CHAPTER 7

General discussion
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As endosomal cargo transport is indispensable for a broad spectrum of cellular processes, it is not surprising that anomalies in the endocytic system lead to numerous disorders, including various neurodegenerative diseases and hypercholesterolemia (1, 2). In this thesis we have provided novel molecular insights into the endosomal sorting machinery in the context of cholesterol metabolism using sophisticated in vivo models. Our findings implicate an intricate network of large protein complexes that provides specificity in endosomal cargo sorting. This last chapter will summarize our major findings and place them in the perspective of current knowledge of endosomal sorting in general, and the functions of retromer, WASH, CCC and retriever in particular.

The role of the COMMD family of proteins in endosomal cargo sorting
Together with a core of CCDC22, CCDC93 and, questionably C16orf62/VPS35L, the CCC complex is composed of a combination of proteins of the COMMD family (3). The founder of this family, COMMD1, was initially identified as a protein essential to maintain hepatic copper homeostasis (4). Hepatic COMMD1 deficiency results in hepatic copper accumulation, likely due to aberrant trafficking of ATP7B, a copper transporting protein that mediates the excretion of hepatic copper into the bile (5). We have recently shown that COMMD1 also mediates the trafficking of the low-density lipoprotein receptor (LDLR), and that hepatic depletion of Commd1 leads to hypercholesterolemia (Table 1), as well as an increased susceptibility to hepatic copper accumulation (2, 6). Through the COMM domain, all ten COMMD proteins can interact with each other (7, 8), and form large protein complexes (9), with preferential, but not exclusive formation of COMMD heterodimers (9, 10). Plasticity in COMMD-COMMD protein interactions has been suggested to allow the COMMD proteins to function as adaptors within the CCC complex (9, 10). The different combinations of COMMDs within the CCC complex might be essential for the specificity of this complex in the recognition of cargos. However, the true role of the individual COMMD proteins in the CCC complex and in CCC-mediated cargo sorting is yet to be determined.

To obtain better insights into the specific role of each COMMD protein in cargo sorting, we studied different mouse models in which we specifically ablated one member of the COMMD family in hepatocytes (Commd1 (2), Commd6 (Chapter 4), Commd9 (Chapter 4) and Commd10 (unpublished)). Surprisingly, hepatic deficiency of a specific COMMD protein (COMMD1, COMMD6 or COMMD9) also blunted the protein expression of the CCC core proteins (CCDC22, CCDC93 and C16orf62) (Chapter 4). In addition to the core components,
the protein expression of all COMMD proteins was strongly reduced, but not all to the same extent (Chapter 4). Although not all COMMD proteins may be equally important for each other’s function, hepatic ablation of each COMMD protein (COMMD1, 6, 9, or 10) resulted in a similar increase in plasma cholesterol levels (Chapter 4, unpublished data, (2)). Furthermore, our unpublished data imply that all COMMD proteins are required to maintain hepatic copper homeostasis, as hepatic depletion of Commd1, Commd6 or Commd9 leads to an increase in hepatic copper levels after dietary copper supplementation (Fig. 1A and (6)). Together these results suggest that in mouse livers all COMMD proteins are equally important in endosomal sorting of cargos in cholesterol and copper transport.

Conflicting results have been obtained on individual functions of COMMDs in endosomal cargo trafficking using in vitro models. Systematic siRNA-mediated downregulation of each COMMD in HEK293T cells revealed that the cell surface expression of Notch2 is dependent only on COMMD5 and COMMD9, and not on the other COMMDs (10). In addition, in two different kidney cell lines the trafficking of EGFR relies only on COMMD5 but not on COMMD1 (11), suggesting that each COMMD protein has a specific role in the recognition

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of cargos during endosomal cargo trafficking. This specific role in cargo recognition is further supported by the finding that not all COMMD proteins can physically interact with ATP7B (12).

In contrast, our in vivo models imply that all COMMDs in hepatocytes are equally important for the endosomal transport of cargos such as LDLR and ATP7B (Chapter 4, unpublished data). These contradicting results may be due to different effects of the down regulation of a COMMD protein on the protein expression of the CCC core components (CCDC22, CCDC93 and C16orf62/VPS35L). In various cell lines, deficiency of a single COMMD protein does not always adversely affect the protein levels of CCDC22, CCDC93 and C16orf62 (10,11), whereas in mouse hepatocytes, loss of any COMMD results in a decrease of protein levels of the CCC core components. It is therefore likely that the effect of the loss of a COMMD on plasma cholesterol levels is indirect and caused by the disruption of the complete CCC complex upon ablation of any COMMD. Thus, these data imply that the integrity of the CCC complex in hepatocytes is completely dependent on each individual COMMD protein, whereas this might be different in other cell types. To understand the mechanism by which this cell specific effect is regulated, future systematic investigation of the CCC complex formation in cargo sorting is warranted.

**Participation of WASH and the CCC complex in cargo-specific molecular pathways**

Recent in vitro studies have indicated that WASH and the CCC complexes act together, as depletion of either WASH or CCC components in HeLa cells results in mislocalization of the copper transporting protein ATP7A (3). Our in vivo data support this model; depletion of either protein complex results in hypercholesterolemia due to compromised recycling of the lipoprotein receptors LRP1, LDLR and SR-B1 (Chapters 4 and 5, Table 1). Combined hepatic ablation of both WASH and CCC does not further increase plasma cholesterol levels in mice; this further supports the notion that both complexes act in a linear pathway (Chapter 5). Interestingly, our preliminary data suggest that the CCC complex and the WASH complex do not contribute equally to biliary copper transport. Loss of the CCC complex (Fig. 1A and (6)) results in a ±10 fold increase in hepatic copper levels after high copper feeding, whereas hepatic depletion of Washc1 leads only to a ±3-fold increase (Fig. 1B). These results imply that although both complexes are essential to maintain hepatic cholesterol and copper transport, and the level of contribution to biliary copper excretion of both complexes may differ. Further research is therefore needed to fully understand the roles of both complexes in copper homeostasis. In addition, it is currently unclear which other cargos in hepatocytes rely on both complexes and whether a complex-specific set of cargos exists. Moreover,
the exact role of the CCC complex in the CCC-WASH axis remains unclear. Initially, CCC was thought to act as an adapter to provide an extra layer of fine-tuning in selective cargo transport, but it is also possible that the CCC complex plays a role in regulating the function of WASH to promote Arp2/3-induced F-actin accumulation on endosomes, as hepatic loss of the CCC complex increases the protein level of several WASH components (Chapter 4).

Do retriever and retromer act as independent protein complexes in cargo sorting?
The CCC complex has originally been characterized as a protein complex consisting of CCDC22, CCDC92, C16orf62/VPS35L and the ten COMMD proteins. The first description of the CCC complex in this composition was by Phillips-Krawczak et al, who showed that all proteins of the CCC complex readily coprecipitate (3). Later studies advocated other compositions of the CCC complex. Computational approaches based on co-evolution and characterization of the human interactome suggested that the CCC complex also contains DSCR3 (later renamed VSP26C), a protein homologous to the retromer component VPS35 (13). Since C16orf62/VPS35L shows high homology with the retromer component VPS35, it has been suggested that C16orf62/VPS35L and DSCR3/VPS26C might have a function parallel to that of retromer, with the COMMD proteins providing cargo selection specificity (13). This hypothesis was further developed by McNally et al, who suggested that C16orf62/VPS35L and DSCR3/VPS26C in combination with retromer subunit VPS29 form a separate retromer-like heterotrimeric complex, termed retriever (14). Retriever facilitates endosomal sorting of sorting nexin 17 (SNX17) cargos independently of retromer (14). According to this model the CCC complex recruits retriever to the endosomes and retrieves cargos from the degradative pathway in a WASH-dependent manner (14).
Figure 2. Hypothetical models of endosomal sorting of lipoprotein receptors. A) Endosomal sorting of LDLR/LRP1. After binding of LDL to LDLR/LRP1, sorting nexin 17 (SNX17) recognizes the NPxY domain in the cytoplasmic tail of LDLR or LRP1 and, together with DSCR3/VPS35C, retrieves the receptors from lysosomal degradation after endocytosis. The WASH complex is recruited to the endosomes, which might occur via retromer, or via an unknown retromer-independent mechanism. Subsequently, WASH recruits the CCC complex via an interaction of WASHC2 with CCDC22/CCDC93. DSCR3/VPS35C couples LDLR/LRP1 to the WASH:CCC axis, after which the WASH complex facilitates deposition of branched actin patches, enabling the formation of vesicles for transport of LDLR/LRP1 back to the plasma membrane.

B) Retromer- and retriever-independent recycling of SR-B1. SR-BI facilitates uptake of HDL cholesterol, and is recognized by an unknown adaptor protein and is coupled to the WASH:CCC axis that facilitates the recycling of SR-BI back to the plasma membrane.

C) Retromer-mediated retrograde transport of Sortilin (SORT1). Recognition of SORT1 by retromer at the early endosomes facilitates retrograde transport to the Golgi apparatus, where it facilitates VLDL production and secretion. Additionally, SORT1 facilitates LDLR independent uptake of LDL. Trafficking of SORT1 to the plasma membrane has not yet been well established and might either occur directly from the early endosomes or indirectly via the Golgi apparatus.
In contrast to the hypothesis that retriever functions as an independent protein complex, we have previously shown that deficiency of CCC components results in a dramatic downregulation of C16orf62/VPS35L (Chapter 4), whereas ablation of DSCR3/VPS26C in HeLa’s did not show this effect (3). Deficiency of both CCDC22 and CCDC93 in HeLa cells did not lead to a decrease in C16orf62/VPS35C levels (14). In this thesis we aimed to obtain further insights into the function of retriever as an independent protein complex in cargo transport. In addition to the CCC-dependent protein stability of C16orf62/VPS35L, we showed that hepatic DSCR3/VPS26C protein levels also rely on the CCC complex, as hepatic depletion of Commd1 resulted in a marked reduction in DSCR3/VPS26C levels (Chapter 5). To further determine the role of retriever in cholesterol metabolism, using the somatic CRISPR-Cas9 gene editing approach to target hepatic Dscr3/Vps26c, we observed that only plasma LDL-C levels were increased (Chapter 5, Table 1). This observation implies that DSCR3/VPS26C has a specific function within the CCC:WASH-axis and facilitates the trafficking of LDLR but not SR-B1. This would support the notion that DSCR3/VPS26C specifically assists the trafficking of SNX17 cargos (14). Through its FERM-domain, SNX17 recognizes NPxY/NxxY motifs in cargos, such as the receptors of the LDLR family (15).

An increase in plasma LDL-C, and not HDL-C, was also seen in hepatic VPS35 deficient mice (Table 1), which would imply that, like retriever, retromer also facilitates endosomal LDLR but not SR-B1 transport. Interestingly, however, McNally and colleagues suggested that retriever and retromer act independently to maintain the surface levels of cargos (14). Here the authors showed that loss of VPS35 affected neither the endosomal localization of retriever nor a large pool of WASHC2. These authors suggested that retriever:CCC:WASH and retromer:CCC:WASH pathways are functionally distinct. Our data do not completely support this model, as plasma LDL-C are increased in both models (hepatic DCSR3/VPS26C and VPS35-deficient models) (Table 1), indicating that LDLR recycling is retriever:SNX17:CCC:WASH:retromer-dependent (Fig. 2A). However, based on the plasma lipid phenotype of the different models (Chapters 4 and 5), SR-B1 is CCC:WASH-dependent but retriever and retromer-independent (Fig. 2B).

Alternatively, the increased plasma LDL-C levels in hepatic VPS35 deficient mice may be caused by a different mechanism. This is supported by the finding that plasma TG was also reduced in these mice, whereas this was not observed in the knockout models for CCC, WASH and DSCR3/VPS26C. The endosomal trafficking of sortilin (SORT1) is coordinated by retromer (16) and SORT1 has also been shown to play an essential role in lipid metabolism (17, 18). It regulates VLDL production and is involved in hepatic LDL-C cholesterol uptake in
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an LDLR-independent manner (17-20). Thus, the regulation of LDL-C levels by retromer may be independent of the retriever:SNX17:CCC:WASH pathway and partially phenocopy the CCC and WASH deficient models (Fig. 2C).

Taken together, these results lead to our current hypothesis that DSCR3/VPS26C functions as an adaptor protein, which couples SNX17 with the CCC:WASH-axis to mediate the recycling of cargos, such as LDLR and LRP1 (15, 21). SR-BI is recognized independently of retromer and retriever, by an as yet to be identified adaptor which connects SR-BI to the CCC:WASH pathway. Sorting of SR-BI into the CCC:WASH pathway prevents lysosomal degradation and results in trafficking of the receptor back to the cell surface. Additional studies are needed to assess whether retromer participates in the retriever:SNX17:CCC:WASH axis to coordinate LDLR recycling or in another pathway to control plasma LDL-C levels. In addition, since plasma TG level is an additional risk factor for cardiovascular disease (22, 23), it would be of interest to further elucidate the mechanism by which VPS35 controls plasma TG levels.

Role of WASH components outside the pentameric complex

The WASH complex is a pentameric protein complex, consisting of WASHC1-5, that regulates sorting and trafficking of cargos (24-27), but recently debate has arisen about the function of WASH components outside of their pentameric context. The first indication of individual roles of WASH components was that whereas WASHC2 puncta remained localized at the endosomes in HeLa cells, independently of WASHC1 expression, other WASH components were abolished (26). Analysis of the protein stability of all 5 WASH components in Jurkat T-cells confirmed this finding, and added that WASHC1 and WASHC3 protein levels are decreased by knock down of all other WASH components, whereas WASHC2, WASHC4 and WASHC5 are decreased only by each other’s depletion, and not by depletion of WASHC1 and WASHC3 (28). In pancreas cells, WASHC1 downregulation does not affect WASHC2, whereas WASHC2 downregulation results in diminished protein levels of WASHC1, suggesting the existence of a separate WASHC2 pool (29). In MEFs, however, depletion of Washc1 resulted in substantial decrease of all WASH subunits (27). We confirmed the latter findings in vivo, and showed that hepatic depletion of Washc1 in mice resulted in a significant decrease in protein levels of all WASH components in the liver (Chapter 5).

Next to their collective role in endosomal sorting, individual WASH components have separate functions. Independent of pentameric WASH, WASHC2 can be located in the nucleus, where it results in diminished NF-κB target gene activation (29). WASHC1 covers the WASHC2s nuclear localization signal, thereby preventing nuclear localization of WASHC2.
when incorporated in the endosomal pentameric WASH complex (29).

In addition, a heterotrimeric WASH complex is located at the centrosome (30). Here, HSBP1 promotes the assembly of WASHC1, WASHC2 and WASHC3. This trimeric WASH complex was shown to be critical for tumor cell invasion in breast cancer (30). The authors of this study suggest that all typical WASH functions are carried out by ternary WASH complex, as HSBP1-depleted cells have a phenotype identical to that of WASH-depleted cells. In accordance with this hypothesis, Washc5 depleted cells still contain active WASHC1-4 and F-actin clusters at the endosomal surface, which supports the existence of WASH subcomplexes (31).

Overall these data suggest that WASH components have ambiguous functions and can participate in multiple processes, either as individual proteins, or by forming subcomplexes composed of different WASH subunits. Studies strongly suggest that these WASH subcomplexes are cell type specific, as we implied for the CCC complex, but whether these WASH and CCC subcomplexes act together remains to be investigated.

**Endosomal recycling machinery as therapeutic target to treat hypercholesterolemia**

Defects in the endocytic recycling machinery underlie many phenotypes, including hypercholesterolemia, multiple neurological diseases, and cancer types (32, 33). Hypercholesterolemia can be caused by disruption of multiple facets of cholesterol metabolism, including decreased LDL uptake due to non-functioning LDLR, or increased LDLR degradation due to overactive PCSK9 (34). Characterization of molecular pathways can be crucial for the understanding of disease processes, and subsequently to applying this fundamental knowledge in treatment, or possibly tailored treatment. One of the most potent novel therapies to lower plasma LDL-C involves inhibition of PCSK9 (35). PCSK9 targets LDLR for lysosomal degradation and inhibition of PCSK9 increases LDLR levels, subsequently ameliorating plasma LDL-C clearance (36). However, when LDLR cannot be properly recycled back to the plasma membrane, and taking into account that during its lifespan LDLR can be recycled up to 100 times (37), one can envision that preventing degradation of LDLR might not be sufficient to improve plasma cholesterol levels when endosomal LDLR recycling is impaired. Combining knowledge of molecular processes in hypercholesterolemia with individual screening of hypercholesterolemia patients may help in development of optimal treatment strategies for patients.

Since impairment of the endosomal sorting machinery lies at the base of multiple diseases, amelioration of endosomal cargo sorting can be considered a potential therapeutic target,
for instance by stabilizing proteins of the endosomal sorting machinery to improve recycling of specific cargos. Such a strategy has been studied for both retromer and the CCC complex.

Stabilization of retromer has been tested as a potential strategy in the treatment of Alzheimer’s disease. Neuronal retromer facilitates recycling of amyloid-precursor protein (APP), both via retrograde trafficking or direct trafficking to the cell surface (38). Retromer deficiencies result in accumulation of APP at endosomes, where it will be cleaved to amyloid-β (Aβ), the pathogenic fragment of Alzheimer’s disease. The pharmacological chaperone R33 was developed to bolster the core structure of the trimeric retromer complex, thereby increasing complex stability and enhancing retromer function (39). Incubation of human induced pluripotent stem cells (hiPSCs) with chaperone R33 resulted in reduced Aβ-secretion and TAU phosphorylation, both drivers of Alzheimer pathology (40). This study suggests that pharmacological intervention to stabilize retromer could be an interesting therapeutic strategy to ameliorate Alzheimer’s disease.

Increased functioning of the CCC complex has recently been associated with lower plasma LDL-C levels (41). GWAS were analyzed to identify loci of the endosomal sorting machinery associated with low LDL-C and low total cholesterol plasma levels. This strategy resulted in the identification of a missense variant rs17512204 in CCDC93. Carriers with this variant present with reduced myocardial infarction prevalence, and in vitro studies demonstrated that this variant increased the protein stability of CCDC93. These results suggest that pharmacological agents that improve the functioning of the CCC complex might be applicable to ameliorate LDLR recycling and subsequent hepatic LDL-C uptake. Interestingly, increased LDL-C uptake was also seen in cells overexpressing SNX17 (42), further supporting the potential of the endosomal sorting machinery as a therapeutic target.

These initial studies show promising effects for the stabilization of the endosomal sorting machinery to treat disease; however, the versatile nature of endosomal sorting should be carefully considered when developing therapeutics, as the endosomal sorting machinery facilitates recycling of large groups of cargos.

**Concluding remarks**
In recent years, advances in understanding the mechanisms of endosomal cargo sorting have been realized by unraveling the molecular organization of the endosomal sorting pathways and the identification of the cargos sorted by these pathways. Endosomal sorting processes have been investigated primarily in vitro (continuous cell cultures), which has
hindered relating this fundamental knowledge to its physiological significance. In the studies addressed in this thesis, we combined classical methods with state-of-the-art gene editing technologies to study the physiological role of the different complexes of the endosomal sorting machinery in vivo, with a focus on cholesterol metabolism. Our studies have provided novel insights into how the intracellular trafficking of different lipoprotein receptors (e.g. LDLR, LRP1, and SR-BI) is molecularly coordinated. We have expanded our understanding of the intricate interplay between the different protein complexes of endosomal sorting machineries. Although our in vivo and recent in vitro data show some inconsistencies in the molecular organization of the different complexes, we strongly believe that combining different research disciplines, such as cell biology, physiology, genetics and proteomics, will increase our understanding of the molecular organization of endosomal sorting pathways and their contribution to (patho)physiological processes. This knowledge can pave the way for better prognosis, diagnosis and realization of innovative therapies to treat diseases caused by damaged endosomal sorting processes, such as hypercholesterolemia, as well as copper-storage and neurological diseases.
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