CHAPTER 5

The hepatic WASH complex is required for efficient clearance of plasma LDL and HDL cholesterol

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Abstract

The evolutionary conserved Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) complex is one of the crucial multi-protein complexes that facilitate endosomal recycling of transmembrane proteins. In humans, defects in the components of the WASH complex have been associated with inherited developmental and neurological disorders. A recent in vitro study suggested that the WASH complex is also involved in the metabolism of low-density lipoproteins (LDL). Here we show that, in mice, hepatic ablation of the WASH component Washc1 increases plasma concentrations of cholesterol in both LDL and high-density lipoproteins (HDL), without affecting hepatic cholesterol content or hepatic very low-density lipoprotein (VLDL) synthesis. Elevated plasma LDL cholesterol was related to reduced hepatic surface levels of the LDL receptor (LDLR) and the LDLR related protein (LRP1). Hepatic WASH ablation also reduced the surface levels of scavenger receptor class B type I (SR-B1) and, concomitantly, selective uptake of HDL-cholesterol into the liver. Our data also suggest that WASH-mediated LDLR recycling depends on the endosomal sorting complexes retriever and retromer, however, these two protein complexes are not required for the endosomal trafficking of SR-B1. Altogether, these findings identify the WASH complex as a regulator of LDL as well as HDL metabolism, and provide evidence for endosomal trafficking of SR-B1 in hepatocytes. Here, we also reveal novel molecular insights into the specific role of the endosomal sorting machinery in cholesterol metabolism.

Keywords: Atherosclerosis; Cardiovascular disease; Cholesterol; Hepatology; Metabolism
Introduction

The endosomal network plays a crucial role in the delivery of integral membrane proteins (also referred to as cargo proteins) either to lysosomes for proteolysis, to the trans-Golgi network, or to the plasma membrane. In recent years, the Wiskott-Aldrich syndrome protein and SCAR homologue (WASH) complex has emerged as one of the key multi-protein complexes facilitating the endosomal sorting of cargos (1-3), such as the epidermal growth factor receptor (EGFR) (4), copper-transporting P-type ATPase ATP7A (5), and β2-adrenoreceptor (6,7). The WASH complex consists of five subunits: WASHC1 (WASH1), WASHC2 (FAM21), WASHC3 (CCDC53), WASHC4 (SWIP) and WASHC5 (Strumpellin or KIAA0196), and governs the destination of cargos by activating the Arp2/3 complex (8,9). Arp2/3 forms actin-enriched sub-domains on the endosomal membrane whence cargos are sorted and transported (10,11).

It has been well established that localization of the WASH complex to the endosomes is directed by retromer, a cargo-selective multiprotein complex consisting of VPS35, VPS26 and VPS29 (9,12,13), but recent work has found a retromer-independent pathway of WASH-mediated endosomal cargo recycling (14). This recent study showed that WASH forms together with the COMMD-CCDC22-CCDC93 (CCC) complex an evolutionary conserved endosomal cargo sorting pathway, which likely acts partially independently of retromer (5,14,15). This same study suggests that C16orf62, which was originally considered to be a component of the CCC complex, participates in retriever, a separate heterotrimeric protein complex consisting of C16orf62 (VPS35L), DSCR3 (VPS26C) and VPS29 (14), which acts in parallel to retromer in cargo recycling.

Mutations in several components of the WASH complex have been identified in developmental and neurological disorders; mutations in WASHC4 have been found in patients with non-syndromic autosomal recessive intellectual disability (16), while defects in WASHC5 have been reported in patients with autosomal dominant hereditary spastic paraplegia (HSP) (17,18) or Ritscher-Schinzel/3C syndrome (RSS) (19). Our recent in vitro work also implicated a role for the WASH complex in the metabolism of low-density lipoprotein (LDL) cholesterol (LDL-C). We found that WASHC1 deficiency impairs endosomal trafficking of the LDL receptor (LDLR), leading to LDLR accumulation in endosomes, reduced LDLR surface levels, and impaired LDL uptake in mouse embryonic fibroblasts (MEFs) (15). Furthermore, we showed that hepatic destabilization of the WASH-associated CCC (COMMD-CCDC22-CCDC93) complex increases plasma LDL-C levels in mice, dogs and humans, and accelerates atherosclerosis in mice.
 Altogether, these data strongly indicate the involvement of the WASH complex in the regulation of cholesterol homeostasis. Accordingly, we reported a familial RSS patient presenting hypercholesterolemia (15), beyond developmental malformation and intellectual disability. However, due to the clinical complexity of RSS and our assessment of the plasma cholesterol levels being limited to only one RSS patient, the exact contribution of the WASH complex to the regulation of plasma cholesterol levels remained unclear.

We therefore generated liver-specific WASH-deficient mice to establish the role of the WASH complex in the regulation of cholesterol metabolism. In addition, we examined the contribution of retromer and retriever in WASH-mediated cholesterol metabolism in vivo. We found that ablation of the WASH complex leads to elevated plasma levels of cholesterol in both LDL and high-density lipoprotein (HDL) fractions. We also observed that WASH is important not only for the cell surface expression of the LDLR family members, i.e. LDLR and LDLR related protein 1 (LRP1), but also for the scavenger receptor class B type I (SR-BI), the main receptor that mediates selective uptake of cholesteryl esters (CE) from HDL into hepatocytes. Kinetic studies showed that selective HDL-CE uptake was impaired in hepatic WASH-deficient mice. To further understand the role of retromer in WASH-mediated lipoprotein receptor recycling, we depleted retromer in the liver by somatic CRISPR/Cas9-mediated editing of hepatic Vps35. Interestingly, in contrast to WASH depletion, Vps35 deletion resulted only in accumulation of cholesterol in LDL, and not in HDL particles. In addition, we found that hepatic loss of retromer but not WASH reduces plasma triglyceride (TG) levels. Moreover, CRISPR/Cas9-mediated ablation of hepatic Dscr3/Vps26c resulted only in an increase in plasma LDL-C, without affecting plasma TG levels.

Altogether, our results imply that, in addition to its role in developmental and neurological processes, the WASH complex in the liver is required for hepatic uptake of LDL-C and HDL-C. Furthermore, our data suggest that WASH/CCC-mediated LDLR trafficking is both retromer- and DSCR3/VPS26C-dependent, whereas SR-BI surface expression is only WASH and CCC-dependent.

Results

Depletion of Washc1 destabilizes the WASH complex and adversely affects the lysosomal architecture in hepatocytes

To decipher the role of the hepatic WASH complex in cholesterol homeostasis, we generated a liver-specific Washc1 knockout model (Washc1ΔHep) by cross-breeding mice carrying floxed
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WASHC1 deficiency destabilizes the WASH complex and perturbs lysosomal structures in primary hepatocytes. (A) Protein expression of the WASH complex components in liver homogenates of WT and Washc1^{ΔHep} mice as determined by immunoblotting. (B) Quantification of immunoblot results depicted in A. (C) Electron microscopy pictures of WT and Washc1^{ΔHep} livers. (D) Quantification of WT (n=57) and Washc1^{ΔHep} (n=63) lysosomal areas in liver sections. (E) Electron microscopy picture depicting aberrant lysosomal structures in a Washc1^{ΔHep} hepatocyte. Inset shows magnified area with tubulated lysosomes. For visualization of lysosomal tubulation, lysosomal perimeters were selected using Lasso tool in Adobe Photoshop and the selected organelles were colored. Data are presented as the mean ± SEM, **p < 0.01, ***p < 0.001 as determined by Student’s T-test.

Washc1 alleles (Washc1^{fl/fl}) (4) with transgenic mice expressing Cre recombinase under the control of the Albumin (Alb) gene promoter (Alb^{Cre}). Successful ablation of hepatic WASHC1 was confirmed by immunoblotting (Fig. 1A, B). In line with previous findings in MEFs (4), hepatic WASHC1 deficiency strongly reduced the abundance of all WASH components (Fig.
1 A, B), illustrating the importance of WASHC1 in the formation of a stable WASH complex in the mouse liver. Washc1ΔHep mice were born at the expected Mendelian ratio (Fig. S1A) and showed no overt changes in body weight, liver weight or general liver histology compared with littermate controls (from now on referred to as wild-type (WT)) (Fig. S1B, C). WASH binds to many different multiprotein complexes, such as the CCC complex, retriever and retromer, to coordinate the endosomal trafficking of cargos (5,9,12-15,21). Ablation of the WASH complex did not affect the abundance of any subunit of these complexes (Fig. S1D).

In vitro studies have shown that WASH deficiency alters the morphology of endosomal and lysosomal structures (4,8-10). We therefore examined these structures by electron microscopy in liver sections of WT and Washc1ΔHep mice. Hepatic ablation of the WASH complex decreased the size of the lysosomes (Fig. 1C, D) and aggravated lysosomal tubulation (Fig. 1E), but did not noticeably alter the morphology of other organelles such as Golgi, endoplasmic reticulum, and mitochondria. These results demonstrate that we successfully deleted Washc1 in mouse livers, resulting in destabilization of the WASH complex and changes in lysosomal architecture in mouse hepatocytes in vivo.

Loss of hepatic WASH results in elevated plasma LDL and HDL cholesterol levels
To determine the effect of hepatic WASH deficiency on circulating lipids, we measured total plasma cholesterol (TC) and triglyceride (TG) levels in chow-fed WT and Washc1ΔHep mice. Hepatic inactivation of the WASH complex increased TC without affecting TG levels (Fig. 2A). We found no changes in hepatic cholesterol and TG contents, nor in the production of very low-density lipoprotein (VLDL) (Fig. S2A, B, C). Using targeted proteomics (20) we observed increased plasma levels of apolipoproteins ApoE and ApoB in Washc1ΔHep mice compared with WT mice (Fig. 2B). In line with increased ApoB levels, the cholesterol content of the LDL fraction isolated by fast protein liquid chromatography (FPLC) was increased (Fig. 2C and D). We also found the HDL fraction to be enriched with cholesterol and increased in size (Fig. 2C). Although total plasma ApoA-I levels were not affected (Fig. 2B) in Washc1ΔHep mice, we observed a shift of ApoA-I toward earlier eluted FPLC fractions (Fig. 2E). These fractions also contained ApoE (Fig. 2F). Altogether, the shift of ApoA-I, ApoE and HDL-C into earlier eluted FPLC fractions indicates that HDL particles in Washc1ΔHep mice are larger than in WT mice (22-24).

In contrast to humans, mice do not express cholesteryl ester transfer protein (CETP), which exchanges HDL cholesterol esters for triglycerides in (V)LDL (25). As a consequence, mice carry plasma cholesterol mainly in HDL rather than in LDL (26). To assess the effect of hepatic
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Figure 2. Loss of hepatic WASHC1 results in increased plasma LDL and HDL cholesterol. (A) Total plasma cholesterol and triglyceride levels in WT and Washc1ΔHep mice. (B) Plasma apolipoprotein levels in WT and Washc1ΔHep mice measured by targeted proteomics, indicated by fold change vs WT controls. (C) Cholesterol levels in fast-performance liquid chromatography (FPLC) fractionated plasma pools of the experimental groups of mice. (D-F) Apolipoproteins in pooled FPLC fractionated plasma of WT and Washc1ΔHep mice depicted in C determined by targeted proteomics, indicated as percentage relative to the total apolipoprotein levels in these fractions, n=5-7. BDL=below detection limit. Data are presented as the mean ± SEM, ***p < 0.001 as determined by Student's T-test.

Washc1 deletion on plasma LDL and HDL cholesterol levels in mice with a more human-like lipoprotein profile, we injected both Washc1ΔHep and WT mice with an adenovirus-expressing human CETP (Ad-CETP). Similar hepatic levels of human CETP expression were observed in
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Washc1ΔHep and WT mice (Fig. 3A). Total plasma cholesterol levels were significantly higher in CETP-expressing Washc1ΔHep compared with WT mice (Fig. 3B). Hepatic expression of human CETP resulted in the expected shift of cholesterol from HDL towards LDL in both Washc1ΔHep and WT mice (Fig. 3C). However, in Washc1ΔHep both LDL-C and HDL-C levels were increased, compared with WT mice (Fig. 3C). Altogether, these data indicate that the WASH complex plays an important role in the regulation of plasma LDL-C and HDL-C levels.

The WASH complex is required for normal expression of LDLR and LRP1 at the cell surface.

We have recently shown that WASH mediates endosomal recycling of LDLR back to the cell surface in MEFs (15). Therefore, we hypothesized that the increased LDL-C levels in Washc1ΔHep mice are due to reduced surface levels of LDLR caused by impaired LDLR recycling. To test this, we determined the cell surface levels of LDLR using a biotinylation assay. We found that the levels of LDLR were reduced on the plasma membranes of WASH-deficient primary hepatocytes compared with WT cells (Fig. 4A, B). Previously, we showed that the WASH-associated CCC complex also facilitates the trafficking of LRP1 to the plasma membrane (20). Consistent with this, we found that WASH is also required for normal levels of LRP1 at the cell surface (Fig. 4A, B). In addition to the surface levels, the total protein levels of LDLR and LRP1 were also reduced in WASH-deficient primary hepatocytes (Fig. 4A, B). This is likely due to impaired retrieval of these receptors from the lysosomal proteolytic fate into the recycling pathway (4,14,15).

Figure 3. Hepatic WASHC1 deficiency results in increased plasma LDL and HDL cholesterol in mice expressing CETP. (A) Hepatic CETP mRNA expression after Ad-CETP administration. (B) Total plasma cholesterol levels three days after Ad-CETP administration in WT and Washc1ΔHep mice. (C) Cholesterol levels of FPLC fractionated pooled plasma of the experimental groups of mice after Ad-CETP administration. n=5-6. Data are presented as the mean ± SEM, ***p < 0.001 as determined by Student’s T-test.

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Figure 4. The WASH complex is required for normal cell surface expression of LDLR and LRP1. (A) Whole cell and cell surface protein levels of LDLR and LRP1 in WT and Washc1ΔHep primary hepatocytes. (B) Quantification of immunoblot depicted in A. (C) Immunoblotting of LDLR and LRP1 three weeks after AAV-PCSK9-D377Y administration. (D) Total plasma cholesterol levels in WT and Washc1ΔHep mice before and three weeks after AAV-PCSK9-D377Y administration. (E) Total cholesterol levels of FPLC fractionated pooled plasma of WT and Washc1ΔHep after AAV-PCSK9-D377Y administration. CR=chylomicron remnants. (F) Relative plasma apolipoprotein levels in WT and Washc1ΔHep mice after AAV-PCSK9-D377Y administration as measured by targeted proteomics. (G) Coomassie blue staining of ApoB100 and ApoB48 in WT and Washc1ΔHep plasma before and after AAV-PCSK9-D377Y administration. n=6-7. Data are presented as the mean ± SEM, **p < 0.01, ***p < 0.001 as determined by Student’s T-test.
In mice, hepatic LRP1 is redundant in the clearance of plasma cholesterol, as LDLR can compensate for the loss of LRP1 (27). To address the role of WASH in LRP1-mediated plasma cholesterol uptake we expressed a human gain-of-function variant of PCSK9 (D377Y) in mouse livers, using an AAV expression system, to ablate LDLR expression (20,28). In both Washc1\textsuperscript{ΔHep} and WT mice, the expression of PCSK9-D377Y blunted hepatic LDLR levels but not the levels of LRP1 (Fig. 4C). We found that hepatic Washc1 depletion exacerbated hypercholesterolemia in PCSK9-induced hepatic LDLR-deficiency (Fig. 4D). Consistent with increased plasma chylomicron remnant/VLDL and LDL lipoprotein fractions (Fig. 4E), plasma ApoB48 and ApoB100 levels were elevated in mice deficient in hepatic WASH and LDLR, compared with mice lacking only hepatic LDLR (Fig. 4F, G). Hepatic WASH/LDLR deficiency mimics the plasma lipid phenotype in hepatic LRP1-deficient mice with an Ldlr knockout background (27), suggesting that the WASH complex is also required for hepatic LRP1-mediated cholesterol uptake \textit{in vivo}. Altogether, these data demonstrate that hepatic depletion of WASH impairs the functioning of both LDLR and LRP1 in the uptake of plasma cholesterol, thereby leading to hypercholesterolemia.

**HDL cholesterol uptake is impaired in hepatic WASH-deficient mice due to reduced surface SR-BI levels**

In addition to accumulation of cholesterol in LDL, we also found plasma levels of HDL-C to be increased in Washc1\textsuperscript{ΔHep} mice (Fig. 2C). In mice, HDL-C is cleared mainly by SR-BI, a membrane receptor that takes up CE selectively from HDL rather than internalizing the holoparticle (29). To assess whether the accumulation of plasma HDL cholesterol in Washc1\textsuperscript{ΔHep} mice is due to impaired endosomal trafficking of SR-BI to the plasma membrane, we applied a biotinylation assay to examine the cell surface levels of SR-BI in primary hepatocytes. We found the cell surface abundance of SR-BI to be markedly decreased in WASH-deficient hepatocytes compared with WT cells (Fig. 5A). It has been shown that the enzyme hepatic lipase (HL) is also involved in hepatic HDL-CE uptake (30-33). However, as we found no difference in plasma HL levels as measured by targeted proteomics (Fig. 5B), it is unlikely that HL contributes to the increased plasma HDL-C levels in hepatic WASH-deficient mice.

To establish the consequences of the reduced hepatocyte SR-BI surface levels in hepatic WASH-deficient mice in more detail, we compared the uptake of HDL-associated CE and HDL holo-particles between WT and Washc1\textsuperscript{ΔHep} mice upon injecting these mice with HDL particles containing \textsuperscript{3}H-labeled CE as well as \textsuperscript{125}I-labeled proteins. Selective HDL cholesterol uptake was calculated from the difference in the uptake of both tracers. Plasma decay of radioiodine- reflecting holo-particle HDL was not different between WT and Washc1\textsuperscript{ΔHep}
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Figure 5. Hepatic uptake of HDL cholesterol is impaired by reduced surface levels of SR-BI in hepatic WASH-deficient mice. (A) Immunoblot of whole cell and cell surface SR-BI levels in WT and Washc1 ΔHep primary hepatocytes. (B) Plasma hepatic lipase levels of WT and Washc1 ΔHep mice as determined by targeted proteomics (C) HDL protein (125I) and HDL cholesterol (3H) plasma decay in WT and Washc1 ΔHep mice. (D) Fractional catabolic rate of plasma HDL holo-particle (125I), HDL cholesterol (3H) and selective HDL-cholesterol clearance (3H/125I) of WT and Washc1 ΔHep mice. (E) HDL holo-particle (125I), HDL cholesterol (3H) and selective HDL-cholesterol uptake (3H/125I) in liver of WT and Washc1 ΔHep mice. n=6-7. Data are presented as the mean ± SEM, *p < 0.05, **p < 0.01, as determined by Student’s T-test.

mice (Fig. 5C). However, 3H-HDL-CE clearance was delayed upon hepatic WASH inactivation (Fig. 5C). From the decay curves, we calculated plasma fractional catabolic rates (FCR) and observed that HDL-CE clearance in hepatic WASH-deficient mice was significantly reduced compared with WT mice, whereas holo-particle uptake was not changed (Fig. 5D). As a consequence, the difference reflecting selective HDL-CE uptake by Washc1 ΔHep livers was
significantly decreased compared with WT livers (Fig. 5E). The kidney is the main organ for the catabolism of small, poorly lipidated ApoA-I molecules and does not express SR-BI (29,34). In line with previous studies (35), the renal uptake rate of HDL-CE was much lower than the hepatic HDL-CE uptake, and no differences were observed between WT and Washc1ΔHep mice (data not shown). Overall, these results indicate that the hepatic uptake of HDL-CE is impaired in Washc1ΔHep mice, likely due to reduced expression of SR-BI at the plasma membrane of hepatocytes.

Previous and current analyses indicate that the WASH and CCC complexes act in concert to regulate plasma lipid levels. To genetically confirm this, we inactivated both protein complexes in mouse livers by cross-breeding Washc1ΔHep mice with mice carrying Commd1 floxed alleles (15,36) (Washc1/Commd1ΔHep) (Fig. 6A). Hepatic ablation of both complexes did not further increase total plasma cholesterol levels when compared with hepatic Washc1 and Commd1 single knockout mice (Fig. 6B). In addition, hepatic ablation of both complexes resulted in increased plasma LDL-C (Fig. 6C, D) and larger HDL-C particles (Fig. 6D) to an extent similar to that seen in single knockout models (Fig. 2, (15,20)). Furthermore, targeted proteomics analysis of FPLC fractions showed a leftward shift of ApoA-I and ApoE (Fig. 6E), similar to that seen in hepatic Washc1 single knockout mice (Fig. 2D-F). Altogether, these data support the notion that the WASH and CCC complexes together form a sorting pathway that is crucial to maintain normal plasma cholesterol levels.

**Retromer plays a partial role in WASH-mediated endosomal lipoprotein receptor sorting**

It has been well established that recruitment of WASH to the endosomes relies on retromer through a direct interaction between the retromer subunit VPS35 and the WASH component WASHC2 (FAM21) (12,13,37). However, a recent study has proposed a model whereby endosomal localization of the WASH complex is partially dependent on retromer and may be to a certain degree facilitated through a direct binding of WASH to phospholipids (14).

To determine whether endosomal WASH-mediated lipoprotein receptor trafficking relies on retromer, we depleted Vps35 in mouse livers using somatic CRISPR/Cas9 gene editing technology (38,39). Three gRNA’s were designed to target Vps35 (Fig. 7A), and an adenovirus expression system was used to express the gRNA’s in the liver of hepatic Cas9 expressing mice (Cas9Hep) (38). As control mice, we used Cas9Hep mice injected with adenovirus that does not express gRNA’s (Ad-EV). To ensure randomization of both groups we collected blood and measured plasma total cholesterol levels one week before virus administration (Fig. S3A). Three weeks after virus injection, we collected blood and tissues for analysis. We assessed the protein expression of VPS35 in liver homogenates by immunoblotting, and
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observed that expression of Vps35-gRNA’s in Cas9<sup>Hep</sup> mice resulted in a marked decrease in VPS35 levels compared with control mice (Fig. 7B). Using quantitative targeted proteomics, we showed that the level of VPS35 was reduced by ±70%, accompanied by a strong decrease in protein levels of the retromer subunits VPS26A and VPS29 (Fig. 7C). Hepatic inactivation of retromer altered neither body weight nor liver to body weight ratio (Fig. S3B, C)

Hepatic Vps35 depletion resulted in a plasma TC increase of ±15% and a TG decrease of
Figure 7. VPS35 is required for hepatic LDL-C uptake but not HDL-C uptake. (A) Schematic representation of the single-vector AV system to target Vps35 in parenchymal cells of hepatic Cas9-expressing mice. Annotated are the target sequences (red) and PAM sequences (blue) of the gRNAs. (B) Western blot of VPS35 after CRISPR/Cas9-mediated Vps35 editing. (C) Protein levels of retromer components VPS35, VPS26A and VPS29 in mice targeted with gRNA-Vps35 as quantified by targeted proteomics. (D) Plasma cholesterol and (E) TG levels after CRISPR/Cas9-mediated Vps35 editing. (F) Cholesterol levels of FPLC fractionated plasma of mice targeted with gRNA-Vps35. (G) Plasma apolipoprotein levels, as measured by targeted proteomics. (H) TG levels of FPLC fractionated plasma. n=4-5. Data are presented as the mean ± SEM, *p < 0.05, ***p < 0.001 as determined by Student’s T-test.
±40% (Fig. 7D, E). In contrast to WASH depletion, hepatic VPS35 deficiency resulted in an increase only in plasma LDL-C (Fig. 7F), which was supported by elevated plasma ApoB levels (Fig. 7G). The TG concentration was reduced in the VLDL fraction of hepatic VPS35 deficient mice (Fig. 7H), and in line with this decline we observed a significant decrease in plasma levels of ApoC3, a component of VLDL (Fig. 7G).

Together these data may suggest that retromer is required for WASH-mediated endosomal LDLR trafficking, whereas WASH-mediated trafficking of SR-BI to the cell surface is retromer independent. These results further indicate that retromer is also involved in triglyceride metabolism, independent of WASH. Currently, additional studies are being performed to further assess the interrelationship of VPS35 and WASH in endosomal trafficking of LDLR and SR-BI in mouse hepatocytes.

**Plasma LDL cholesterol but not HDL cholesterol is increased by depletion of Dscr3/Vps26c**

McNally and coworkers have identified retriever as a retromer-like multiprotein complex that acts in conjunction with WASH and CCC to orchestrate retromer-independent endosomal cargo trafficking (14). Retriever is composed of DSCR3 (VPS26C), VPS29 and C16orf62 (VPS35L) and shares structural similarities with retromer. Remarkably, C16orf62 has previously been recognized as a core component of the CCC complex (5,20,40). The strong reduction in C16orf62 levels upon ablation of either the CCC component CCDC22 or COMMD proteins (20), but not upon suppression of DSCR3/VPS26C (14), favors the hypothesis that C16orf62 is assembled into the CCC complex and may not act as a distinct multiprotein complex in the WASH:CCC axis. These inconsistent data led us to examine the role played by DSCR3/VPS26C in the WASH-CCC axis to control cholesterol metabolism. Using targeted proteomics, we first determined the protein levels of DSCR3/VPS26C in hepatic COMMD1 deficient mice and found that loss of COMMD1 reduced the protein levels of DSCR3/VPS26C (Fig. 8A) in a way similar to that of other components of the CCC complex (20). Second, we ablated Dscr3/Vps26c in hepatocytes of mouse livers, using an approach similar to the one we used to ablate hepatic Vps35 (Fig. 8B, S4A). Hepatic expression of Dscr3-gRNA’s in Cas9Hop mice resulted in marked reduction in Dscr3/Vps26c mRNA levels (Fig. 8C), without affecting body weight or liver to body weight ratio (Fig. S4C). The protein levels of C16orf62, CCDC22 and CCDC93 were unaffected by Dscr3/Vps26c depletion (Fig. 8D), but hepatic ablation of Dscr3/Vps26c led to an increase in plasma TC levels without affecting plasma TG levels (Fig. 8D, E). Cholesterol accumulation in hepatic DSCR3/VPS26C deficient mice was seen only in LDL fractions (Fig. 8F).
Overall, these data suggest that DSCR3/VPS26 might be involved in trafficking of LDLR but not of SR-BI, which indicates that DSCR3/VPS26C partially participates in WASH/CCC-mediated cargo recycling.
Discussion

Mutations in subunits of the WASH complex have been correlated with developmental and neurological defects in humans (16-19,41), and here we report that the WASH complex also plays a pivotal role in the maintenance of plasma cholesterol levels. Furthermore, our data indicate that WASH acts in concert with the CCC complex to facilitate the endosomal trafficking of LDLR/LRP1 and SR-BI, but that retromer and DSCR3/VPS26C (retriever) are involved only in endosomal trafficking of LDLR.

In agreement with our in vitro studies (15), hepatocyte-specific WASH-deficiency leads to reduced LDLR and LRP1 levels at the plasma membrane, a reduction which likely underlies elevated plasma LDL-C levels in Washc1ΔHep mice. Surprisingly, we also found cholesterol accumulation in HDL, leading to larger HDL particles in Washc1ΔHep mice. SR-BI is the main receptor in hepatic HDL-C uptake in mice (42,43). The diminished surface levels of SR-BI, accompanied by reduced selective hepatic HDL-CE uptake, suggest that, in addition to LDLR and LRP1, the functioning of SR-BI is also dependent on the WASH complex. The importance of hepatic SR-BI for HDL-C metabolism has also been shown in humans (44). Individuals who are heterozygous for a loss-of-function variant in SCARB1 (P376L), the gene encoding SR-BI, have increased levels of plasma HDL-C (44). Furthermore, common genetic variants near SCARB1 have been found to be correlated with plasma HDL-C levels (45,46), underlining the importance of SR-BI in HDL-C metabolism in both species.

Notably, besides increased plasma LDL-C levels, plasma HDL-C levels were also reported to be relatively high in an RSS patient with a homozygous hypomorphic WASHC5 allele (15). As in the RSS patient, both LDL-C and HDL-C are elevated in patients with X-linked intellectual disability (XLID) caused by mutations in the gene encoding the CCC-complex component CCDC22 (15). Our current and previous mouse models mimic the dyslipidemic phenotype of RSS and XLID patients, as both plasma LDL-C and HDL-C levels are elevated upon either WASH or CCC ablation in mouse livers (15,20), suggesting that both the CCC and WASH complexes are required for efficient hepatic LDL-C and HDL-C uptake in humans and mice. In vitro studies have shown that the CCC complex is physically associated with the WASH complex through the binding of the CCC components CCDC93 and CCDC22 to the WASH subunit WASHC2 (FAM21) (5). This interaction is required for the localization of the CCC complex to endosomes and subsequently for endosomal trafficking of integral membrane proteins. Furthermore, we have shown that loss of the CCC complex led to increased expression of several WASH subunits, including WASHC1 (20). Here, we provide genetic evidence that
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the WASH and CCC complexes act in concert in the endosomal cargo recycling pathway, as simultaneous inactivation of both complexes in mouse livers does not exaggerate the hypercholesterolemic phenotype.

The findings of our current study are particularly important as very little is known about the mechanisms regulating endosomal trafficking of SR-BI. Several studies have shown that SR-BI is endocytosed (47,48) and localized in recycling endosomes (48). Our results indicate that the localization of SR-BI at the plasma membrane largely relies on the WASH-CCC axis. Based on the role of the WASH-CCC pathway in endosomal cargo recycling, we hypothesize that, after internalization, SR-BI is further transported to the endosomes from whence it is directed back to the plasma membrane. The function behind the endocytosis and recycling of SR-BI remains unclear, as the selective uptake of HDL-CE by SR-BI is independent of endocytosis (34,49,50). It has been suggested, however, that SR-BI recycling might be involved in the retro-endocytosis of HDL, and might facilitate transcytosis and transport of HDL-C into the bile canaliculus (48). Our results and model may thus offer relevant insights into the biology of the intracellular trafficking pathway of SR-BI in cholesterol homeostasis.

In the classical view, WASH recruitment to the endosomes is retromer-dependent (9,12,13). However, it has recently been demonstrated that a significant proportion of WASHC2 (FAM21) is still localized to endosomes in VPS35-deficient HeLa cells (14), suggesting that the WASH complex may also exert retromer-independent functions. Our in vivo data support retromer-independent recruitment of WASH, as depletion of hepatic VPS35 results in a more moderate increase in plasma total cholesterol levels compared with hepatic WASH depletion. This can be explained by the fact that hepatic VPS35 ablation increases only plasma LDL-C levels, whereas WASH deficiency in hepatocytes leads to an increase in both plasma LDL-C and HDL-C. These results may suggest that WASH-dependent endosomal sorting of LDLR and LRP1 relies on retromer, whereas endosomal SR-BI trafficking is WASH-dependent but retromer-independent. Furthermore, in contrast to hepatic depletion of WASH, retromer depletion results in decreased plasma TGs, implying that retromer also has a role in TG metabolism, independent of WASH.

Previous studies implied that C16orf62 participates in the CCC-complex, as deficiency of CCDC22 or COMMD proteins blunts the protein expression of C16orf62 in hepatocytes (20). However, it was recently speculated that C16orf62 is part of the retriever complex, together with DSCR3/VPS26C and VPS29 (14). Interestingly, we found here that hepatic deficiency of the CCC complex leads to a reduction in hepatic levels of DSCR3/VPS26C, whereas hepatic
DSCR3/VPS26C deficiency leads to reduced protein expression neither of C16orf62, nor the CCC components COMMD1, CCDC22 and CCDC93. Although DSCR3/VPS26C ablation does not affect the integrity of the CCC complex, our data indicate that in mice DSCR3/VPS26C participates in the CCC/WASH-mediated LDLR recycling, as loss of hepatic DSCR3/VPS26C increases plasma LDL-C. However, SR-BI-mediated hepatic HDL-C uptake does not rely on DSCR3/VPS26C, as we have found for WASH and CCC. These results suggest that DSCR3/VPS26C partially participates in the WASH/CCC axis to orchestrate the recycling of cargos, and is probably involved in selective recognition of cargos. However, more research is needed to understand the molecular organization of the endosomal cargo sorting pathways.

In conclusion, this study extends our understanding of the mechanistic basis for the increased plasma cholesterol levels in humans with defects in WASH, the CCC complex, retromer and retriever, and appeals for extra medical attention to plasma lipid levels and atherosclerotic cardiovascular disease risk in patients showing mutations in components of these endosomal multi-protein sorting complexes.

Materials and Methods

Animals
All animal studies were approved by the Institutional Animal Care and Use Committee, University of Groningen (Groningen, the Netherlands). Washc1Δ Hep C57BL/6 mice (4) were crossed with Alb-Cre expressing mice (The Jackson Laboratory stock#003574) to generate liver-specific Washc1 knockout mice. The following primers were used to genotype the mice: floxed Washc1 forward (5’- CGCATTGATCTTCCTATACGC-3’) and reverse (3’-TGTCAGTCCTATGCTTAGTG-5’); Alb-Cre forward (5’-GCGGTCTGGCAGTAAAAACTATC-3’) and reverse (5’-ACGAACCTGGTCGAAATCAGTG-3’). Rosa26-LSL-Cas9 knock in mice (#024857, The Jackson Laboratory) were crossed with Alb-Cre mice to generate liver-specific Cas9-expressing mice, and were used to target Vps35 and Dscr3 in mouse livers through CRISPR/Cas9 gene editing technology (20). The liver-specific Commd1 knockout mice were generated as previously described (36). Mice lacking both COMMD1 and WASHC1 in the liver were obtained by intercrossing liver specific Commd1 and Washc1 knockout mice.

To ablate hepatic LDLR expression of Washc1Δ Hep mice and WT littermates, as previously, we expressed PCSK9-D377Y in the liver of these mice by retro-orbital injection of 3.0x10^11 vector genomes of AAV-PCSK9-D377Y (20). CETP expression was induced by injection of 5.0x10^11 Ad-CETP particles via the orbital vein adjusted to 100 μl with sterile phosphate-
buffered saline (PBS). Ad-CETP virus was kindly provided by Uwe Tietge.

All experiments were performed with male mice (n=4-7). Mice were housed individually and fed ad libitum with a standard rodent chow diet (RMH-B, AB Diets, the Netherlands). In all experiments littermates were used as wild type (WT) controls. Before sacrifice, mice were fasted for 4 hours. 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.

**Immunoblotting**
For immunoblotting, 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 were 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**
A list of commercially acquired antibodies is provided in Table S1. Rabbit polyclonal antibodies against WASH1 (1:1000) (9), FAM21 (1:100) (9), CCDC53 (WASHC3, 1:1000) (51) and Strumpellin (WASHC5, 1:1000) (51) have previously been described.

**Transmission Electron Microscopy sample preparation and analysis**
For EM analysis, mice were fasted for 18 hours and perfusion fixed with 4% formaldehyde in 0.1 M phosphate buffer. Their livers were rapidly removed, cut into small pieces (~2 mm³), and placed and stored in glass vials with 4% formaldehyde until further processed. Samples were further fixed at room temperature by adding Karnovsky fixative (2.5% glutaraldehyde and 2% formaldehyde (Electron Microscopy Sciences) in 0.2 M Cacodylate buffer, pH 7.4) for 2 h at room temperature. They were then postfixed with 1% OsO4/1.5% K3Fe(III)(CN)6 in 0.065 M phosphate buffer for 2 h at 4°C and finally 1 h with 0.5% uranyl acetate. After fixation, samples were dehydrated and embedded in Epon epoxy resin (Polysciences). 60 nm ultrathin sections were made and contrasted with uranyl acetate and lead citrate using the AC20 (Leica) and examined with a Technai T12 electron microscope (FEI Thermo Fischer). Images were made of 2 different blocks per mouse and per condition. 25 random images
per block were collected for each mouse per condition (100 random images per condition).

All measurements of the lysosome size were done using Fiji. For quantification of the lysosome area, the perimeters of lysosomes were selected one-by-one using the freehand selection tool in Fiji, and the encircled area was measured. For visualization of lysosomal tubulation, lysosomal perimeters were selected using the Lasso tool in Adobe Photoshop, and the selected organelles were colored.

**Fast-performance liquid chromatography (FPLC)**
Plasma samples of each group of mice were pooled and fractionated by FPLC as previously described (52), 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. The total cholesterol and triglyceride content of the fractions was determined as described below.

**Hepatic lipid extraction**
Liver homogenates prepared as 15% (w/v) solutions in PBS were subjected to lipid extraction according to the Bligh & Dyer method (53). In short, 600 ml of demi-water were mixed with 200 ml of liver homogenate and 3 ml of chloroform. After 30 min of incubation, 1.2 ml of H₂O and 1 ml of chloroform were added, mixed and subsequently centrifuged (10 min, 1,500 r.p.m. at room temperature). The chloroform layer was transferred into a new glass tube, and evaporated using nitrogen at 50 °C. Lipids were resolved in 1 ml of chloroform and used for further determination of cholesterol and TG content.

**Cholesterol and triglyceride analysis in plasma and liver**
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.

**Targeted LC-MS analysis**
To quantify the protein concentrations of ApoA1, ApoB and ApoE in mouse plasma we used targeted proteomics assay as previously described (20). The protein levels of hepatic lipase (HL) in non-heparinized plasma were quantified using the isotopically-labeled
standard peptide LSPDDANFVDAHTFTR. Isotopically labeled standards were generated by concatenating all target peptides into synthetic proteins (Polyquant GmbH, Germany) containing $^{13}$C-labeled lysines and arginines (so-called QconCATs). The known concentrations of these ‘standard’ peptides were used to calculate the concentrations of the endogenous equivalents of the peptides (in amount of protein $\mu$g or mg) per volume (dL) plasma or FPLC fraction.

In-gel digestion and liquid chromatography-mass spectrometry analyses were performed as described previously (20), with the following alterations: for plasma samples, 1 $\mu$L plasma plus 20 ng QconCATs were loaded onto the gel and an equivalent of 100 nL plasma plus 2 ng QconCATs was injected into the LC-MS for analysis.

For the FPLC fractions, 15 $\mu$L of the pooled FPLC fractions were loaded onto the gel. The QconCATs were mixed after digestion, and the cleanup step after the digestion was excluded. An equivalent of 3 $\mu$L sample plus 0.4-1 ng QconCATs was injected into the LC-MS for analysis.

**Gene expression analysis**

100 mg of mouse liver 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 were used for quantitative real-time PCR (qRT-PCR) analysis, using the FastStart SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR System (Applied Biosystems). The following primer sequences were used: CETP forward (5’-GAATGTCTCAGAGGACCTCCC-3’) and reverse (5’-CTTGAACTCGTCTCCCATCAG-3’), DSCR3 forward (5’- TGCGGGTACCGTCGACGAGGGCCTATTTCC-3’) and reverse (5’-AGACGGATGCGCGGACCCAGCAGAAGG-3’), and PPIA forward (5’- TCCCTCTCCATTCAAACTATTCA-3’) and reverse (5’-CCGCCAGTGCCATTATGG-3’). 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 $\Delta\Delta$CT method of calculation. PPIA expression was used as an internal control.

**Biotinylation assay**

Culture of primary hepatocytes and biotinylation assay was performed as previously
described (Lagace 2006). Cells were washed 3x with ice-cold PBS-CM buffer (PBS, 1 mM MgCl2 and 0.1 mM CaCl2), and subsequently 0.5 mg/ml biotin reagent solution (EZ-Link Sulfo-NHS-SS_Biotin, Thermo Scientific) in biotinylation buffer (10mM triethanolamine, pH 8.0, 150 mM 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, 150 mM Tris-Cl, pH 7.4 and 192 mM Glycine) for 30 min at 4°C. Next, cells were washed 2x with PBS-CM and 1x with TBS-C (50 mM Tris-Cl, pH 7.4, 100 mM NaCl and 2 mM CaCl2). Cells were collected by scraping in TBS-C. Cells were centrifuged (1,000g, 5 min, 4°C), lysed in biotin lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 5 mM EDTA and 5 mM EGTA), sonicated (10% output for 10 s) and incubated on ice for 15 min, and subsequently centrifuged for 15 min at 12,000 g. Protein concentration was determined; 30 mg were used as input; 300 mg were used diluted in biotin lysis buffer (500 μl); 30 μl Neutravidin beads (Neutravidin plus ultralink beads, Thermo Scientific) were added and incubated for 4 h at 4°C. The beads were collected by centrifugation (500 g, 5 min), and washed 3x with Biotin lysis buffer, 1x with high-salt buffer (50 mM Tris-Cl, pH 7.4 and 500 mM NaCl) and 1x with low-salt buffer (10 mM Tris-Cl, pH 7.4 and 500 mM NaCl) and 1x with low-salt buffer (10 mM Tris-Cl, pH 7.4 and 500 mM NaCl). Finally, the beads were resuspended in 30 μl 2x loading buffer.

Radioactive labeling of HDL
Total human HDL (1.063<d<1.21 g/ml) was obtained by sequential ultracentrifugation, as previously described (54). HDL was labeled with 125I by a modification of the iodine monochloride method (55). To label HDL with 3H-cholesteryl-ether (3H-CE), 500 μCi of 3H-CE resuspended in 50 μl of ethanol were added to a solution containing heat-inactivated human lipoprotein deficient serum (200 mg protein) and iodinated HDL (6 mg protein). Radiolabeled HDL was incubated overnight at 37°C with gentle shaking, followed by re-isolation of the labeled HDL by sequential ultracentrifugation. Animals were injected with 8333 Bq 3H-CE with an activity of 357 Bq/μg HDL, and 33333Bq 125I-HDL with an activity of 2465 Bq/ μg HDL.

Adenovirus generation for somatic gene editing by CRISPR/Cas9 technology
Synthetic genes were ordered, comprising three U6-crRNA-tracrRNA cassettes flanked by Sal-I and Not-I sites (GeneArt genesynthesis, Thermo Fisher Scientific), targeting exon 1 and exon 3 of murine Vps35 (Fig. 7A), or exon 1 of Dscr3 (Fig. 8A). The 1.1 kb Not-I/ Sal-I fragments were cloned into the pENTR2B vector (Invitrogen, # 11816-014) and the adenoviral expression construct was formed by a LR recombination reaction (Invitrogen, #11791-020 following manufacturer’s protocol) with pAD/pL-dest (Invitrogen, V49420). For
virus production, 4 ug of PacI-digested expression vector were transfected into HEK293A cells (Invitrogen # R705-07). Visible virus production was observed after 9 days. Reproduction and upscaling of the virus took place 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 into 9–10 week old mice expressing Cas9, specifically in the liver (see material and methods 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.

**VLDL production assay**

After a 4-h morning fasting, mice were intraperitoneally injected with 100mg/ml poloxamer 407 (BASF) in PBS (1 g per kg body weight). Blood was drawn by retro-orbital puncture at time points 0, 30, 60, 120 and 240 min. Plasma samples were used for TG determination.

**Statistical analysis**

In vitro data were obtained from three independent experiments ± standard error of the mean (SEM). Mouse data show average values ±SEM, n=4-7. Analyses were performed using GraphPad version 6.05 (GraphPad software). Unpaired 2-tailed 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.

**Acknowledgements**

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References


Supplementary material

A.

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B.

Supplementary figure 1. Characterization of Washc1ΔHep mice. (A) Mendelian inheritance of Washc1ΔHep genotype. (B) Body weight and liver weight of Washc1ΔHep and WT mice. (C) Liver histology of Washc1ΔHep and WT mice. (D) Protein expression of the members of the CCC complex, retriever and retromer in Washc1ΔHep and WT livers as determined by immunoblotting. n=5-7. Data are presented as the mean ± SEM.
Supplementary figure 2. Cholesterol production and excretion are unaffected in Washc1ΔHep mice. Hepatic cholesterol (A) and triglyceride levels (B) in WT and Washc1ΔHep livers. (C) VLDL production 240 minutes after P407 injection in Washc1ΔHep and WT mice. n=5-8 Data are presented as the mean ± SEM.
**Supplementary table 1. List of used antibodies**

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