authors suggest that C16orf62 does not belong to the CCC complex but participates in retriever as a separate complex in the CCC-WASH axis (33). This is an interesting observation, as our study implies that C16orf62 does participate in the CCC complex since ablation of either \textit{Ccdd22} or a \textit{Commd} gene leads to a dramatic reduction in C16orf62 levels. In contrast,
a decrease in C16orf62 levels has not been seen in HeLa cells deficient for both CCDC22 and CCDC93 (33). The exact reason for these opposite results is unclear, but it might be that loss of the CCC complex results in mislocalization of C16orf62 - as the recruitment of the retriever to the endosomes relies on the CCC and WASH complexes (33) - that may eventually lead to proteolysis of this protein in hepatocytes. Further research is therefore needed to fully understand the molecular organization of the SNX17-retriever-CCC-WASH axis in coordinating the endosomal recycling of receptors, including LDLR and LRP1 (Fig. S9).

We speculate that PCSK9 prevents LDLR to be recognized by the CCC-WASH axis and directly targets LDLR for proteolysis, a process that is independent of the endosomal sorting complexes required for transport (ESCRT) machinery (38). Receptors that are not retrieved and recycled by the CCC-WASH axis are sorted into the lysosome for proteolysis, a process likely mediated by ESCRT (Fig. S9A) (33). Therefore, we hypothesize that the PCSK9-mediated LDLR degradation and CCC-dependent LDLR recycling pathways are acting independently of each other, but further research is needed to understand the relation between these two pathways.

Interestingly, our data show that hepatic ablation of Commd1 (8) and Commd6 in mice fed a chow diet also lead to elevated plasma HDL cholesterol levels. Plasma HDL cholesteryl esters uptake is mediated by the Scavenger Receptor Class B Member 1 (SR-B1). Different studies have shown that SR-B1 can be endocytosed (39), which may indicate that the CCC complex also plays a role in SR-B1-mediated HDL cholesteryl esters uptake, but further studies are warranted.

Collectively, our study has uncovered a previously unknown role for the evolutionarily conserved COMMD proteins in maintaining the integrity and function of the hepatocyte CCC complex during endosomal LDLR and LRP1 trafficking.

Materials and Methods

A brief description of the methods is provided below. For a detailed description of the methods please refer to the data supplement.

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee, University of Groningen (Groningen, the Netherlands). Detailed description of the different
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mouse models can be found in the data supplement.

Hepatic lipid extraction
Lipid was extracted from liver homogenates and prepared as 15% (w/v) solutions in PBS, as previously described (40). Samples were used to determine total cholesterol and triglyceride content.

Fast-performance liquid chromatography (FPLC)
Plasma samples of each group of mice were pooled and fractionated by FPLC as previously described (41), with minor modifications. In brief, the system contained a PU-980 ternary pump with an LG-980-02 linear degasser and a UV-975 UV/VIS detector (Jasco, Tokyo). EDTA plasma was diluted 1:1 with Tris-buffered saline, and 300 μL sample/buffer mixture was loaded onto a Superose 6 HR 10/300 column (GE Health care, Lifesciences division, Diegem) for lipoprotein separation at a flow rate of 0.5 mL/min. Total cholesterol and triglyceride content of the fractions was determined as described above.

Cholesterol and triglyceride analysis in plasma and liver homogenates
Total cholesterol (TC) levels were determined using colorimetric assays (11489232, Roche) with cholesterol standard FS (DiaSys Diagnostic Systems GmbH) as a reference. Triglyceride (TG) levels were measured using Trig/GB kit (1187771, Roche) with Roche Precimat Glycerol standard (16658800) as a reference.

Cell lines
HEK293T, Huh7, and HepG2 cells were cultured in Dulbecco’s modified Eagle medium GlutaMAX™, supplemented with 10% fetal calf serum (FCS) and 1% penicillin streptomycin (pen/strep) solution. RAW 264.7 cells were cultured in RPMI medium GlutaMAX™, supplemented with 10% FCS and 1% pen/strep. All cell lines were cultured at 5% CO₂ and 21% O₂. HEK293T, Huh7, HepG2 and RAW264.7 cells, in which COMMD1 and COMMD6 were stably silenced using shRNA (detailed below), were selected by 1μg/ml puromycin. Primary hepatocytes were cultured as described previously (8).

RNA interference
COMMD1 was stably silenced in HEK293T and HepG2 cells using shRNA as previously described (17). A plasmid encoding short hairpin RNA against COMMD6 was generated by cloning a target sequence specific for human COMMD6 (AATGACGATTCCACAGTTTCA) or mouse COMMD6 (TGACAATTCCACAATTTC) into the pLKO-TRC vector. HEK293T, HepG2 and
RAW264.7 cell lines were infected with lentiviral particles carrying the pLKO-TRC or pLKO-shCOMMD6 vector.

**Sucrose Gradients**

Male mice (WT and Commd1ΔHep) were fasted for 4 h and sacrificed. Livers were isolated, and processed as described previously (8).

**Immunoprecipitation analysis**

Immunoprecipitation (IP) experiments were performed as described before (8). In this study HEK293T cells and primary hepatocytes were used. Mouse anti-COMMD1 (MAB7526, R&D Systems) antibodies were used to immunoprecipitate COMMD1, rabbit anti-COMMD6 (custom made (24)) was used to immunoprecipitate COMMD6. Normal IgG was used as a negative IP control.

**Gene expression analysis**

Cells were grown to 70% confluency and lysed with QIAzol Lysis Reagent (Qiagen). 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. One microgram of RNA was used to prepare cDNA with the Transcriptor Universal cDNA Master kit (Roche), according to the manufacturer’s protocol. Twenty nanograms of cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using the FastStart SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR System (Applied Biosystems).

**Western blot**

For Western blot, total cell lysates and liver homogenates were obtained using NP40 buffer (0.1% Nonidet P-40 (NP-40), 0.4 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA) supplemented with protease and phosphatase inhibitors (Roche). Protein concentration was determined using the Bradford assay (Bio-Rad). Thirty micrograms of protein was separated using SDS-PAGE and transferred to Amersham™ Hybond™-P PVDF Transfer Membrane (GE Healthcare; RPN303F). Membranes were blocked in 5% milk in tris-buffered saline with 0.01% Tween20 and incubated with the indicated antibodies. Proteins were visualized using a ChemiDoc™ XRS + System (Bio-Rad) using Image Lab software version 5.2.1 (Bio-Rad).

**Antibodies**

Antibodies used for Western blot and immunofluorescence are described in the data.
supplement.

**Targeted proteomics**

To quantify the protein concentrations of the COMMDs, components of retromer, the CCC and WASH complexes, LDLR, LRP1, ApoA1, ApoB100, and ApoB48 in the samples of the different mouse models we developed targeted proteomics assay. Detailed description is provided in the data supplement.

**Statistical analysis**

*In vitro* data were obtained from three independent experiments ± standard error of the mean (SEM). Mouse data show average values ±SEM, n=7–12. Analyses were performed using GraphPad version 6.05 (GraphPad software). The Student’s t-test was used to test for statistical significance between two groups. For all experiments, a P-value of <0.05 was considered statistically significant.

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**Disclosures**

The authors report no conflicts of interest
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Supplementary material

Online Methods

Animals
Commd6 conditional knockout mice were generated using the multi-purpose targeting vector pNTKV1901-frt/loxP (1). Cloning of the targeting construct was performed as previously described (1). Using homologous recombination in embryonic stem (ES) cells, we introduced a neomycin (neo) cassette with a cryptic acceptor and donor site in intron 3, and loxP sites flanking exon 3 (Fig. 1A). The neo cassette was flanked with frt sites (Fig. 1A). The NotI-linearized targeting vector was electroporated into TL1 129Sv/E ES cells. After selection with G418 and expansion, the presence of the conditional Commd6 allele was confirmed by Southern blot analysis (Fig. 1A). We produced chimeric mice by microinjecting two independent ES cell-targeted clones into C57BL/6 blastocysts. Next, we crossed chimeric males with C57BL/6 females and the mutated Commd6 allele was successfully transmitted to their offspring. We crossed the mutated Commd6 mice with ROSA26::FLPe mice (The Jackson Laboratory stock#009086) to excise the neo gene cassette. Then, mice were backcrossed to a C57BL/6 genetic background for at least six generations, before crossing with Alb-Cre mice (The Jackson Laboratory stock#003574) to generate liver-specific Commd6 knockout mice. The following primers were used to genotype the mice: floxed Commd6 forward (5′- AGGGCTTGAGTATGGGACAG-3′) and reverse (3′-GTGAGAAATACCACTGCCTTG-5′); Alb-Cre forward (5′-GCGGTCTGGCAGTAAAAACTATC-3′) and reverse (5′-ACGAACCTGGTCGAAATCAGTG-3′). Commd6 conditional knockout mice have been submitted to The Jackson Laboratory (stock#031057).

Liver-specific Commd1 knockout mice were previously described (2). Commd9 ablation in hepatocytes was accomplished by crossing floxed Commd9 mice (3) with Alb-Cre mice (#003574, The Jackson Laboratory). Rosa26-LSL-Cas9 knock in mice (#024857, The Jackson Laboratory)(4) were crossed with Alb-Cre mice to generate liver-specific Cas9-expressing mice, and were used to target Ccdc22 in mouse livers through CRISPR/Cas9 gene editing technology.

Except for the atherosclerosis study, in any other studies we used male mice. Mice were housed individually and fed ad libitum with either a standard rodent chow diet (RMH-B, AB Diets, the Netherlands) or, starting at 10 weeks of age, a high-fat, high-cholesterol (HFC) diet (45% calories from butter fat, containing 0.2% cholesterol, SAFE Diets), n=5–9. Mice were
fed a HFC diet for 1 week, and mice were fasted for 4 hours before the mice were sacrificed. In all experiments, littermates, which are homozygous floxed mice without the Cre-allele, were used as wild type (WT) controls.

To generate a hepatic LDLR-deficient background, Commd1\(^{ΔHep}\) mice and WT littermates were injected via the orbital vein with 3.0x10\(^{11}\) vector genomes of AAV-PCSK9-D377Y (5) (n=9-10). Volumes of injection were adjusted to 100 μl with sterile phosphate-buffered saline (PBS).

For atherosclerosis measurements, ten weeks old female ApoE3*Leiden;Commd1\(^{ΔHep}\); Alb-cre (APOE3*L; Commd1\(^{ΔHep}\)) and ApoE3*Leiden;Commd1\(^{ΔF}\) (ApoE3*L) mice were fed a HFC diet for 12 weeks (n=9-12). After 12 weeks of HFC diet, mice were sacrificed, blood was collected, and the heart was fixed by cold isopentane for histology. Cross-sections of 6μm were made throughout the aortic root area of the heart and sections were stained with Toluidin blue. Slides were scanned with a Hamamatsu slide scanner. The average lesion size was determined from ten sections for each animal with a 24 μm interval. Plaque size was measured in a blinded fashion using image scope software (Leica Aperio Imagescope). The aortic arch was dissected, fixed in formalin and stained for lipid deposits using Oil Red-O, and pinned on silicon-coated dishes. Oil Red O positive areas were quantified using Image J.

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 cardiac puncture, and plasma was collected after centrifugation at 3000 rpm for 10 min at 4°C. All animal studies were approved by the Institutional Animal Care and Use Committee, University of Groningen (Groningen, the Netherlands).

**Fast-performance liquid chromatography (FPLC) of single plasma samples**

Cholesterol concentrations in the main lipoprotein classes (VLDL, LDL and HDL) were determined using fast protein liquid chromatography (FPLC) as described previously with some minor modifications (6). The system contained a PU-980 ternary pump with an LG-980-02 linear degasser, FP-920 fluorescence and UV-975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra PU-2080i Plus pump (Jasco, Tokyo, Japan) was used for in-line cholesterol PAP or Triglyceride enzymatic reagent (Roche, Basel, Switzerland) addition at a flowrate of 0.1 ml/min. Plasma lipoproteins were separated with a Superose 6 HR 10/30 column (GE Healthcare Hoevelaken, The Netherlands) using TBS pH 7.4, as eluent at a flow rate of 0.31 ml/min. Quantitative analysis of the chromatograms was carried out with ChromNav.
chromatographic software, version 1.0 (Jasco, Tokyo, Japan). Commercially available lipid plasma standards (low, medium and high) were used for quantitative analysis (SKZL, Nijmegen, the Netherlands) of the separated lipoprotein fractions.

**RNA interference**

*COMMD1* was stably silenced in HEK293T and HepG2 cells using shRNA as previously described (7). A plasmid encoding short hairpin RNA against COMMD6 was generated by cloning a target sequence specific for human COMMD6 (AATGACGATTCCACAGTTTCA) or mouse COMMD6 (TGACAATTCCACAATTCCA) into the pLKO-TRC vector. HEK293T, HepG2 and RAW264.7 cell lines were infected with lentiviral particles carrying the pLKO-TRC or pLKO-shCOMMD6 vector. To produce lentiviral particles, HEK293T cells were transfected with pLKO-TRC or pLKO-shCOMMD6 together with pMDLG-pRRE, pH-CMV-G and pRSV-Rev packaging plasmids using FuGENE-6 transfection reagent (Promega) at a Fugene to DNA ratio of 3:1. Virus-containing supernatant culture medium was filtered (0.45 micron, Corning), mixed with polybrene (4 mg/ml) and used for infection for three consecutive 12 hour periods. Twenty-four hours after the third infection, the culture medium was supplemented with puromycin (1μg/ml).

**Sucrose Gradients**

Male mice (WT and *Commd1ΔHep*) were fasted for 4 h and sacrificed. Livers were isolated, and processed as described previously.

In short, 200 mg of liver was homogenized in 800 μl homogenization buffer (50 mM Tris-HCl, ph 7.4, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, 3 mM imidazole, Roche protease inhibitor mixture) with 20 strokes in a Dounce homogenizer. Homogenates were centrifuged (1000xg, 10 min at 4°C) to remove nuclei and other debris. A unit of 300 μg of liver homogenates was loaded on a 3.7-ml continuous 10-40% sucrose gradient and centrifuged using a Beckman Coulter SW55 Ti rotor for 16 h at 40,000 rpm. 285 μl fractions were collected from the bottom by puncture the bottom of the tube with a 20-gauge needle. 1/10 of each fraction were mixed SDS sample buffer and used for Western blot analyses.

**Immunoprecipitation analysis**

Immunoprecipitation experiments were performed as described before (7). Cells were lysed in NP-40 buffer (0.4MNaCl, 0.1% NP-40, 10mM Tris-HCl, pH 8.0 and 1mM EDTA), supplemented with protease inhibitors. In this study HEK293T cells and primary hepatocytes were used. Mouse anti-COMMD1 (MAB7526, R&D Systems) antibodies were
used to immunoprecipitate COMMD1, rabbit anti-COMMD6 (custom made (8)) was used to immunoprecipitate COMMD6. Normal IgG was used as a negative IP control.

**Immunofluorescence**

HEK293T cells were cultured for 24 h on coverslips, fixed in ice-cold fixative (4% paraformaldehyde and 0.5% glutaraldehyde in PBS) and incubated for 18 min in the dark at room temperature, followed by permeabilization for 4 min with 0.2% Triton X-100 in PBS. Cells were subsequently incubated with primary antibodies at 4°C in a humidified chamber. Following three washes in PBS, cells were incubated with secondary antibodies (1:500 dilution in blocking buffer) for 1 h at room temperature or overnight at 4°C in a humidified chamber. After three washes in PBS, coverslips were mounted on slides with Vectashield mounting medium containing DAPI (Vector Laboratories; H-1200). Images were obtained with a Zeiss Axio Imager2 with Apotome 2 with a Plan-APoChromat 63x/1.4 oil objective, using ZEN software (Zeiss).

**Adenovirus generation for somatic gene editing by CRISPR/Cas9 technology**

Three guide RNAs, targeting either exon 1 or exon 2 of murine *Ccdd22* (Fig. 6A), were separately cloned into a *Bbs*I-digested pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene plasmid# 48139) to create U6-guideRNA-sgRNA scaffold cassettes. These cassettes were amplified by PCR using the following primers: forward sg#1: 5ʹ-GTCGACgagggcctatttccatgat-3ʹ; reverse sg#1: 5ʹ-GGATCCAAAAAgcaccgactcggtg -3ʹ; forward sg#2: 5ʹ-GGATCCgagggcctatttccatgat-3ʹ; reverse sg#2: 5ʹ-tctagaAAAAAgcaccgactcggtg-3ʹ; forward #3: 5ʹ-TCTAGAAgagggcctatttccatgat-3ʹ; reverse sg#3: 5ʹ-gcggccgcAAAAAgcaccgactcggtg-3ʹ, and were ligated into a *Sal*I-NotI-linearized pENTR2B entry plasmid (Invitrogen #11816-014). The construct sequence is available upon request. A LR recombination reaction (Invitrogen #11791-020, following manufacturer’s protocol) with pAd/pl-Dest was performed to obtain the adeno-expression construct. For virus production, 4 μg of *Pac*I-digested expression vector was transfection into HEK293A cells (Invitrogen # R705-07). Visible virus production was observed after 9 days. Reproduction and upscaling of the virus was performed in five steps, and produced up to 7500 cm² infected HEK293A cells. Viruses were purified using cesium chloride density gradients, and stored at -80°C at a concentration of 7.45x10^{12} particles per ml.

AV particles were injected intravenously in 9–10 week old mice expressing Cas9 specifically in the liver (see material and method section: animals). All AV doses were 1x10^{11} particles and adjusted to 100 μl with sterile phosphate-buffered saline (PBS). Blood was collected
by retro-orbital bleeding one week before virus administration, and mice were sacrificed 3 weeks after administration.

**COMMD6-V5 tag fusions by CRISPR/Cas9 gene editing technology**

To fuse a V5-tag to endogenous *COMMD6* of HEK293T cells we used CRISPR/Cas9 gene editing technology. The homology construct for specific integration of the V5-tag was assembled in pBluescriptII KS+ vectors. The left (225 bp) and right (280 bp) homology arms and the V5-tag flanking the stop codon were cloned into the vector using standard cloning techniques. Primer sequences are available upon request. gRNAs were designed using the online MIT CRISPR designer tool, and cloned into the pX459 vector (Addgene plasmid #48139). The guide sequence and the homology construct were validated by Sanger sequencing. gRNA efficiency was tested as previously described (9). HEK293T cells were transfected with the pX459-sgRNA vector and the V5-tag flanked with the short homology regions (Fig. S2A). Forty-eight hours after transfection, single cells were seeded to form colonies. Two week later, colonies were collected and cultured individually in 96-well plates. Single-cell clones were cultured and correct incorporation of the V5-tag was determined by PCR amplification and sequencing (Fig S2B, C). Western blot analysis with an anti-V5 antibody detected proteins with the expected molecular weight in targeted HEK293T cells but not in control cells (Fig. S2D). In addition, COMMD6 fused with V5-tag was specifically observed by immunofluorescence in gene edited cells and not in control cells (Fig. S2E).

**Gene expression analysis**

Cells were grown to 70% confluency and lysed with QIAzol Lysis Reagent (Qiagen). 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. One microgram of RNA was used to prepare cDNA with the Transcriptor Universal cDNA Master kit (Roche), according to the manufacturer’s protocol. Twenty nanograms of cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using the FastStart SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR System (Applied Biosystems). The PCR reaction was performed as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60°C. Expression data were analyzed using SDS 2.3 software (Applied Biosystems), using the ‘standard curve’ method of calculation. GAPDH was used as an internal control for human cell lines (HEK293T and HepG2). PPIA expression was used as an internal control for mouse samples and RAW 264.7 cells. Primer sequences are available upon request.
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Antibodies

We used the following antibodies: rabbit anti-COMMD1 (11938-1-AP, Proteintech Group, 1:1000), mouse anti-COMMD1 (MAB7526, R&D Systems, 1:100), rabbit anti-COMMD3 (ab176583, Abcam, 1:1000), rabbit anti-COMMD4 (ab115169, Abcam, 1:1000), rabbit anti-COMMD5 (10393-1-AP, Proteintech Group, 1:1000), rabbit anti-COMMD6 (custom made(8), 1:100), rabbit anti-COMMD9 (192-AP, custom made, Starokadomskyy, 2013, 1:1000), rabbit anti-COMMD10 (GTX121488, GeneTex, 1:1000), rabbit anti-LDLR (PAB8804, Abnova GmbH, 1:1000), rabbit anti-CDC22 (16636-1-AP, Proteintech Group, 1:2000), HRP-conjugated goat anti-rabbit IgG (H + L) (170-6515, Bio-Rad, 1:10000), mouse anti-Flag-M2-HRP (A8592, Sigma, 1:2000), HRP-conjugated goat anti-rabbit IgG (H+L) (170-6516, Bio-Rad, 1:10000), mouse anti-b-actin (A5441, Sigma-Aldrich, 1:5000); rabbit anti-tubulin (ab4047, Abcam, 1:2000), rabbit anti-CCDC22 (16636-1-AP, Proteintech Group, 1:100), rabbit anti-CCDC93 (20861-1-AP, Proteintech, 1:5000), goat anti-VPS35 (ab10099, Abcam, 1:100), mouse anti-V5 (46-0705, Invitrogen, 1:1000), and rabbit anti-V5 (ab9116, Abcam, 1:500). Rabbit polyclonal antibodies against WASH1 (1:1000) and FAM21 (1:1000) have previously been described (10). Anti-apoB100 (1:1000) and rabbit anti-apoA1 (1:1000) antibodies were a gift from Dr. A.K. Groen. For generation of the monoclonal antibody against mouse COMMD6, approximately 50 µg of GST-COMMD6 fusion protein dissolved in PBS was emulsified in an equal volume of incomplete Freund’s adjuvant and C57BL/6J mice were immunized subcutaneously (s.c.) and intraperitonially (i.p.). 6 weeks after immunization, a 50 µg boost injection was applied i.p. and s.c. three days before isolation and fusion of the splenic B cells with the myeloma cell line P3X63Ag8.653 (performed using polyethylene glycol 1500 according to standard protocols) (11). a Hybridoma supernatants were tested by solid-phase enzyme-linked immunoassay (ELISA) using GST-COMMD6 fusion protein and verified by Western blot. The hybridoma cells of COMMD6-reactive supernatant 26F7 were cloned twice by limiting dilution. The IgG subclass clone 26F7 was determined with ELISA assay as mouse IgG2a kappa light chain. All experiments targeting mouse COMMD6 were performed with this antibody.

Isotopically labeled standard for targeted proteomics

To quantify the protein concentrations of the COMMDs, components of retromer, the CCC and WASH complexes, LDLR and LRP1 in the liver homogenates and APOA1 and APOB in plasma of the different mouse models we developed targeted proteomics assay. Isotopically labeled peptide standards were used to develop targeted LC-MS assays through selection of optimal MS settings for two peptides per targeted protein. Quantotypic peptides were selected for both mouse and human orthologs, based on selection criteria described previously (12). All target peptides were concatenated into synthetic proteins (Polyquant
GmbH, Germany) containing $^{13}$C-labeled lysines and arginines (so-called QconCATs). The mouse specific peptide targets for this study are listed in Supplementary Table S2. Additional $^{13}$C$^{15}$N-labeled lysine containing synthetic peptides (AQUA QuantPro, Thermo Scientific) were added for COMMD1 to include peptide variants resulting from missed cleavage (sequence motifs causing incomplete digest of COMMD1), since the initially selected peptide for COMMD1 (without missed cleavages) did not yield a LC-MS response above the detection limit (Supplementary Table S2).

**In-gel digestion and sample cleanup for mass spectrometry analysis**

For immunoprecipitated (IP) enriched protein samples in SDS loading buffer (0.01% bromophenol blue, 2% SDS, 4% glycerol, 1% β-mercaptoethanol in 150 mM Tris buffer (pH 6.8)), 20 µL total volume was loaded on the gel. For plasma samples 1 µL plasma plus 1 ng QconCATs were loaded on the gel. For liver tissue samples: 40 µg total protein plus 28.7 fmol QconCAT were loaded on the gel and samples were run briefly into a precast 4–12% Bis-Tris gel (Novex, ran for a maximum of 5 min at 100 V). The sample was run briefly into a precast 4-12% Bis-Tris gel (Novex, ran for maximally 5 min at 100V). The gel was stained with Biosafe Coomassie G-250 stain (Biorad) and after destaining with milliQ, one band containing all proteins was excised from the gel.

The gel was stained with Biosafe Coomassie G-250 stain (Bio-Rad). After destaining with milliQ, one band containing all proteins was excised from the gel. To quantify the concentrations of the COMMD proteins in liver lysates the same samples were also loaded into a precast 4–12% Bis-Tris gel (Novex), and were run for approximately 15 min at 100 V. In this way, the small proteins were separated from the larger, more abundant proteins. The gel was stained with Biosafe Coomassie G-250 stain (Bio-Rad) and after destaining with milliQ, gel bands containing proteins (including COMMD proteins) with a molecular weight between 5–25 kDa were excised from the gel.

Each excised gel band was sliced into small pieces, washed subsequently with 30% and 50% v/v acetonitrile with 100 mM ammonium bicarbonate, and incubated at RT for 30 min while mixing (500 rpm). Then, 100% acetonitrile was added for 5 min and the gel pieces were dried at 37°C. The proteins were reduced with 20 µL 10 mM dithiothreitol (30 min, 55 °C) and alkylated with 20 µL 55 mM iodoacacetamide (30 min, in the dark at RT). Then, gel pieces were washed for 30 min with 50% acetonitrile containing 100 mM ammonium bicarbonate while mixing (500 rpm) and dried at 37°C before overnight digestion with 20 µL trypsin (1:100 g/g, sequencing grade modified trypsin V5111, Promega) at 37 °C. The next day, the
residual liquid was collected and proteins were eluted from the gel pieces with 20 μL 75% v/v acetonitrile containing 5% v/v formic acid (incubation 20 min at RT, mixing 500 rpm).

For liver tissue and plasma samples, the elution fraction was combined with the residual liquid and diluted to 1 mL with 0.1% v/v formic acid for cleanup on a C18-SPE column (SPE C18-Aq 50 mg/1ml, Gracepure). This column was conditioned with 2x1 ml acetonitrile containing 0.1% v/v formic acid and re-equilibrated with 2x1 mL 0.1% v/v formic acid before the samples were loaded. Bound peptides were washed with 2x1 mL 0.1% v/v formic acid and eluted with 2x0.4 mL 50% v/v acetonitrile plus 0.1% v/v formic acid. The eluted fractions were dried under vacuum and resuspended in 20 μL 0.1% v/v formic acid. For immunoprecipitated samples and fractions enriched for the COMMD proteins the additional cleanup step was omitted, samples were dried under vacuum immediately and resuspended in 10 μL 0.1% v/v formic acid.

**Targeted LC-MS analysis**

From the IP samples 3 μL was injected in the LC-MS after addition of 6.5 fmol pre-digested QconCAT and 225 amol COMMD1-peptides. 2 ug total protein of liver homogenates plus 1.4 fmol QconCAT were injected into the LC-MS. In case the relative small proteins (5–25 kDa) were analyzed, an equivalent of 11 μg total protein was injected in the LC-MS after addition of 3.8 fmol pre-digested QconCAT and 133 amol COMMD1-peptides. For plasma an equivalent of 25 nL plasma plus 0.5 ng QconCAT was injected.

Targeted LC-MS analyses were performed on a triple quadrupole mass spectrometer with a nano-electrospray ion source (TSQ Vantage, Thermo Scientific). Chromatographic separation of the peptides was performed by liquid chromatography on a nano-UHPLC system (Ultimate 3000, Dionex) using a nano-LC column (Acclaim PepMap100 C18, 75 μm x 500 mm, 2 or 3 μm, 100 Å, Dionex). Samples were injected using the μL-pickup method with 0.1% v/v formic acid as a transport liquid from a cooled autosampler (5°C) and loaded onto a trap column (μPrecolumn cartridge, Acclaim PepMap100 C18, 5 μm, 100 Å, 300 μm x 5 mm, Dionex). Peptides were separated on the nano-LC column using a linear gradient from 3–60 % v/v acetonitrile plus 0.1% v/v formic acid for 100 min at a flow rate of 200 nL/min. The mass spectrometer was operated in positive mode at a spray voltage of 1500 V, a capillary temperature of 270°C, a half maximum peak width of 0.7 for Q1 and Q3, a collision gas pressure of 1.2 mTorr and a cycle time of 1.2 ms. Optimal collision energies (CE) were predicted using the following linear equations: CE =0.03*m/z precursor ion + 2.905 for doubly charged precursor ions, and CE=0.03*m/z precursor ion + 2.467 for triply charged
precursor ions. For each of the peptides, the optimal precursor charge and three optimal transitions were selected after screening with the QconCAT peptides. The measurements were scheduled in windows of 5 min around the pre-determined retention time. The LC-MS peak assignments were manually curated using Skyline software (13) and the integration peak areas can be used for quantification using the known concentration of the spiked isotopically labelled standard. For qualitative purposes, the targets were considered specifically IP enriched if the isotopically spiked peptides were detected in both IP and control samples, but the endogenous peptide was detected only in the IP sample and not in the control sample. The targets were considered absent (or below the limit of detection) if the endogenous peptides were not detected in both samples, but the isotopically labelled standards were detected in both.

**Biotinylation assay**

Culture of primary hepatocytes and biotinylation assay were performed as previously described (14). Cells were washed 3x with ice-cold PBS-CM buffer (PBS, 1mM MgCl2 and 0.1mM CaCl2), subsequently 0.5mg/ml biotin reagent solution (EZ-Link Sulfo-NHS-SS_Biotin, Thermo Scientific) in biotinylation buffer (10mM triethanolamine, pH 8.0, 150mM NaCl and 2mM CaCl2) was added to the cells for 30 min at 4°C. Biotin reagent was removed and cells were washed 1x with quenching buffer (PBS-CM, 25mM Tris-HCl, pH 7.4 and 192mM Glycine) for 30 min at 4°C. Next, cells were washed 2x with PBS-CM and 1x with TBS-C (50mM Tris-Cl, pH 7.4, 100mM NaCl and 2mM CaCl2). Cells were collected by scraping in TBS-C. Cells were centrifuged (1,000g, 5 min, 4°C), lysed in biotin lysis buffer (50mM Tris-Cl, pH 7.4, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5mM EDTA and 5mM EGTA), sonicated (10% output for 10 s) and incubated on ice for 15 min, subsequently centrifuged for 15 min at 12,000 g. Protein concentration was determined; 30 mg was used as input; 300 mg was used diluted in biotin lysis buffer (500μl); 30μl Neutravidin beads (Neutravidin plus ultralink beads, Thermo Scientific) was added and incubated for 4 h at 4°C. Beads were collected by centrifugation (500g, 5min), beads were washed 3x with Biotin lysis buffer, 1x with high-salt buffer (50mM Tris-Cl, pH 7.4 and 500mM NaCl) and 1x with low-salt buffer (10mM Tris-HCl). Finally, the beads were resuspended in 30μl 2x loading buffer.
Supplementary references


Supplementary Figures

A. Schematic representation of the Commd6 gene targeting strategy to generate a conditional Commd6 knockout allele. A genetic map of Commd6, the targeting vector, the Commd6 locus after homologous recombination, and the final conditional Commd6 knockout allele after Flp-mediated excision of the Neo cassette are illustrated. Depicted are the Commd6 exons (blue boxes), loxP sites (black boxes), FRT sites (white boxes) and neomycin selection gene (Neo). Homologous recombination is marked with dotted lines. (A) Southern blot of EcoRV digested genomic DNA from two ES cell clones and probed for the DNA fragment indicated in (A). The 10.4 kb and 12.2 kb fragments represent the wild type (WT) and Commd6 floxed allele, respectively. (C) PCR genotyping of two WT, WT/loxP and loxP/loxP mice without the Neo cassette. C represents a negative control (H2O). (D) Commd6 mRNA expression in livers of Commd6<sup>step</sup> and WT mice (n=7–9). (E) Immunoblot analysis showing COMMD6 protein levels in the liver of WT and Commd6<sup>step</sup> mice. Group averages and SEM are shown.

Supplementary Figure 1. Generation of hepatocyte-specific Commd6 knockout mice. (A) Schematic representation of the Commd6 gene targeting strategy to generate a conditional Commd6 knockout allele. A genetic map of Commd6, the targeting vector, the Commd6 locus after homologous recombination, and the final conditional Commd6 knockout allele after Flp-mediated excision of the Neo cassette are illustrated. Depicted are the Commd6 exons (blue boxes), loxP sites (black boxes), FRT sites (white boxes) and neomycin selection gene (Neo). Homologous recombination is marked with dotted lines. (B) Southern blot of EcoRV digested genomic DNA from two ES cell clones and probed for the DNA fragment indicated in (A). The 10.4 kb and 12.2 kb fragments represent the wild type (WT) and Commd6 floxed allele, respectively. (C) PCR genotyping of two WT, WT/loxP and loxP/loxP mice without the Neo cassette. C represents a negative control (H2O). (D) Commd6 mRNA expression in livers of Commd6<sup>step</sup> and WT mice (n=7–9). (E) Immunoblot analysis showing COMMD6 protein levels in the liver of WT and Commd6<sup>step</sup> mice. Group averages and SEM are shown.
Supplementary figure 2. Body weight, liver weight and liver histology are unaffected by hepatic COMMD6 deficiency. Body weight (A) and liver weight as percentage of body weight (B) for WT and Commd6<sup>ΔHep</sup> mice (n=7–9). (C) H&E and ORO staining of hepatic tissue from WT and Commd6<sup>ΔHep</sup> mice fed a chow or a HFC diet for 1 week. Representative images shown, scale bar = 100 µm. Total cholesterol levels of FPLC fractionated pooled plasma sample of the experimental groups of mice fed either a chow (D) or a HFC (E) diet (n=7–9). Immunoblotting of ApoB100 and ApoA1 was performed on fractions #13–26. Group averages and SEM are shown.
**Supplementary figure 3. Generation of a V5 tagged COMMD6 allele in HEK293T cells.** (A) Schematic representation of the construct used to edit endogenous COMMD6. Annotated are the gRNA target sequence (red), stop codon (orange) and PAM sequence (blue). The repair construct consisted of a V5 tag (green), which was introduced right before the stop codon (orange) of COMMD6, and two homology arms (HA-L and HA-R) for homologous recombination (blue lines). F1, R1 and R2 represent the primers used to confirm V5 insertion via PCR. (B) PCR analysis of wild type and V5-tagged HEK293T monoclonal cell lines (B1 and D12). (C) Western blotting of V5 and β-actin in wild type and COMMD6-V5 HEK293T cell lysates. (D) Sequence throughout the V5-targeted region to confirm correct fusion of the V5-tag to the last codon of COMMD6. (E) Immunofluorescence staining of COMMD6-V5 in HEK293T wild type and COMMD6-V5 monoclonal cell lines (B1).
Supplementary figure 4. Subcellular localization of COMMD6 overlaps with COMMD1, WASH complex and retromer. (A) Cellular localization of COMMD6-V5 (green), COMMD1 (red), WASH1 (red), FAM21 (red) and VPS35 (red) in HEK293T cells expressing endogenous V5-tagged COMMD6 determined by indirect immunofluorescence staining. DAPI (blue) was used to stain nuclear DNA. Representative images are shown. Scale bar = 10 µm. Overlap coefficient of COMMD6 (B) and COMMD1 (C) with each other, the WASH complex subunits WASH1 and FAM21, and retromer component VPS35. Group averages and SEM are shown (n≥30).
COMMD proteins regulate plasma lipid levels

Supplementary figure 5. Subcellular localization of COMMD1 overlaps with the WASH complex and retromer. (A) Cellular localization of COMMD1 (green), WASH1 (red), FAM21 (red) and VPS35 (red) in the HEK293T COMMD6-V5 monoclonal cell line, determined by immunofluorescence staining. Representative images shown, scale bar = 10 µm. (B) Whole cell and cell surface levels of LDLR and LRP1 in WT and Commd6−/− primary hepatocytes.
Supplementary figure 6. Hepatic COMMD9 regulates plasma cholesterol. (A) Plasma cholesterol and triglyceride levels of WT and Commd9ΔHep animals after one week of HFC feeding (n=6). (B) Total cholesterol levels of pooled FPLC fractionated plasma samples of mice fed a HFC diet for one week (n=6). (Inset B) Plasma ApoB100 and ApoA1 levels of Commd9ΔHep and WT, indicated by fold change versus WT controls (n=6) (C) Body weight and liver weight as a percentage of body weight of WT and Commd9ΔHep animals after one week of HFC feeding (n=6). (D) H&E and ORO staining of hepatic tissue from WT and Commd9ΔHep mice after one week of HFC feeding. Representative images shown, scale bar = 200 µm. Group averages and SEM are shown. ***p<0.001 (compared to controls).
**Supplementary figure 7.** Body weight, liver weight and liver histology are not affected by hepatic CCDC22 insufficiency. (A) Schematic representation of the single-vector AV system to target Ccdc22 in parenchymal cells of hepatic Cas9-expressing mice. Annotated are the target sequences (red) and PAM sequences (blue) of the gRNAs. (B) Experimental timeline of the CRISPR/Cas9-mediated gene editing approach. (C) Fold change of protein levels of CCC core complex components and COMMD proteins after CRISPR/Cas9-mediated Ccdc22 editing (n=4) (D) Body weight and liver weight as a percentage of body weight of WT and Cas9 animals injected with AV-gRNA-Ccdc22 (n=5-6). (E) H&E staining of hepatic tissue of WT and Cas9 animals injected with AV-gRNA-Ccdc22. Representative images shown, scale bar = 200 µm.
Supplementary figure 8. Lipid-rich plaques in the aortic arch of ApoE3*L and ApoE3*L;Commd1<sup>ΔHep</sup> animals. (A) Total liver protein levels of LDLR and LRP1 in ApoE3*L and ApoE3*L;Commd1<sup>ΔHep</sup> animals, as determined by immunoblotting. (B) Representative images of Oil Red-O stained aortic arch of ApoE3*L and ApoE3*L;Commd1<sup>ΔHep</sup> animals.
Supplemental figure 9. Hypothetical model of the CCC-WASH axis in endosomal LDLR and LRP1 trafficking.

(A) On entering the endosomal network LDLR and LRP1 are subjected to one of two fates decisions, either the receptors are sorted into the lysosome for proteolysis, likely through the endosomal sorting complexes required for transport (ESCRT), or the receptors are retrieved and are recycled back to cell surface to take up the next cargo. We speculate that SNX17 is a LDLR and LRP1-specific adaptor allowing the receptors to enter into retrieval and recycling pathway, which is coordinated by retriever (DSCR3, C16orf62, VPS29), the CCC complex and WASH complex. The recruitment of the CCC and WASH complexes to the endosomes may be dependent on the retromer subunit VPS35. (B) Depletion of one component (either a COMMD protein or a CCC core component) of the CCC complex destabilizes the complete CCC complex leading to impaired endosomal trafficking of LDLR and LRP1 resulting in enhanced ESCRT-mediated sorting into the lysosomes, decreased surface levels of LDLR and LRP1 and consequently hypercholesterolemia and enhanced atherogenesis.
Supplementary table 1: Relative protein levels of the proteins studied

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**Supplementary table 2.** List of peptides used for targeted proteomics.

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### Supplementary table 2 - continue

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* proteins that were targeted in total liver lysate via excision of the small protein band
# The APOB48 concentration was calculated via the difference between the two APOB peptides
COMMD proteins regulate plasma lipid levels