Identification of a novel multiprotein complex in cargo sorting that preserves metabolic pathways in the liver
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CHAPTER 5

Generation and characterization of hypomorphic Commd6 mice

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In Preparation


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Abstract

The COMMD family of proteins consists of ten members and has multiple functions. Studying of their biological role in mice is, however, limited, as deletion of individual Commd genes results in embryonic lethality at an early stage in development. We previously showed that deletion of Commd6, specifically in hepatocytes, results in reduced levels of a subset of COMMD proteins, including COMMD1. This reduction in protein levels is accompanied by elevated circulating low-density lipoprotein (LDL) cholesterol and increased susceptibility to hepatic copper accumulation. To be able to study the role of COMMD6 at the organismal level we generated mice with a hypomorphic Commd6 allele. Combining this hypomorphic allele with either a wild type or knockout allele, a series of live-born mice were generated in which the Commd6 expression was gradually reduced. The combination of a Commd6 null allele with a hypomorphic allele resulted in an 80% reduction in Commd6 expression. Despite the decreased levels of Commd6 mRNA we observed no significant effect on COMMD1 protein levels. Moreover, we saw no difference in plasma cholesterol levels between wild type and hypomorphic mice. Altogether, we successfully generated a
hypomorphic Commd6 mouse model, but observed that a decreased expression of Commd6 does not cause an overt phenotype in mice.

Introduction

COMMD1, previously known as MURR1, was initially identified as a gene involved in copper homeostasis\(^1\); based on a unique region in the carboxy terminus nine factors homologous to MURR1 were identified \(^2\). This domain was called the Copper Metabolism MURR1 Domain, and MURR1 was renamed as COMMD1\(^2\). The ten COMMD proteins form the COMMD family, which throughout evolution are conserved in different species, starting from lower metazoans, where eight of the COMMD genes can be found (excluding COMMD1 and COMMD9), to vertebrate species, where all ten COMMD genes are present \(^2\). This strong conservation between species indicates that the COMMD proteins have unique and important functions. In contrast to the shared COMM domain in the carboxy terminus, the amino terminus of COMMD proteins is unique. For instance, human COMMD1 and COMMD10 are only 34\% conserved in the non-COMMD region \(^2\).

Currently, COMMD1 is the best-studied member and is a protein with pleiotropic functions \(^3,4\). Along its role in inflammation \(^3,5,7\) and hypoxia signaling \(^8,9\) we showed that COMMD1 preserves copper \(^1\) and cholesterol homeostasis \(^10\) by facilitating the endosomal trafficking of copper transporters ATP7A/7B \(^11\) and low-density lipoprotein receptor (LDLR) \(^10\), respectively. About the functions of other COMMD family members much less is known. COMMD5 and COMMD7 have been associated with regulation of cell proliferation and cell cycles \(^12,13\). COMMD5 controls cell growth and differentiation through p21 transactivation \(^12\). Additionally, COMMD5 has a role in kidney repair after injury by means of its effect on renal cell migration and TGF-β secretion \(^14\). COMMD7 was reported to affect hepatocellular carcinoma progression by reducing cell apoptosis and preventing cell cycle arrest \(^13\). Like
COMMD1, COMMD3 and COMMD9 can mediate sodium transport by altering the expression of the epithelial sodium channel (ENaC) at the cell surface. Recently, COMMD5 and COMMD9 were also shown to regulate the endosomal sorting of Notch family members, a transmembrane family that mediates multiple cell differentiation processes during embryonic and adult life.

Investigating the function of the COMMD proteins in mice has been limited because deletion of individual Commd genes results in embryonic lethality at an early stage in their development. In dogs this is not the case, but mice lacking COMMD1 die around day 9.5-10.5 of embryogenesis due to abnormal placental vascularization, likely caused by increased activity of the hypoxia inducible factor 1α (HIF-1α). As with Commd1, loss of Commd9 or Commd10 in mice causes early death; both models die within 11-12 days of embryogenesis (and personal communication E.Burstein). Homozygous deletion of a genomic region (Acrg minimal region) that contains 4 genes, including Commd6, also leads to arrest in embryonic development. Two genes in this region (Lmo7 and Uchl3) were excluded as candidate genes for embryonic arrest at day 8.5 of embryogenesis, leaving Commd6 as the most likely gene required for embryogenesis.

Despite the fact that the COMMD proteins interact with each other to regulate various transmembrane proteins (Chapter 4), the different phenotypes of Commd knockout mice indicate that besides their joint action each member has its own specific function. Therefore, it would be of interest to uncover the biological functions of COMMD proteins at an organismal level. To do so we decided to apply a gene targeting methodology, which allows us to generate a series of mice with progressively reduced levels of protein, starting from normal to zero. Since COMMD6 is the most interesting member of the COMMD family, as it consists practically only of the COMM domain and is required for the expression
of almost all COMMD proteins (Chapter 4), we generated a hypomorphic Commd6 allele, which resulted in a mouse model expressing a fraction of its normal Commd6.

**Material and methods**

**Animals**

Hypomorphic Commd6 mice were generated as described in the Materials and Methods section of Chapter 4. These mice were individually housed males, fed ad libitum with either a standard rodent chow diet (RMH-B, AB Diets, the Netherlands) or, starting at 10 weeks of age, a high-fat, high-cholesterol (HFC) diet (45% calories from butter fat) containing 0.2% cholesterol (SAFE Diets), n=8-14. HFC feeding lasted for 12 weeks. Mice were sacrificed following a 4-hour morning fasting period. Tissues for mRNA and protein expression analysis were snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Blood was drawn by means of heart puncture, and plasma was isolated by centrifugation at 3000 rpm for 10 min. at 4°C. All animal studies were approved by the Institutional Animal Care and Use Committee, University of Groningen (Groningen, the Netherlands).

**Cholesterol and triglyceride analysis in plasma**

Colorimetric assay (11489232, Roche) with cholesterol standard FS (DiaSys Diagnostic Systems Gmbh) was used to determine total plasma cholesterol (TC) levels. Triglyceride (TG) levels were measured using Trig/GB kit (1187771, Roche) with Roche Precimat Glycerol standard (16658800) as a reference.

**Gene expression analysis**

Pieces of murine liver of approximately 100 mg were homogenized in 1 ml QIAzol Lysis
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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 (Roche), according to the manufacturer’s protocol. 20 ng cDNA was used for subsequent quantitative real-time PCR (qRT-PCR) analysis using FastStart SYBR Green Master (Roche) and 7900HT Fast Real-Time PCR System (Applied Biosystems). The following PCR program was used: 50 °C/2 minutes, 95 °C/10 minutes, 40 cycles of 95 °C/15 seconds and 60 °C/1 minute. Expression data were analyzed using SDS 2.3 software (Applied Biosystems), applying the ‘standard curve’ method of calculation. PPIA expression was used as an internal control for mouse samples. Primer sequences are listed in Table S1.

Western blot analysis
As described in Chapter 4 we performed Western blot analysis, using the following antibodies: rabbit anti-COMMD1 (11938-1-AP, Proteintech Group), mouse anti-β-Actin (A5441, Sigma-Aldrich), HRP-conjugated goat anti-rabbit IgG (H + L) (170-6515, Bio-Rad), HRP conjugated goat anti-mouse IgG (H + L) (170-6516, Bio-Rad).

Statistical analysis
Mouse data show mean values ±SEM. Analyses were performed using GraphPad version 6.05 (GraphPad software). The Student’s t-test was used to test significance. For all experiments a P-value of <0.05 was considered statistically significant.

Results and Discussion
To study the biological role of COMMD6 at the organismal level we generated mice with a
hypomorphic *Commd6* allele using a multifunctional targeting strategy. Generation of the mice carrying a hypomorphic allele is described in Chapter 4. The mechanism of a hypomorphic allele used here is illustrated in Fig. 1A. A neomycin resistance (*Neo*) gene was inserted into intron 3 of *Commd6* by homologous recombination. This neo gene harbors a cryptic exon that affects normal splicing of pre-mRNA.

During pre-mRNA splicing this cryptic exon is either spliced into mRNA product (80-95%) or spliced out (5-20%). The mRNAs carrying the cryptic exon will be translated into truncated protein because the neo cassette will introduce stop codons in all 3 reading frames. The limited amount of normal mRNA will be translated into a wild type protein. According to previous publications the reduction in protein expression varies between 75% and 90%.

We examined the expression of *Commd6* mRNA in a series of mice carrying different combinations of wt (+), hypomorphic (H), and knockout (-) alleles, using a primer set located in exon 2 (p1) and exon 4 (p2) (Fig. 1A). Gene expression analysis showed that mice homozygous for the hypomorphic allele (H/H) have approximately a 60% reduction in *Commd6* mRNA levels in different tissues, compared to wild type (wt) mice (Fig. 1B). We also generated mice heterozygous for a *Commd6* null allele (+/-) (as described in Chapter 4). As expected, heterozygous *Commd6* null mice showed a 50% reduction in *Commd6* expression, and combining a hypomorphic allele with a knockout allele (-/H) resulted in *Commd6* expression of only 20% in various tissues (Fig. 1B). In contrast to *Commd6*/H mice, which were born according to Mendelian ratio without an overt phenotype, *Commd6* null mice were not born.
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A. Schematic representation of Commd6 targeting strategy to generate a Commd6 hypomophic allele. Homologous recombination marked with crosses. Location of primer binding sites marked with arrows. B. Commd6 mRNA levels in mice with different genotypes. wt = wild-type, - = knockout allele, H = hypomorphic allele (n=5-8). Quantitative RT-PCR performed with primer combination p1+p2.

Figure 1. Generation of Commd6 hypomorphic mice.
In Chapter 4 we reported that ablation of Commd6 in hepatocytes results in reduced protein levels of COMMD1 accompanied by elevated circulating LDL cholesterol. To assess whether COMMD6 insufficiency also causes elevated plasma cholesterol concentrations we compared the total plasma cholesterol levels of high-fat/high-cholesterol (HFC, cholesterol 0.2%) fed Commd6−/− mice with HFC-fed wild type mice. Twelve weeks of HFC feeding resulted in lipid deposits in both groups, but we observed no significant differences between the two groups (Fig. 2A). Moreover, we saw no changes in body, liver weights (Fig 2B,C), or total plasma cholesterol and plasma triglyceride concentrations (Fig. 2 D, E). Altogether, these results demonstrate that 80% reduction in Commd6 expression does not cause hypercholesterolemia, as was reported for hepatic Commd6 null mice (Chapter 4).

We recently reported that myeloid COMMD1 suppresses inflammation in different inflammatory disease models, including non-alcoholic fatty disease (NASH) 23,24. Furthermore, loss of COMMD6 causes decreased COMMD1 levels in bone marrow derived macrophages and in a mouse macrophage cell line (RAW 264.7) (personal communication AF&BS, and chapter 4). These observations prompted us to investigate the consequences of Commd6 insufficiency on diet-induced liver inflammation. After 12 weeks of HFC feeding we determined the hepatic mRNA levels of pro-inflammatory (Il-1α, Il-1β, TNF-α, Mcp1, Ccl5) and anti-inflammatory (A20, IκB-α) genes (Fig. 3), but we saw no differences between wild type and Commd6−/− mice. These data indicate that reduced Commd6 expression does not exacerbate diet-induced liver inflammation (Fig. 3), such as previously demonstrated for myeloid Commd1 deficiency 23.
Figure 2. COMMD6 hypomorph mice do not show elevated total plasma cholesterol levels.

A. H&E and ORO straining of hepatic tissue from 4-hour fasted chow and HFC-fed mice. H&E staining performed on paraffin-embedded samples and ORO staining on snap-frozen hepatic cryo-sections. B. Body weight (BW) and C. liver weight (LW), represented as % of the BW, of wild-type (WT) (n=8) and Comm6⁻/⁻ mice (-/-H) (n=14) after 12 weeks of high-fat/high-cholesterol (0.2%) (HFC) diet feeding. D. Total plasma cholesterol, and E. plasma triglyceride (TG) levels of WT and -/-H mice fed a HFC diet for 12 weeks. All group averages presented with ±SEM.
Figure 3. COMMD6 insufficiency does not augment diet-induced liver inflammation.

Relative liver mRNA expression of pro-inflammatory cytokines and NF-κB target genes IL-1α, IL-1β, TNF-α, Mcp-1, Ccl5, A20, IκB-α in liver of WT (n=8) and -/H mice (n=14), as determined by quantitative RT-PCR. All values per group shown as mean ± SEM.

As Commd6-/- mice do not show elevated circulating cholesterol levels and enhanced diet-induced liver inflammation, this indicates that Commd6 insufficiency does not result in a reduced level of COMMD1, a critical factor for recycling of LDLR and suppressing of NF-κB activity (reviewed in 3). To test this notion we determined the protein levels of COMMD1 in numerous tissues of chow-fed Commd6-/- mice.

Although protein levels of COMMD1 are decreased in Commd6-/- livers and kidneys, the levels of reduction are quite variable, regardless of the marked reduction of Commd6 expression in these samples (Fig.4 A, B). These results suggest that 20% Commd6 expression in mice is sufficient to control the levels of COMMD1 needed to facilitate the endosomal trafficking of LDLR, and to inhibit NF-κB-mediated inflammation.
Figure 4. COMMD1 levels are not markedly affected by COMMD6 insufficiency.

A. Protein levels of COMMD1 in liver, kidney and spleen lysates of WT (n=3) and -/H mice (n=8) determined by immunoblot analysis.

B. mRNA levels of Commd1 and Commd6 in liver, kidney and spleen of WT (n=3) and Commd6^{-/-} mice (n=8), as determined by quantitative RT-PCR. All values per group shown as mean ± SEM; significance tested against control group: ***p<0.001.
To conclude, in this study we have successfully generated a series of mice in which $\text{Commd6}$ expression is gradually reduced from normal to 20%. In contrast to $\text{Commd6}$ null mice, mice with an 80% reduction in $\text{Commd6}$ expression are viable and are born without a clear phenotype. We found that 20% $\text{Commd6}$ expression is sufficient to manage adequate levels of COMMD1 in various tissues, levels necessary to control various biological processes such as cholesterol homeostasis and inflammation. Although our strategy did not result in a model with $\text{Commd6}$ expression below a threshold resulting in an overt phenotype, it is still very likely that under certain conditions other biological processes are affected by decreased $\text{Commd6}$ expression. For example, a gene expression study showed that the expression of $\text{COMMD6}$ in monocytes of healthy humans was significantly upregulated in an acute hypoxia response (personal communication PZ and BS). In this study $\text{COMMD6}$ was the most strongly upregulated gene (>5 fold), and its increased expression was positively correlated with the expression of genes encoding ribosomal proteins, indicating that COMMD6 might be involved in protein synthesis under hypoxic conditions.

Altogether, using a multi-purpose gene-targeting vector we successfully generated a hypomorphic $\text{Commd6}$ allele that also allows us to generate a conditional and a knockout allele to study the biological function of COMMD6 in mice.
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References


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Table S1. qRT-PCR primer sequences.

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<th>Reverse 5’→3’</th>
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