Identification of a novel multiprotein complex in cargo sorting that preserves metabolic pathways in the liver
Fedoseienko, Alina

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General discussion

The COMMD proteins together form an evolutionarily highly conserved family of proteins. With the exception of COMMD1, the founding member of this family, the functions of the other members remain largely unknown. In this thesis, we aimed to uncover the biological role of the other COMMD proteins, in particular COMMD6. We focused on COMMD6 because this member consists predominantly of the COMM domain, a unique domain that is characteristic of the COMMD family. Using cellular and mouse models this research uncovered for the first time that the COMMD proteins together form a multiprotein complex to control various biological processes, including copper homeostasis and cholesterol metabolism. Our findings also implicate the existence of different COMMD-associated subcomplexes controlling various cellular processes, which vary between cell types. In this chapter, I will discuss my findings in relation to the current knowledge of COMMD1 and will give future perspectives.

COMMD proteins in endosomal cargo trafficking

COMMD1 was initially discovered as a protein regulating copper homeostasis in mammals. Until now, the mechanism of regulation remained unclear (reviewed in 1). Phillips-Krawczak and colleagues showed that COMMD1 is a component of the multiprotein complex CCC (COMMD/CCDC22/CCDC93) and that, in conjunction with the WASH complex, it facilitates the endosomal trafficking of the copper transporting protein ATP7A². In the absence of the CCC complex, ATP7A trafficking is impaired, resulting in compromised response to copper, and eventually in intracellular copper accumulation². In hepatocytes cellular copper homeostasis is dependent on ATP7B, a copper transporting protein that is highly homologous to ATP7A.
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Figure 1. Simplified model of the ATP7B and LDLR trafficking pathway in hepatocytes. Under normal conditions, ATP7B localizes to the trans-Golgi network (TGN). Upon elevated copper levels, ATP7B relocates to vesicular compartments at the cell periphery. From these peripheral vesicles, ATP7B can reach the plasma membrane to excrete copper into the bile canaliculus. From the Golgi LDLR is directed to the cell surface. At the cell surface LDL cholesterol (LDL) binds to LDLR, and LDL-LDLR complex is internalized via endocytosis and is sorted at endosomes. From the endosome LDLR can be recycled back to the cell surface or be directed to lysosomes for proteolysis. Through the interaction of FAM21 with the retromer component VPS35, WASH and CCC are recruited to the endosomes (marked CCC, WASH and retromer). Subsequently, CCC and WASH form a protein complex with LDLR; WASH mediates branched actin on endosomes, these acting patches defines regions from which LDLR is sorted back to the cell surface. CCC, COMMD1/CCDC22/CCDC93/C16orf62; WASH, WASH1, FAM21, strumpellin, KIAA1033 (SWIP), CCDC53; Retromer, VPS26, VPS29, VPS35.

Therefore, one can speculate that in hepatocytes COMMD1 assists the trafficking of ATP7B in a similar fashion as has been reported for ATP7A (Figure 1). A role for COMMD1 in endosomal sorting in hepatocytes is corroborated by our recent findings showing that COMMD1 also regulates the endosomal trafficking of LDLR $^3$ (Figure 1). We found that
both COMMD1 and the WASH complex bind to LDLR, and loss of either COMMD1 or WASH impairs LDLR sorting, resulting in decreased LDLR levels at the cell surface, and eventually reduced LDL uptake. In line with our in vitro results, inactivation mutations in COMMD1 lead to hypercholesterolemia in mice and dogs. Loss-of-function mutations in the CCC component, CCDC22, and the WASH component Strumpellin are both associated with hypercholesterolemia in humans. Taken together, these data clearly demonstrate the significance of the CCC and WASH complexes for the preservation of cholesterol homeostasis.

Although all COMMD proteins can interact with each other and have the ability to associate with CCDC22\textsuperscript{4,5}, it remains unclear whether they all participate in the CCC-complexes to facilitate the trafficking of ATP7B and LDLR. Therefore, we decided to generate various mouse models to study COMMD proteins, including COMMD6 (Chapter 4). We expected that COMMD6 would be the most valuable member of the family to provide insights into the biological role of the COMMD family, as COMMD6 only consists of the COMM domain\textsuperscript{6}. This domain is characteristic for the COMMD family, and is fundamental for protein-protein interactions\textsuperscript{4,6-8}, including binding to components of the CCC complex (CCDC22 and CCDC93)\textsuperscript{5}.

In this thesis we show that ablation of Commd6 in hepatocytes results in decreased levels of a subset of COMMD proteins, including COMMD1, 3, 4, 5, 9 and 10, as well as the CCC components CCDC22 and CCDC93 (Figure 4A-Chapter 4 and Table1). Similar effects were seen upon Commd1 deletion in hepatocytes (Figure 4C-Chapter 4 and Table 1); moreover, like COMMD1 deficiency \textsuperscript{3,9}, COMMD6 inactivation leads to hypercholesterolemia and increased susceptibility to hepatic copper accumulation in mice. Interestingly, although COMMD6 silencing in various cell lines results in COMMD1 insufficiency, downregulation of COMMD1 does not affect the protein levels of COMMD6.
Figure 2. The organization of the COMMD proteins within the CCC complex in endosomal LDLR sorting in hepatocytes. This hypothetical complex consists of COMMD1, COMMD3, COMMD4, COMMD5, COMMD6, COMMD9 and COMMD10. By ablation of COMMD1, COMMD6 or COMMD9, other components of the complex become unstable and are directed for proteolysis. Only COMMD1 was shown to interact directly with LDLR and WASH complex; the effect of COMMD6 and COMMD9 on cholesterol metabolism is probably indirect (through regulation of COMMD1 stability). CCC, COMMD proteins/CCDC22/CCDC93/C16orf62; WASH, WASH1, FAM21, strumpellin, KIAA1033 (SWIP), CCDC53.

These data suggest that COMMD6 is required for the integrity and subsequently the function of the CCC complex, which is likely composed of multiple COMMD proteins (Figure 2). We showed that COMMD6 interacts with all COMMD proteins and other proteins of the CCC-complex (CCDC22, CCDC93, C16orf62), but we could not identify a physical interaction and colocalization between COMMD6 and LDLR, COMMD6 and WASH, and COMMD6 and retromer, as was demonstrated for COMMD1. These data indicate that COMMD6 indirectly controls cholesterol homeostasis. Furthermore, we do not know whether COMMD6 interacts with ATP7B, but considering that COMMD6 is likely not a component of the CCC complex associated with WASH and retromer suggests that COMMD6 preserves copper homeostasis, also indirectly. Our current model is that COMMD6 is needed for the assembly of the CCC complex that is composed of a subset of COMMD proteins (in hepatocytes COMMD3, 4, 5, 9 and 10), CCDC22, CCDC93 and C16orf62 (Figure 2). Our
data indicate that COMMD6 is not present in the final CCC complex, which recognizes specific cargos, and in conjunction with WASH, facilitates the endosomal sorting of cargos, such as LDLR.

Taken together, our data suggest that COMMD6 indirectly regulates cholesterol and copper homeostasis by mediating the integrity of the CCC complex that consists of a subset of COMMD proteins. To get better insights into the composition of the CCC-complex formation additional antibodies have to be generated to allow co-localization and protein-protein interaction studies. Furthermore, it would be of great interest to assess whether the endosomal trafficking of other cargos is dependent on the COMMD proteins. Additional COMMD knockout mice models would be essential to confirm the role of other COMMD proteins (COMMD3-4-5-10) in LDLR and ATP7B trafficking.

**COMMD proteins in inflammation and cancer**

Prior studies indicate the existence of different COMMD-CCDC22 complexes involved in NF-κB signaling, and as myeloid COMMD1 suppresses inflammation in different inflammatory disease models\(^{10,11}\) we therefore assessed the function of other COMMD proteins in inflammation. In vitro studies reported that COMMD6 also inhibits NF-kB activity through an interaction with the NF-kB subunit p65\(^4,6\). However, it has never been established whether COMMD6 controls inflammation in vivo. As total body knockout of COMMD6 is lethal\(^{12,13}\) (this study), we generated a series of mice in which Commd6 expression was gradually reduced from normal (wild type mice) to 20% (Commd6\(^{-/H}\) mice) (Chapter 5). We expected that such dramatic decrease of COMMD6 levels in Commd6\(^{-/H}\) mice would subsequently impair the integrity of the CCC complex in different tissues, as was the case in liver specific Commd6 knockout mice (Chapter 4). However, here we discovered that although the protein levels of COMMD1 were slightly decreased in tissues of Commd6\(^{-/H}\)
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mice, the levels of reduction were quite variable. Furthermore, no indication of enhanced diet-induced liver inflammation or elevated circulating cholesterol was found in these mice. These results suggest that 20% expression of *Commd6* in mice is sufficient to maintain the levels of COMMD1 that are needed to control NF-κB-activity\textsuperscript{10,11} and to facilitate the endosomal trafficking of LDLR.

In this study, we showed for the first time that the composition and functions of the CCC complexes are tissue/cell-type specific (Table 1).

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KO = knockout; — = protein levels unchanged; ↓ = protein levels are decreased; ND = not determined

The steady-state levels of COMMD proteins that are affected by COMMD6 depletion various between tissues/cell types. For example, *Commd6* ablation in macrophages results in reduced levels of COMMD1 and COMMD10, but is not associated with enhanced inflammatory response (unpublished data), whereas myeloid COMMD1 deficiency results in a significant decline in COMMD1, 5, 9 and 10, and a slight decrease in COMMD3 and COMMD4 (Table 1). This effect of myeloid COMMD1 deficiency on the COMMD
proteome is associated with increased inflammatory response\textsuperscript{10,11}. These phenotypic differences between myeloid COMMD1 and myeloid COMMD6 deficient mice could be explained by the reduction of COMMD5 and/or COMMD9 levels in COMMD1 deficient macrophages. To that end, we assessed the role of myeloid COMMD9 in inflammation by depleting \textit{Commd9} specifically in the myeloid lineage (Chapter 6). We excluded any role for myeloid COMMD9 in inflammation, as the inflammatory response of BMDM is not affected by \textit{Commd9} ablation, and in contrast to myeloid COMMD1, diet-induced liver inflammation is not aggravated by the loss of myeloid COMMD9. Proteome analysis of the COMMD family in COMMD9 deficient BMDM shows that COMMD9 is not required for the levels of other COMMD proteins, as in the case of COMMD1 and COMMD6 (Table 1); this suggests that myeloid COMMD9 participates in a protein complex distinct from COMMD1 and COMMD6\textsuperscript{5,14}. This observation is in line with prior studies showing the presence of COMMD-CCDC22 complexes with different functions. For example COMMD1-CCDC22 and COMMD8-CCDC22 are two discrete protein complexes in NF-κB signaling (Figure 3). COMMD1 suppresses NF-κB by promoting the ubiquitination and subsequently the proteasomal degradation of p65 (Figure 3A)\textsuperscript{7,15}. COMMD1 tethers the components of the Cullin2-RING ubiquitin ligase into a functional complex, while COMMD8 and CCDC22 both participate in the Cullin1-RING ligase complex that promotes the degradation of the NF-kB inhibitor IκB\textsuperscript{16} (Figure 3B). The observation that COMMD9 is the only member of the COMMD family that does not interact with a Cullin protein\textsuperscript{17} further supports our finding that COMMD9 is not involved in NF-κB-mediated inflammation and it exerts its function in different protein complexes. Indeed, of the ten COMMD proteins only COMMD9 and COMMD5 act together with CCDC22 to regulate Notch signaling\textsuperscript{2}.  

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Figure 3. Simplified model of the COMMD proteins in NF-κB signaling. A. The interaction of COMMD1-CRL2 complex with p65 is promoted by phosphorylation at serine 468 of p65$^{25}$, and results in ubiquitination and subsequent proteasomal degradation of p65. B. COMMD8 is in complex with CCDC22 and CRL1 and promotes the degradation of IκBα, hereby stimulating NF-κB activity. CRL1, Cullin1-RING ubiquitin ligase; CRL2, Cullin2-RING ubiquitin ligase; Ub, ubiquitin; P, phosphorylated serine.

Remarkably however, depletion of Commd9 in hepatocytes leads to a decrease in COMMD1, CCDC22 and CCDC93 levels, accompanied by hypercholesterolemia; this suggests that in hepatocytes COMMD9 is a part of the CCC complex that mediates endosomal LDLR sorting (Figure 2), but whether it is also involved in copper homeostasis needs to be investigated.

Previously the action of COMMD1 with WASH in endosomal cargo sorting seemed to be unrelated to its role as a negative regulator of NF-κB. However, recent evidence points to a possible overlap between these two pathways. In addition to COMMD1, also the WASH component FAM21 interacts with the NF-κB subunits p50 and p65$^{17}$. FAM21 represses NF-κB-dependent gene transcription by affecting p65 chromatin binding. FAM21-depletion decreases the recruitment of p65 to several NF-κB-target chromatin regions. The nuclear
levels of FAM21 are controlled by a nuclear localization signal sequence (NLS), as well as a CRM1/exportin1-dependent nuclear export signal (NES). Furthermore, FAM21 depletion sensitizes pancreatic cancer cells to chemotherapeutic drugs gemcitabine and 5-fluorouracil, an effect which is probably mediated by the inhibiting action of FAM21 on NF-κB\textsuperscript{18}. Like FAM21, COMMD1 also has a role in chemotherapy sensitivity in various cancers\textsuperscript{19,20}, including ovarian cancer. In chapter 3 we demonstrated that nuclear COMMD1 affects the sensitivity of ovarian cancer to platinum based therapy. We found that in a subset of cancer tissue samples the nuclear expression of COMMD1 was elevated; this increase in nuclear COMMD1 levels is correlated with an improved response to chemotherapy, possibly by inhibiting the NF-κB-mediated expression of the antiapoptotic genes $\text{XIAP}$ and $\text{BCL2}$. Elevated nuclear COMMD1 in a subset of ovarian cancers is a fascinating phenomenon, as in most cell types COMMD1 is expressed predominantly in the cytoplasm\textsuperscript{2,21,22}. Although COMMD1 contains NES, the molecular mechanism that controls its nuclear levels remains unclear. It would therefore be of interest to assess whether COMMD1 and other COMMD proteins act in complex with FAM21 to regulate NF-κB signaling in cancer cells, and whether COMMD1 requires FAM21 to enter the nucleus\textsuperscript{22}.

We confirmed a role for nuclear COMMD1 in sensitizing ovarian cancer to cisplatin in the ovarian cancer cell line A2780 (Chapter 3). We speculated (summarized in Figure 4D, Chapter 3) that nuclear COMMD1 inhibits BRCA1-NF-κB mediated transcription of the antiapoptotic genes $\text{BCL2}$ and $\text{XIAP}$ and thereby sensitizes ovarian cancer cells to cisplatin-induced apoptosis. However, additional studies are required to better understand the mechanism by which nuclear COMMD1 improves cisplatin sensitivity in ovarian cancer.

**COMMD subcomplexes.**
The existence of distinct COMMD-CCDC22 complexes has been established\(^5\) (Figure 1), as corroborated by our \textit{in vivo} studies. Hepatocellular distribution and the reduced levels of COMMD proteins in the COMMD6\textsuperscript{Hep} and COMMD1\textsuperscript{Hep} livers indicate that the CCC complex in hepatocytes consists of COMMD1, 3, 4, 5, 9 and 10 (Figure 2). However, it remains unclear whether all these members form one large protein complex as depicted in Figure 2. It is very likely that there are different subCCC complexes to regulate the trafficking of particular cargos, as recently indicated for COMMD5 and COMMD9 in Notch signaling\(^{23}\).

Interestingly, in contrast to myeloid COMMD9, hepatic COMMD9 inactivation resulted in reduction of COMMD1 (Chapter 6 Figure 4E), together with other components of the CCC complex (CCDC22 and CCDC93)\(^3\). As expected, this inactivation of the CCC complex resulted in elevated plasma cholesterol levels, but the level of increase of plasma cholesterol by \textit{Commd1} ablation is higher than by \textit{Commd9} deletion. Unfortunately, we were not able to determine the levels of all COMMD proteins in COMMD9 depleted liver samples, so it is unclear whether other members of the family (COMMD4, 5 and 10) are also reduced to the same extent as with COMMD1 deficient hepatocytes. In contrast to hepatic \textit{Commd1} ablation, the levels of COMMD3 are not affected upon COMMD9 inactivation. It is possible that the level of COMMD1 reduction in COMMD9 deficient hepatocytes does not result in a complete loss of COMMD1, which is indispensable to have an impact on the levels of COMMD3 (and maybe other COMMD proteins). In chapter 3 we showed that COMMD3 is present in the same sucrose gradient fractions as CCDC22, WASH, FAM21 and LDLR (Chapter 4 Figure 5A), a fact which indicates that COMMD3 is a part of the CCC complex that acts together with WASH in the endosomal sorting of LDLR. It is therefore tempting to speculate that in COMMD9 deficient hepatocytes COMMD3 can partially take over the function of COMMD1, resulting in a more moderate increase in plasma cholesterol levels.
Altogether, our data indicate that COMMD proteins can form multiprotein complexes comprised of different combinations of COMMD proteins in a tissue-specific manner to regulate particular cellular processes \(^2,^5,^23\) (Figure 2). Assembly of large stable protein complexes built of one protein family is not unique. For example, inactivation of a member of the Conserved Oligomeric Golgi (COG) family of proteins, an evolutionarily conserved Golgi-associated tethering complex (Cog1 – Cog8), disrupts the integrity of the COG complex\(^24\). Some subunits of COG complex are essential for the targeting of the complex to the Golgi membranes, and loss of these subunits results in degradation and/or mislocalization of other components of the complex\(^24\). If we draw a parallel between COMMD and the COG family, it is highly possible that a loss of one particular COMMD protein can also lead to mislocalization of family members without affecting their steady-state levels. As an example, COMMD1 deficiency affects not only the COMMD3 levels but also its colocalization with the WASH complex (Chapter 4 Figure 5A). Further research is needed to unravel the structural organization and functions of these COMMD-associated protein complexes.

**Concluding remarks and future directions**

The COMMD family of proteins was described a decade ago, and here we provide novel insights into the biology of these proteins. To our knowledge COMMD proteins do not contain any enzymatic activities, and therefore we consider that they act as scaffold proteins to fine-tune essential cellular processes such as inflammation, cholesterol and copper homeostasis. It has previously been hypothesized that COMMD proteins have unique functions, as they are evolutionarily conserved and non-redundant. However, our results show that COMMD proteins not only act alone, such as COMMD1 in the E3 ubiquitin ligase complex\(^7\) (Figure 3), but also together in large multiprotein complexes.
As a component of the CCC complex, COMMD1 has been shown to regulate copper and cholesterol homeostasis and inflammation in mammals. Here we show that also other COMMD proteins, such as COMMD6 and COMMD9, play an important role in cholesterol and copper homeostasis (COMMD6), but not in inflammation. We report that COMMD proteins are required for the steady-state levels of the CCC complex in a tissue dependent manner. However, further research is needed to unravel the compositions and functions of the different CCC complexes. Additional proteomic and biochemical analyses should be conducted to uncover the exact compositions of these complexes, to identify the cargos sorted by the CCC complexes, and to assess whether cargos are regulated by a specific subset of COMMD proteins, as shown for COMMD5 and 923. It is still unclear how cargo specificity is regulated: are additional proteins needed, or do the COMMD proteins have unique sequences that are involved in cargo recognition? Furthermore, the role of the CCC complex in WASH mediated cargo trafficking has still to be resolved.

Taken together our work excavated the COMMD proteins as a novel family of proteins in endosomal cargo sorting. Better understanding of these pathways will advance therapeutic research to treat hypercholesterolemia and copper disorders.

References


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CHAPTER 7


Discussion


