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Novel insights in the molecular pathogenesis of human copper homeostasis disorders through studies of protein-protein interactions

Nieuwe inzichten in de moleculaire pathogenese van humane koper homeostase ziekten verkregen door eiwit-eiwit interactie studies
(met een samenvatting in het Nederlands)

Proefschrift

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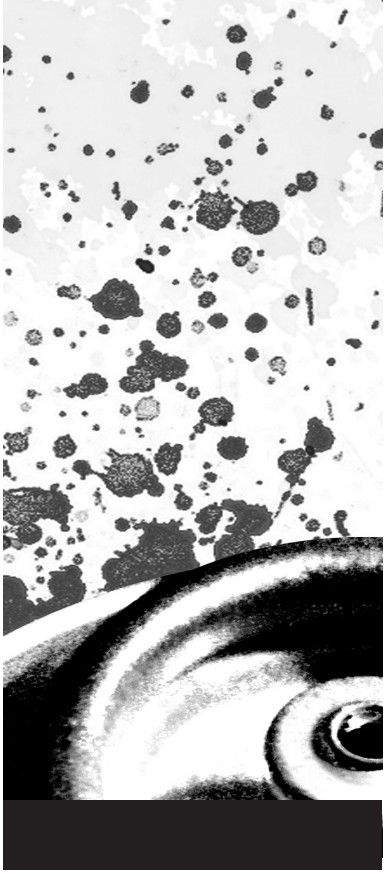
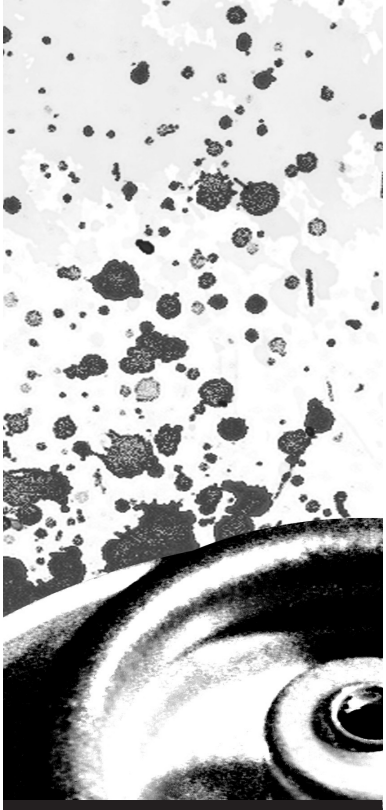


Outline of this thesis

Copper is an essential element for living organisms, yet it is very toxic when present in amounts exceeding cellular needs. Delicate mechanisms have evolved to ensure proper copper homeostasis is maintained for the organism, as well as at a cellular level, and perturbations in these mechanisms give rise to several severe diseases of copper homeostasis. However, the exact mechanisms through which such disorders of copper homeostasis develop are not completely understood and the work in this thesis therefore aims to dissect the molecular pathogenesis of human copper homeostasis disorders. Copper deficiency, as caused by mutations in *ATP7A* in Menkes disease, manifests with neurological degeneration, growth defects and several other symptoms that directly relate to dysfunction of copper-dependent enzymes. Copper overload is observed in several different disorders in man. Wilson disease, for example, is caused by mutations in *ATP7B* and manifests with copper accumulation in the liver and brain, resulting in hepatic and neurological defects. Mutations in ATP7A or ATP7B, causing Menkes and Wilson disease respectively, affect the molecular functions of these proteins in various ways. The relation of these disease-causing mutations and the pathogenesis of Menkes and Wilson disease is discussed further in **chapter 1**. Other copper overload disorders comprise Indian childhood cirrhosis (ICC), endemic infantile Tyrolean cirrhosis (ETIC) and idiopathic copper toxicosis (ICT), caused by both excessive copper intake and an uncharacterized genetic predisposition. Previous studies in our department identified COMMD1 as a novel protein involved in copper homeostasis; a deletion in *COMMD1* underlies the development of copper toxicosis in Bedlington terriers, a disorder that shares many pathophysiological features with ICC, ETIC, and ICT. Although copper toxicosis in Bedlington terriers was initially studied as a model for ICC, ETIC, and ICT, no defects in *COMMD1* could be related to these disorders. The experimental work in this thesis aimed to functionally characterize COMMD1 through the study of protein-protein interactions. Identifying novel interacting partners was expected to result in increased understanding of the mechanism through which COMMD1 exerts its function in copper homeostasis, and lead to identification of novel candidate genes for ICC, ETIC and ICT. In addition, over the last few years since the identification of COMMD1, protein-protein interaction studies have implicated COMMD1 in a number of cellular processes extending beyond the pathways of copper homeostasis that include sodium transport, signal transduction and ubiquitin modifications – these are reviewed in **chapter 2**. Using yeast two-hybrid technology, we searched for novel interacting partners of COMMD1. These studies resulted in the identification of the previously uncharacterized protein COMMD6. In **chapter 3**, the interaction of COMMD1 with COMMD6 is characterized further. Together with studies by Burstein et al., this work has contributed to the identification and characterization of the COMMD protein family, a family consisting of nine human homologues of COMMD1. To investigate whether the 10 human COMMD proteins play a role in copper homeostasis, we assessed the potential of COMMD proteins to interact with the copper transporting ATPases ATP7A and ATP7B, as described in **chapters 4 and 5.** As our data implicated three additional COMMD proteins in the hepatic copper excretion pathways, the genes encoding these COMMD proteins were investigated as candidate genes for ICC, ETIC, and ICT (see **chapter 4)**. This study

revealed several single nucleotide polymorphisms that could be involved in the pathogenesis of these disorders. In the studies described in **chapters 5 and 6,** we investigated several potential molecular mechanisms through which COMMD1 regulates ATP7A- and ATP7B-mediated copper transport. Experimental data is provided suggesting that COMMD1 facilitates proteolysis of ATP7B (**chapter 6)**. In addition, **chapter 6** describes a deregulation of the interaction between COMMD1 and ATP7B resulting from Wilson disease-causing mutations in ATP7B, implicating COMMD1 in the molecular pathogenesis of Wilson disease. Finally, in **chapter 7** the results of the studies in this thesis are discussed, providing an integrated view of the implications on our current understanding of the functions of COMMD1 as well as the molecular pathogenesis of human disorders of copper homeostasis.



CHAPTER 1

Molecular pathogenesis of Wilson and Menkes disease; correlation of mutations with molecular defects and disease phenotypes

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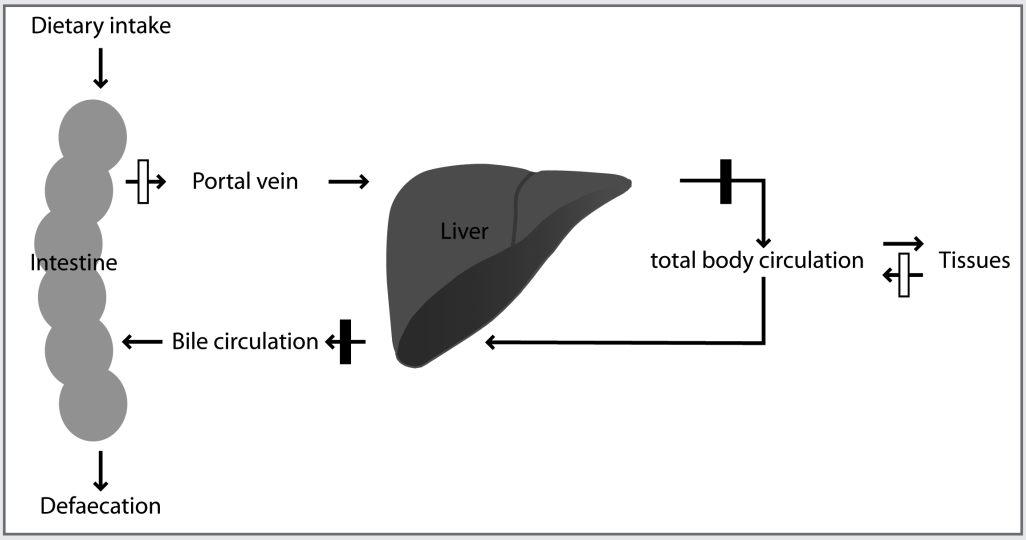
ABSTRACT

The trace metal copper is essential for a variety of biological processes, but extremely toxic when present in excessive amounts. Therefore, concentrations of this metal are kept within a tight balance. Central regulators of cellular copper metabolism are the copper transporting P-type ATPases ATP7A and ATP7B. Mutations in *ATP7A* or *ATP7B* disrupt the homeostatic copper balance resulting in copper deficiency (Menkes disease) or copper overload (Wilson disease), respectively. ATP7A and ATP7B exert their functions in copper transport through a variety of interdependent mechanisms and regulatory events, including their catalytic ATPase activity, copper-induced trafficking, post-translational modifications and protein-protein interactions. Here we will review the extensive efforts that have been undertaken over the last few years to dissect and characterize these mechanisms, and how these are affected in Menkes and Wilson disease. As both disorders are characterized by an extensive clinical heterogeneity, we will discus how the underlying genetic defects correlate with the molecular functions of ATP7A and ATP7B and with the clinical expression of these disorders.

INTRODUCTION

Many trace elements require a delicate homeostatic balance to ensure that the needs for normal cellular processes are met, whereas toxicity due to excessive accumulation of these elements needs to be prevented. Copper is an excellent example of such a trace element. It is required for numerous cellular processes, including mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, connective tissue formation, pigmentation, peptide amidation and iron metabolism (table 1) [1]. In amounts that exceed cellular needs, copper is highly toxic due to its potential to facilitate the production of reactive oxygen species by means of Fenton chemistry [2]. Refined mechanisms have evolved to regulate intake, excretion and the cellular distribution of copper (box 1). The importance of these regulatory mechanisms is underscored by several hereditary human disorders of copper homeostasis. These disorders can broadly be divided in two classes; (i) diseases associated with copper deficiency (Menkes disease (MD); OMIM #309400 [3] and occipital horn syndrome (OHS); OMIM #304150), and (ii) diseases associated with copper excess (Wilson diseases (WD); OMIM #277900 [4], Indian childhood cirrhosis (ICC); OMIM #215600 [5], endemic Tyrolean infantile cirrhosis (ETIC); OMIM #215600 [6] and idiopathic copper toxicosis (ICT); OMIM #215600 [7, 8]). The clinical expression of several of these disorders is highly heterogeneous. In this review we will discuss both the genetics and molecular-functional defects underlying MD and WD. Finally we will discuss the genotype-phenotype correlations of these disorders and how such correlations might be explained by the molecular-functional defects. Additional information of interest to the reader, but not essential to the scope of this review is provided in the text boxes.

BOX 1. COPPER HOMEOSTASIS



Copper is taken up from the diet in enterocytes and subsequently effluxed to the liver through the portal vein. This step is blocked in the copper deficiency disorders Menkes disease (MD) and occipital horn syndrome (OHS), as indicated by the white bar in the figure. From the liver, copper is distributed to the general circulation to provide tissues with required copper. Excretion of copper from the body is mediated for 98% by billiary export, indicating that the liver plays a central role in the regulation of body copper homeostasis [230]. Copper excretion from the liver is defective in Wilson disease (WD), Indian childhood cirrhosis (ICC), endemic Tyrolean infantile cirrhosis (ETIC) and idiopathic copper toxicosis (ICT) (illustrated by the black bars in the figure) resulting in accumulation of excess copper in the liver. To ensure copper homeostasis on the cellular level, refined mechanisms to regulate copper uptake, distribution and excretion have evolved. Copper import in the cell takes place via the copper transporters 1 and 2 (CTR1+CTR2) [102, 231, 232]. After uptake, distribution of intracellular copper is facilitated by a group of proteins called copper chaperones, which function to deliver free copper to its sites of utilization. Several human copper chaperones have been described; the copper chaperone for superoxide dismutase 1 (CCS) delivers copper to superoxide dismutase 1 (SOD1) in the cytosol and mitochondria [233, 234], COX17 delivers copper to the cytochrome C oxidase complex in mitochondria [235, 236], and ATOX1 delivers copper to the copper transporting ATPases ATP7A and ATP7B in the *trans* Golgi complex [164, 165]. These copper transporting ATPases play an essential role in the export of copper from the cell. Dysfunction of these proteins underlies the development of MD, OHS and WD, and forms the topic of this review.

COPPER TRANSPORTING ATPASES AND HUMAN DISEASE

(i) Diseases associated with copper deficiency

MD is an X-linked recessive disorder characterized by a general copper deficiency [3, 9]. The incidence of the disease is estimated to range between 1:40,000 and 1:350,000 [10-12]. Clinical features of MD are a direct consequence of dysfunction of several copper-dependent enzymes (cuproenzymes; table 1), secondary to an inability to load these enzymes with copper. Based on the symptoms, two forms of MD have been described; classical MD, and the less severe mild MD. The clinical features of classical MD typically comprise neurological defects (severe mental retardation, neurodegeneration, seizures), growth retardation, hypothermia, laxity of skin and

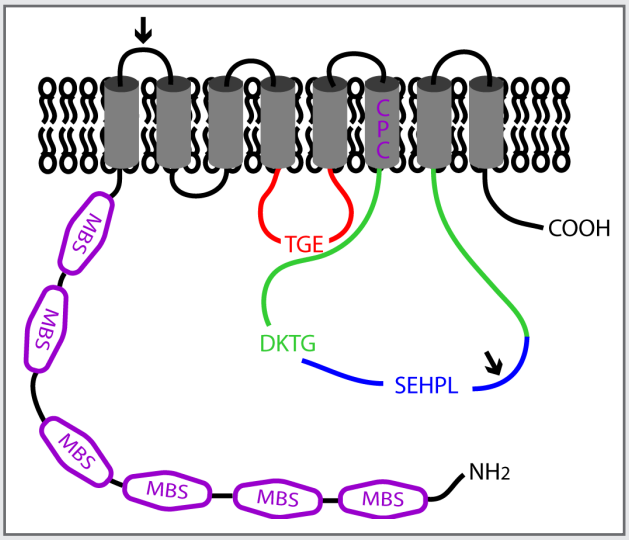
joints, hypopigmentation, and peculiar 'kinky or steely' hair [13-15]. Patients present at 2-3 months of age, and due to the severity of the disorder death usually occurs by 3 years of age. Patients with mild MD display an increased life span, and in these patients the neurological defects are particularly less profound [16-18]. OHS (also known as X-linked cutis laxa, or Ehlers-Danlos syndrome type IX) is allelic to MD and its symptoms are generally overlapping with those observed in MD, with the most notable exception that neurological abnormalities are far less severe, or even absent in OHS. Patients with OHS present with slightly subnormal intelligence, whereas seizures are generally absent. Connective tissue abnormalities represent the predominant features of OHS. Characteristic is the formation of occipital exostoses resulting from calcification of the trapezius and sternocleidomastoid muscles at their attachments to the occipital bone [13, 19]. Treatment of MD consists of copper replacement therapy in which the following fundamental issues should be taken in consideration: (i) the block in intestinal absorption of copper must be bypassed, (ii) patients must be identified and treatment started as early in life as possible, (iii) circulating copper must be delivered to the brain, and (iv) copper must be available within cells for cuproenzyme biosynthesis [20, 21]. The only currently available treatment option consists of administration of copper-histidine, a naturally occurring copper-amino acid complex in serum [22, 23]. Although copper replacement therapy with copper histidine resulted in significant improvement in some patients leading to an increased lifespan, it has not been uniformly benificial and the prognosis of patients with classical MD remains inevitably poor. The age at which treatment is started and the severity of the disease, seem to be among the main determinants for the outcome of this therapeutic approach [20, 24].

Table 1. Functions of copper-dependent enzymes

Enzyme	Function	Consequences of deficiency
Ceruloplasmin	Iron and copper transport	Decreased circulating copper levels,
Cytochrome C oxidase	Mitochondrial respiration	Hypothermia, muscle weakness
Dopamine β-hydroxylase	Catecholamine production	Hypothermia, neurological defects
Lysyl oxidase	Connective tissue formation	Laxity of skin and joint
Peptidylglycine alpha-amidating monooxygenase	Peptide amidation	Neuroendocrine defects
Superoxide dismutase	Antioxidant defense	Diminished protection against oxidative stress
Tyrosinase	Pigment formation	Hypopigmentation of hair and skin

Both MD and OHS are caused by mutations in the *ATP7A* gene [18, 25-27]. *ATP7A* encodes a highly conserved copper translocating P(1B)-type ATPase with orthologues present in eukaryotes, prokaryotes and archaea [28]. *ATP7A* mRNA is expressed in a wide range of human tissues, but its expression is notably low and sometimes even undetectable in the adult

BOX 2. STRUCTURE OF COPPER TRANSPORTING ATPASES



ATP7A and ATP7B are highly homologous and both belong to the heavy metal transporting P(1B)-type ATPases. The basic topology of ATP7A and ATP7B is depicted in the figure. The polypeptide sequences of ATP7A and ATP7B are 54% identical, however two sequence inserts are present in ATP7A that are not present in ATP7B (the position of these sequences are marked with an arrow). Several conserved motifs are present in both ATP7A and ATP7B that are characteristic for the P-type ATPase protein family [125]. These motifs are required for ATP catalysis and include the nucleotide binding domain (N-domain; depicted in blue), the phosphorylation domain (P-domain; depicted in green) and the actuator domain (A-domain; depicted in red). Highly conserved signature residues are present in these motifs; SEHPL in the N-domain, DKTG in the P-domain, and TGE in the A-domain. The specific functions of these domains are discussed in the text. Within the amino terminal tail six metal binding sites (MBSs; depicted in purple) are present, each containing the core sequence MxCxxC. These MBSs bind Cu(I) in a stoichiometry of one atom Cu(I) per MBS [126, 127]. Structural analysis of separate MBSs of ATP7A or ATP7B demonstrated a conserved βαββαβ folding structure [237-243]. The sequence and structure of these MBSs are highly conserved in evolution and to other proteins, such as the copper chaperone ATOX1 [28]. These amino-terminal MBSs in ATP7A and ATP7B are required for several aspects of their function, including copper translocation, incorporation of copper in cuproenzymes, ATPase activity, localization and trafficking, and protein-protein interactions [106, 109, 129, 130, 136, 145, 150, 168, 174, 177, 244-246]. Strikingly however, bacterial and yeast orthologues of ATP7A and ATP7B only contain one or two MBSs, indicating that the six MBSs in ATP7A and ATP7B might be partially redundant. In general, it is believed that P-type ATPases contain binding sites for their substrate ion within their transmembrane regions to facilitate transfer of these ions across the membrane barrier [125]. Transmembrane domain six contains a highly conserved CPC motif (depicted in purple) that is characteristic for heavy metal transporting P-type ATPases [247, 248]. Peptides containing this motif have been shown to bind copper, indicating that the CPC motif could indeed be involved in the actual transfer of copper across the membrane [243].

liver [25, 27]. The ATP7A polypeptide contains several conserved domains required for ATPase function and copper binding (box 2). It has been estimated that one third of MD cases arise from *de novo* mutations [13]. Over 200 MD-causing mutations have been identified, among which small deletions/insertions, nonsense mutations, missense mutations, and splice site mutations are represented with an equal frequency [29, 30]. Small deletions/insertions and nonsense mutations are found throughout the whole gene. In contrast, missense mutations are almost exclusively distributed between the first transmembrane domain and the stop codon. The relative lack of MD- or OHS-causing missense mutations within the six metal binding sites (MBSs) located in the amino terminal tail suggests that functional redundancy exists among these six MBSs. Splice site mutations are mostly clustered between exons 6-8, which encodes a region just

upstream of the first transmembrane domain, and between exons 21-22, a region encoding the last transmembrane domain. Interestingly, splice site mutations appear to be overrepresented in patients with OHS [29, 30].

Several mouse models for MD and OHS have been described, collectively known as mottled mice [31]. The mottled mice phenotypes show a similar variability as observed between MD and OHS patients. The dappled subtype displays the most severe phenotype; affected mice usually die during prenatal development. Blotchy mice on the other hand, display a milder phenotype, reminiscent of OHS. Both dappled and blotchy, as well as other mottled subtypes, are caused by mutations in *Atp7a*, the murine ortholog of *ATP7A* [32-42]. Transgenic expression of ATP7A in mottled mice of the brindled subtype rescued the phenotype and partially restored the copper balance [43]. *Calamity*, a zebrafish mutant defective in the ortholog of ATP7A (atp7a), has recently been established [44]. *Calamity* zebrafish display a general copper deficiency phenotype and form an excellent model to study the function of *atp7a* in the developing zebrafish.

(ii) Diseases associated with copper excess

WD is an autosomal recessive disorder characterized by defective copper excretion, with an estimated incidence between 1:30,000 and 1:100,000 [45]. Clinical features of WD result from toxic accumulation of copper primarily in the liver and the brain, and therefore may include hepatic abnormalities (cirrhosis and chronic hepatitis, culminating in progressive liver failure), neurological defects (Parkinsonian features, seizures), and psychiatric symptoms (personality changes, depression, psychosis) [45, 46]. In some severe cases, patients present with fulminant liver failure. A characteristic feature often observed in WD patients is the Kayser-Fleischer ring, a deposition of copper in Descemet's membrane visible as a gold-brown ring around the periphery of the cornea. In addition, serum levels of the cuproenzyme ceruloplasmin are often found greatly reduced in WD patients as a result of rapid degradation of the copper-free form of ceruloplasmin formed in WD patients [47, 48]. This type of ceruloplasmin deficiency is distinct from aceruloplasminemia, a disorder of iron metabolism caused by mutations in the *ceruloplasmin* gene [49]. The presentation of WD is highly heterogeneous, even among patients with the same mutations. Differences have been observed in age at presentation, severity of the disease and the predominance of hepatic versus neurological symptoms [48]. Treatment of WD focuses on two aspects: (i) copper excretion from the body must be promoted, and (ii) copper absorption from the diet must be reduced [45]. The first aspect is best accomplished by copper chelation therapy using penicillamine [50], trientine [50], or ammonium tetrathiomolybdate [51, 52]. The latter remains under clinical trial, but seems particularly promising as, when taken with meals, this compound also prevents absorption of copper from the diet. Dietary copper absorption is also efficiently inhibited by zinc ingestion, and by omitting copper-rich dietary components. Controversy exists as to which approach constitutes the preferred treatment regimen, and proper randomized control studies are lacking to clarify this issue. In some cases, the preferred method includes initial copper chelation followed by zinc therapy to prevent remission of high

copper concentration in the liver. In general, these treatment options are very effective, however if treatment is ineffective, or in circumstances of fulminant liver failure, liver transplantation provides an effective cure.

The gene mutated in WD, *ATP7B*, encodes a copper transporting P-type ATPase that is highly homologous to ATP7A [53-56]. The expression pattern of *ATP7B* is strikingly different to that of *ATP7A*, as *ATP7B* is most abundantly expressed in the liver. Other tissues that express *ATP7B* include kidney, brain, lung, heart, mammary gland, and placenta [53, 56-58]. Almost 300 mutations in *ATP7B* have been described that are associated with WD development [59]. In contrast to MD, relatively few small insertions/deletions and splice site mutations were identified in WD patients. In fact, almost 60% of all identified WD-causing mutations are missense mutations. The distribution of these mutations over *ATP7B* is similar to the distribution of MD-causing missense mutations in *ATP7A* [30]. Whereas most WD-causing mutations are rare and only reported in single families, some are more common and account for a large portion of WD cases. The most prevalent mutations are H1069Q in Europe and Northern-America, and R778L in South-East Asia [60].

ICC, ETIC and ICT form a second class of copper overload disorders that are distinct from WD [61, 62]. The majority of ICC, ETIC and ICT cases are fatal at an early age due to liver failure as a consequence of decompensated liver cirrhosis. Neurological defects are not observed in ICC, ETIC or ICT. Phenotypic expression of ICC, ETIC and some cases of ICT appears to be associated with both an excessive copper intake and an underlying genetic defect [5, 6, 8, 63]. Attempts to identify the genetic causes for these disorders have remained unsuccessful, although several candidate genes, including *ATP7B*, have been excluded [61, 62].

Several animal models of copper overload diseases have been described [64]. Long-Evans Cinnamon (LEC) rats and toxic milk mice suffer from abnormalities in the *Atp7b* gene, making these valid spontaneous models for WD [65-70]. In addition, an engineered *Atp7b* knockout mouse was recently generated [71]. The hepatic abnormalities observed in both the LEC rat and the toxic milk mouse closely resemble those found in WD, but neurological defects have only been observed in the *Atp7b* knockout mouse, but not in the LEC rat nor in the toxic milk mouse [64, 71]. Another interesting animal model characterized by hepatic copper overload is copper toxicosis in Bedlington terriers (CT) [72]. Pathophysiologically, CT is similar to WD although neurological defects have not been observed and serum ceruloplasmin concentrations are normal. Furthermore, linkage analysis excluded *Atp7b* as a candidate gene for CT, suggesting that CT is more likely to be a model for ICC, ETIC or ICT [73, 74]. Using positional cloning approaches, a deletion in *COMMD1* (formerly *MURR1*), resulting in complete absence of the COMMD1 protein, was identified as a genetic cause for CT [75-77]. This observation initially led to the postulation of *COMMD1* as a candidate gene for human copper overload disorders. However, no disease-causing mutations in *COMMD1* were detected in several cohorts of patients suffering from WD, ICC, ETIC or ICT [61, 78-82]. Possibly, other genetic causes for CT in Bedlington terriers exist, as in several pedigrees with affected dogs no mutation in *COMMD1* could be detected [83, 84]. Nine homologues of COMMD1 have recently been reported, which should be prioritized in search of alternative genetic causes of CT [85].

FUNCTIONS OF COPPER TRANSPORTING ATPASES

The physiological functions of ATP7A and ATP7B can largely be deduced from the observed phenotypes in MD and WD, respectively. In MD, copper transfer across the mucosal barrier is impaired, whereas in WD hepatic excretion of copper into the bile is reduced, suggesting that both proteins are rate limiting for cellular copper export. Biochemical and clinical observations in MD and WD patients suggested an additional role for ATP7A and ATP7B in cuproenzyme biosynthesis, as markedly reduced copper incorporation of several cuproenzymes has been observed in MD and WD [47, 86-90].

Regulation of cellular copper export

Direct evidence for a copper export function of ATP7A and ATP7B came from cell culture studies showing that absence of ATP7A in MD derived patient fibroblasts resulted in cellular copper accumulation and increased copper retention, despite normal copper uptake rates [91]. This copper retention phenotype could be corrected by expression of either ATP7A or ATP7B [92]. Consistent with these observations, overexpression of ATP7A or ATP7B in a variety of cell lines resulted in a decrease in copper accumulation and retention, and in an increased tolerance to elevated environmental copper levels [93-95]. Direct proof for the actual copper translocation function of these proteins came from studies showing that overexpression of ATP7A or ATP7B resulted in increased translocation of ^{64}Cu into isolated membrane vesicles, which was also shown using purified ATP7A reconstituted in soybean asolectin liposomes [96-98]. Taken together, these data suggest that both ATP7A and ATP7B function as ATP-dependent copper export pumps. The difference in phenotype between MD and WD is mainly determined by differences in tissue distribution and cellular localization of ATP7A and ATP7B, as further discussed below. Unfortunately, effects of MD and WD-causing mutations on the copper transport activity of ATP7A and ATP7B have only been determined using complementation assays of Δ*CCC2* yeast as discussed in the paragraph below. Whilst these yeast studies have clearly demonstrated the inability of mutated ATP7A and ATP7B proteins to mediate copper delivery to the secretory pathway, no studies have been performed yet to directly elucidate the effects of disease-associated mutations on the transport activity of ATP7A and ATP7B. Significant effort is currently undertaken to develop several new tools that allow detection of changes in intracellular copper concentrations and bioavailability [99-102]. Among these new tools, an MRE-luciferase reporter developed in our laboratory can be used to assess the ability of ATP7A and ATP7B to exort copper from the cytosol in a sensitive and robust manner, and appears usefull to assess the effects of MD- and WD-causing mutations on cytosolic bioavailable copper (P.V.E. van de Berghe and L.W.J. Klomp, unpublished observations).

Regulation of cuproenzyme biosynthesis

Loss of function of several cuproenzymes is a characteristic feature of both MD and WD. Using fibroblasts isolated from MD patients or mottled mice, it was shown that ATP7A deficiency directly results in reduced activities of the copper-dependent enzymes lysyl oxidase,

tyrosinase, cytochrome C oxidase, extracellular superoxide dismutase and peptidylglycine alpha-amidating monooxygenase [86-90]. Although not consistently observed among all patients, WD is often associated with a dramatic reduction in serum ceruloplasmin levels as a result of rapid degradation of the copper-free form of ceruloplasmin secreted from hepatocytes of WD patients [47, 48].

Table 2. Effects of MD-causing missense mutations on function and regulation of ATP7A

Mutation:	Cuproenzyme biosynthesis	Catalytic ATPase activity	Localization	Post-translational modifications	Protein-protein interactions
A629P	Reduced rescue ΔCcc2 yeast [106]				
S637L	Reduced rescue ΔCcc2 yeast [107]				
R844H			No signal observed, protein absent in patient fibroblasts [108]		
G860V			No signal observed, protein absent in patient fibroblasts [108]		
L873R	No rescue ΔCcc2 yeast [109]	Increased formation of acylphosphate intermediate [109]	Cell periphery and plasma membrane [109], no copper response [109]		
G876R			No signal observed, protein absent in patient fibroblasts [108]		
Q924R			Partial in cell periphery [108]		
C1000R	No tyrosinase activity [110]		Normal, TGN [108, 109], no copper response [109]		
A1007V			Normal, TGN [108]		
G1015D			Normal, TGN [108]		
G1019D	Reduced rescue ΔCcc2 yeast [106]		Partial ER mislocalization [111], copper response present [111]	Impaired glycosylation [111]	
D1044G			Normal, TGN [108]		
K1282E			Normal, TGN [108]		
G1300E			Normal, TGN [108]		
G1302V			Partial in cell periphery [108]		
N1304S	Reduced rescue ΔCcc2 yeast [112]				
N1304K			Normal, TGN [108]		
D1305A			Normal, TGN [108]		
G1315R			Normal, TGN [108]		
A1325V			No signal observed, protein absent in patient fibroblasts [108]		
A1362D	Reduced rescue ΔCcc2 yeast [107]				
A1362V			Normal, TGN, no copper response [113]		
G1369R			No signal observed, protein absent in patient fibroblasts [108]		
S1397F			Normal, TGN [108]		

Table 3. Effects of WD-causing missense mutations on function and regulation of ATP7B

Mutation:	Cuproenzyme biosynthesis	Catalytic ATPase activity	Localization	Post-translational modifications	Protein-protein interactions
G85V			Extensive ER mislocalization [114]		Decreased interaction with ATOX1[115] Increased interaction with COMMD1 [114]
L492S					Decreased interaction with ATOX1[115] Increased interaction with COMMD1 [114]
G591D			Extensive ER mislocalization [114]	Absence of copper-induced phosphorylation [116]	Decreased interaction with ATOX1[115] Increased interaction with COMMD1 [114]
A604P					Increased interaction with COMMD1 [114]
R616W			Normal, TGN [117]		
G710S			Normal, TGN [117]		
P760L			Normal, TGN [117]		
D765N	Normal rescue ΔCcc2 yeast [118]		Partial ER mislocalization [117, 119] Reduced copper response [119]		
M769V	Normal rescue ΔCcc2 yeast [118]		Normal, TGN [117, 119] Copper response unaffected [119]		
L776V	Normal rescue ΔCcc2 yeast [118]		Partial ER mislocalization [119] Reduced copper response [119]		
R778Q	Reduced rescue ΔCcc2 yeast [118]				
R778L	Reduced rescue ΔCcc2 yeast [118]		Extensive ER mislocalization [119] Copper response absent [119]		
W779X			Cytoplasmic clusters [117] Copper response absent [117]		
G943S	Normal rescue ΔCcc2 yeast [118]		Normal, TGN [119] Copper response absent [119]		
R969Q			Normal, TGN [117]		
T997M	No rescue ΔCcc2 yeast [118]				
V995A	Normal rescue ΔCcc2 yeast [118]				
P992L	Reduced rescue ΔCcc2 yeast [118]		Normal, TGN [117]		
E1064A		Absence of ATP binding [120]			
H1069Q	No rescue ΔCcc2 yeast [105] Reduced rescue ΔCcc2 yeast [121]	Absence of ATP binding [120], Absence of ATP hydrolysis activity [122]	Extensive ER mislocalization [117, 123] Mislocalization to aggrosomes [124]		
R1115H		Slightly decreased ATP binding [120]			
N1270S		Reduced ATP hydrolysis activity [122]	Normal, TGN [117] Normal, endosome [124]		

In yeast, the ortholog of ATP7A and ATP7B, Ccc2p, serves a similar function. Ccc2p is required for incorporation of copper into the ceruloplasmin homologue Fet3p, a ferrireductase enabling growth of yeast under iron-deficient conditions [103]. Incorporation of copper into Fet3p takes places within the Golgi apparatus, where Ccc2p resides under basal culturing conditions [104]. Expression of both ATP7A and ATP7B rescued the growth phenotype of *Ccc2* knockout yeast on iron-deficient media, making this an excellent model to study copper transporting ATPase dependent cuproenzyme biosynthesis [105, 106]. Using this *Ccc2* knockout complementation assay, effects of WD and MD-causing mutations on translocation of copper into the TGN lumen

and subsequent incorporation of copper in cuproenzymes can be determined. This approach has been undertaken by several investigators, and indicated that multiple MD- and WD-causing mutations diminish or even completely abrogate the ability of ATP7A or ATP7B to rescue Fet3p biosynthesis or the *Ccc2* knockout growth phenotype (tables 2 and 3). Taken together, these observations imply that ATP7A and ATP7B are critical for both proper delivery of copper to the TGN and subsequent cuproenzyme biosynthesis, and that mutations in ATP7A and ATP7B that perturb this process account for many of the clinical symptoms of MD and WD (table 1).

MOLECULAR MECHANISMS OF ATP7A AND ATP7B FUNCTION AND THE EFFECTS OF DISEASE-CAUSING MUTATIONS

ATP7A and ATP7B exert their functions in copper transport through a variety of interdependent mechanisms and regulatory events. These include catalytic ATPase activity, copper-dependent trafficking, post-translational modifications and protein-protein interactions. In recent years extensive efforts have been undertaken to dissect and characterize these mechanisms, which will be discussed in this section in the context of the pathophysiology of MD and WD. As explained below, these mechanisms are highly interdependent, and specific MD- or WD-causing mutations can exert effects on multiple levels. Therefore, care should be taken in the interpretation of how such effects relate to the pathogenesis of MD and WD.

ATPase catalytic cycle

The ion translocation cycle by P-type ATPases is believed to occur through a general cycling model involving several discrete stages in which ATP hydrolysis drives translocation of the target ion (schematically depicted in figure 1) [125]. These stages include (i) binding of the target ion, (ii) binding of ATP to the N-domain, (iii) ATP hydrolysis and phosphorylation of the P-domain, (iv) translocation of the target ion, and (v) dephosphorylation of the P-domain by the A-domain.

This model suggests that copper plays a key regulatory role in the catalytic cycle of ATP7A and ATP7B. ATP7A and ATP7B contain a number of putative copper binding sites, of which the amino terminal MBSs are the best characterized [126, 127]. The amino terminal tail containing these MBSs interacted with the N-domain of ATP7B, leading to inhibition of ATP binding [128]. This interaction was inhibited by copper, providing a potential mechanism for a copper regulated availability of the N-domain to bind ATP [128]. Consistent with this hypothesis, both ATP hydrolysis and formation of the acylphosphate intermediate (step (iii) in the model) were also dependent on copper [98, 109, 129-131]. The effect of copper on acylphosphate intermediate formation was cooperative, suggesting that the six amino terminal MBSs have a regulatory role in the formation of the acylphosphate intermediate [98, 130, 131]. Indeed, MBSs 5 and 6 are required for the cooperative effect of copper on acylphosphate intermediate formation [130]. However, mutation of all six amino terminal MBSs only mildly affected the rate and extent of catalytic phosphorylation of ATP7A, raising the possibility that other copper binding sites, such as the conserved intramembranous CPC motif, play an important role in the regulation of this process [129].

The next steps in the model predict that copper translocation by ATP7A and ATP7B is dependent on ATP binding and hydrolysis. Indeed, translocation of copper into membrane vesicles isolated from rat liver, and *in vitro* copper translocation activities of ATP7A and ATP7B, were ATP dependent [96-98, 132, 133]. Using purified recombinant protein fragments, ATP binding to both the N- and P-domain of ATP7B were observed [120, 128, 134]. Molecular modeling analysis supported this experimental observation [135]. The exact binding site for ATP in the N-domain has not yet been identified, but analysis of the solution structure of the N-domain of ATP7B implicated a number of residues to be involved in this process. These residues include H1069, G1099, G1101, I1102, G1149 and N1150 [134]. Interestingly, H1069, G1099, G1101 and I1102 are all residues found mutated in WD, and H1069Q represents the most frequent WD-causing mutation in Europe and Northern America [59]. Consistent with a role for this residue in ATP binding, the H1069Q mutation resulted in almost complete absence of ATP binding to ATP7B [120]. However, within the N-domain over 40 different WD-causing mutations have been reported, indicating that more residues might be involved in either direct ATP binding or coordination [59]. This possibility is supported by experiments showing that the WD-causing E1064A mutation results in complete absence of ATP binding to the N-domain of ATP7B [120].

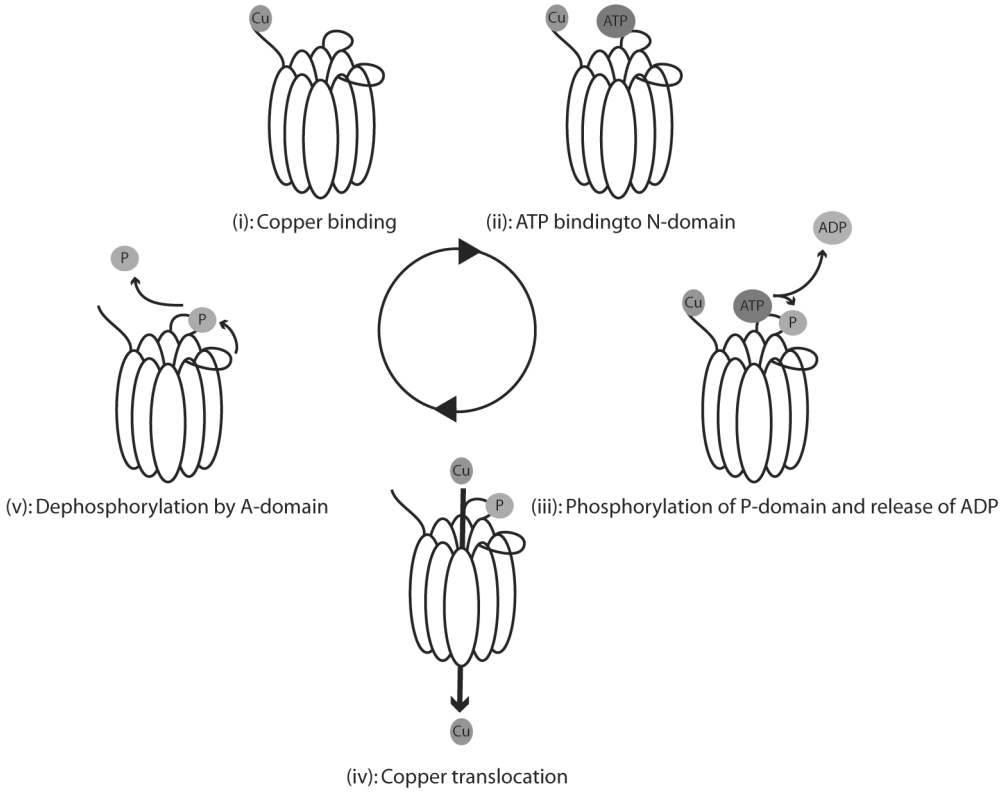


Figure 1: Schematic representation of the general ATPase catalytic cycle
Copper translocation by ATP7A and ATP7B is believed to occur through a general cycling model involving several discrete stages. These stages include (i) binding of the target ion, (ii) binding of ATP to the N-domain, (iii) ATP hydrolysis and phosphorylation of the P-domain, (iv) translocation of the target ion, and (v) deposphorylation of the P-domain by the A-domain.

Molecular modeling analysis suggested that after initial ATP binding to the N-domain of ATP7B, conformational changes take place that bring the ATP binding site within the N-domain in close proximity to the P-domain [135]. This could potentially promote ATP binding and phosphorylation of the P-domain, the third step in the catalytic cycle model [135]. ATP binding in the P-domain of ATP7B has been suggested to take place in the vicinity of D1027 [135]. This residue is part of the DKTG motif, which is highly conserved in all P-type ATPases, and is presumed to be the target of phosphorylation by the γ-phosphate of ATP [125]. Mutation of this aspartic acid residue in either ATP7A or ATP7B completely prevented formation of an acylphosphate intermediate, thereby supporting this hypothesis [109, 129, 131]. Furthermore, this mutation in ATP7A was associated with a complete loss of copper translocation activity, the next step in the general model [129].

Although copper translocation can be measured using isolated membrane vesicles from cells expressing ATP7A or ATP7B, or using purified ATP7A or ATP7B reconstituted in soybean asolectin liposomes, studies have yet to be performed to characterize the effects of MD- and WD-causing mutations on this particular step in the cycling model [96, 98]. Furthermore, no studies have been performed yet to unravel the role of the intramembranous copper-binding CPC motif, which is presumed to play an essential role in this step. However, it was shown that intact MBSs in the amino terminal region of ATP7A are required for proper translocation of ^{64}Cu in isolated membrane vesicles [136].

Dephosphorylation of the aspartic acid residue is mediated by intrinsic phosphatase activity in which the A-domain plays a key role. The signals that induce this phosphatase activity, and the mechanisms behind it, have not yet been elucidated, but an essential role has been ascribed to the conserved TGE motif. Mutation of this motif in ATP7A resulted in hyper-phosphorylation of the protein [109]. Although the TGE motif *per se* has not been reported to be mutated in MD or WD, adjacent residues in ATP7A and ATP7B are known sites of MD- and WD-causing mutations [59, 109]. In fact, the MD-causing L873R mutation, 2 amino acids upstream of the TGE motif, indeed resulted in hyper-phosphorylation of ATP7A [109].

Copper-dependent localization of ATP7A and ATP7B

Under basal conditions, ATP7A and ATP7B are localized within the TGN [105, 137, 138]. This localization is consistent with their function in cuproenzyme biosynthesis, as a number of cuproenzymes are synthesized within the secretory pathway. Some controversy existed about the localization of ATP7B, as it has also been postulated that this protein resides in an endosomal compartment[139, 140]. In addition, a smaller isoform of ATP7B exists that was localized to mitochondria [141]. The current general agreement is however that ATP7B is localized in the TGN, as this has been confirmed by several independent groups, including at ultrastructural resolution in human liver biopsies [105, 117, 119, 142-147].

A key mechanism in the regulation of the copper export function of ATP7A and ATP7B became apparent from studies showing that the subcellular distribution of both proteins was

sensitive to the concentration of copper to which the cell was exposed to (figure 3 and box 3). In response to elevated copper levels, ATP7A reversibly relocalized to a peripheral vesicular compartment as well as to the plasma membrane [138, 148]. In polarized cells, and in intestinal tissue sections, ATP7A specifically localized towards the basolateral membrane upon copper exposure, consistent with its function to transfer copper across the intestinal barrier [149-152]. ATP7A overexpressed in mouse liver tissue was also localized at the hepatocyte basolateral membrane [153]. Specific targeting of ATP7A to the basolateral membrane appears to be mediated through a putative PDZ binding motif present in the carboxy terminal tail of ATP7A. Deletion of this motif resulted in targeting of ATP7A to the apical membrane in response to elevated copper levels [150]. ATP7B undergoes a similar copper-induced relocalization to a peripheral vesicular compartment. Although ATP7B has not been unequivocally detected at the plasma membrane of polarized cells, it can not be excluded that the transporter rapidly recycles between the peripheral vesicular compartment and the plasma membrane [105, 154]. In polarized

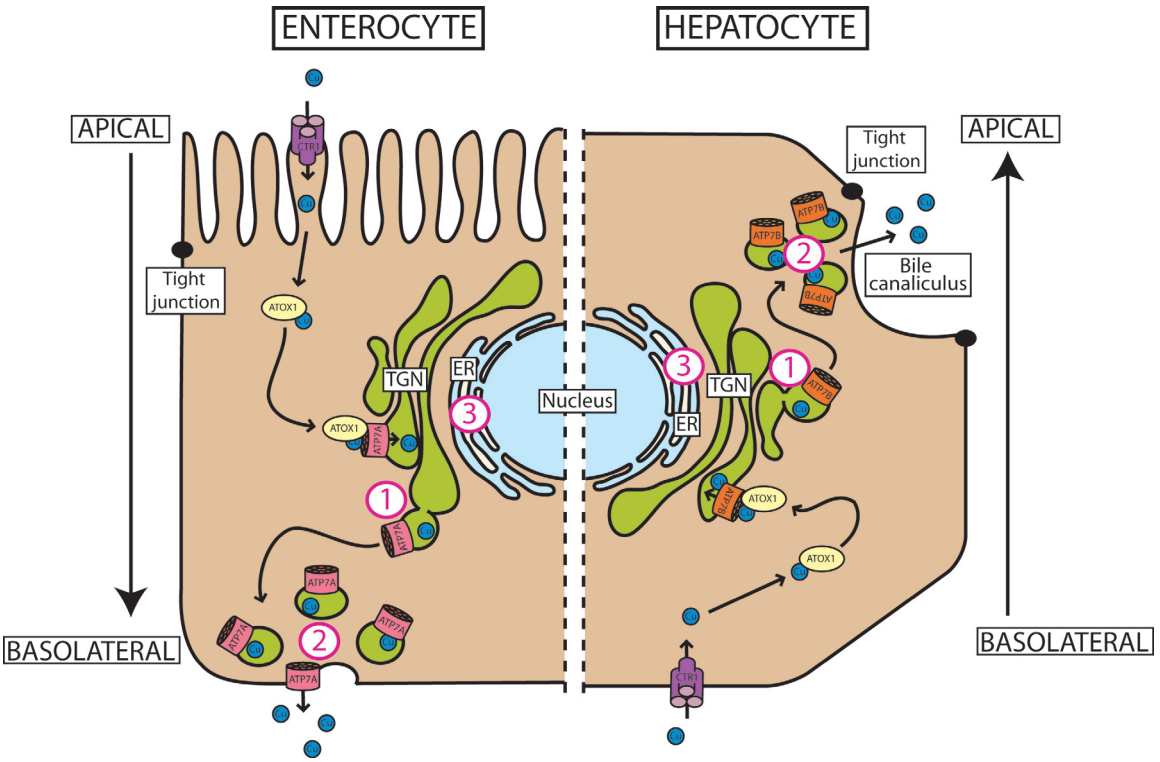


Figure 2: Schematic representation of copper-induced relocalization of ATP7A and ATP7B
Left side depicts an enterocyte, and right side represents and hepatocyte . In both cells, copper enters through the copper transporter 1 (CTR1), and is then distributed via the copper chaperone ATOX1 to ATP7A or ATP7B residing in the TGN. Upon elevated copper concentrations, ATP7A and ATP7B relocalize from the TGN to the cell periphery, and in the case of ATP7A also the plasmamembrane, to facilitate excretion of copper. The main difference in these two copper transport pathways lies in the direction. In the enterocyte, ATP7A facilitates excretion of copper into the bloodstream at the basolateral side, whereas in the hepatocyte copper is excreted at the apical side into the bile. The numbers indicate localization defects of ATP7A and ATP7B due to MD and WD causing mutations respectively; (1) lack of copper responsiveness, resulting in constitutive localization at the TGN, (2) constitutive localization at the cell periphery, and (3) mislocalization at the ER, presumably due to misfolding.

BOX 3. SIGNALS AND MECHANISMS MEDIATING COPPER-INDUCED TRAFFICKING OF ATP7A AND ATP7B

Signals and mechanisms underlying the localization and copper-induced trafficking of ATP7A and ATP7B have been the focus of many studies. Steady state TGN localization of ATP7A was demonstrated to be mediated by a putative TGN-targeting signal within transmembrane domain 3 [156]. Although transmembrane region 3 of ATP7B shows a high degree of conservation with that of ATP7A, it is unknown if it serves the same TGN-targeting function. Copper-induced relocalization of ATP7A and ATP7B appears to be initiated by direct binding of copper to ATP7A and ATP7B. Deletion or mutation of all amino terminal MBS or the putative copper binding CPC sequence resulted in an inability to induce the copper-dependent relocalization [109, 145, 150, 245, 246]. Strikingly however, the presence of only one intact amino terminal MBS was sufficient for normal copper-induced relocalization [145, 245, 246, 249, 250]. This observation indicates that the six different MBSs have redundant functions, at least in copper-induced relocalization of ATP7A and ATP7B. However, some studies have attributed a particularly important role to MBSs five and six for copper-induced relocalization, suggesting that these specific MBSs serve a different function from the others [145, 245, 249]. In addition to copper binding, phosphorylation status plays an important role in the regulation of trafficking of ATP7A and ATP7B. Mutation of the aspartic acid residue in the DKTG motif, which prohibited the formation of an acylphosphate intermediate, abolished copper-induced trafficking of ATP7A and ATP7B from the TGN to the cell periphery [109, 154]. Conversely, hyper phosphorylation of ATP7A or ATP7B induced by mutation of the TGE motif resulted in a constitutive peripheral localization [109]. A combination of mutations of the TGE motif, the six amino terminal MBS and the CPC motif in ATP7B also revealed a constitutive peripheral localization [250]. This fascinating observation can be explained in two ways. It is possible that other metal binding sites are present in ATP7B through which relocalization can be induced by copper. Alternatively, ATP7B constitutively cycles between the TGN and the cell periphery and copper binding serves as a retention signal inhibiting retrograde trafficking to the TGN. Retrograde trafficking of ATP7A from the plasma membrane back to the TGN requires a dileucine motif within the carboxy terminal tail [148, 251, 252]. Although this motif might serve as a classical clathrin-mediated endocytosis targeting motif, other mechanisms play a role in the internalization of ATP7A from the plasma membrane [253, 254]. A trileucine motif is present at the same position of ATP7B, and although ATP7B does not reach the plasma membrane, mutation of this motif also resulted in a constitutive peripheral localization of ATP7B [154].

These studies have provided valuable insights into the mechanisms behind copper-induced trafficking of ATP7A and ATP7B. One of the most important remaining questions is how exactly copper is exported from the cell after this event has occurred, and this should be the focus of future studies.

hepatocytic cell-lines, exposure to high copper concentrations resulted in localization of ATP7B in the proximity of the apical vacuoles, a structure reminiscent of the bile canaliculus [46, 142, 144, 147, 154]. This observation is consistent with its proposed function to excrete copper from the hepatocyte via the bile. Small amounts of ATP7B localized in the proximity of the bile canaliculus were also detected in human liver tissues [144]. Signals mediating the specific targeting of ATP7B towards the apical region seem to be present within the first 63 amino acids, a region that is not present in ATP7A [147]. Taken together, these data indicate that differences in trafficking destinations of ATP7A and ATP7B are not caused by general cell-type specific differences in regulation of polarized membrane protein localization, but are rather determined by intrinsic signals present in their amino acid sequences. The difference in the directionality of copper-induced relocalization of ATP7A and ATP7B illustrates why two distinct, but highly similar, copper export proteins are required to ensure proper copper uptake and excretion in higher organisms. In addition, this difference, resulting in distinct physiological functions of these homologous

proteins to maintain whole-body copper homeostasis, explains the opposed copper transport defects observed in MD and WD.

Several MD- and WD-causing mutations are associated with defects in copper-induced relocalization of ATP7A and ATP7B respectively, suggesting that this is a key event that precedes cellular copper excretion by ATP7A and ATP7B. Therefore such defects might possibly be associated with the pathogenesis of MD and WD. In general, MD- and WD-causing mutations can result in three types of localization defects (tables 2 and 3, and figure 3). The first type displays a normal steady state localization of the protein within the TGN, but responsiveness to copper is lost [109, 110, 113, 119]. One mutation of interest that was shown to result in such a defect is the MD-causing C1000R mutation in ATP7A which disrupts the putative copper binding CPC motif, suggesting that in this case impaired copper binding causes the lack of copper-induced trafficking to the cell periphery [109]. This type of localization defect could potentially explain some of the biochemical symptoms observed in patients with MD or WD. For example, whereas the G943S mutation in ATP7B prohibited copper-induced trafficking of ATP7B, this mutation still permitted cuproenzyme biosynthesis in the Δ*Ccc2* complementation assay [118, 119]. Although this correlation does not exist for all mutations causing this type of localization defect, it could explain normal ceruloplasmin production observed in some patients with WD. Unfortunately, serum ceruloplasmin levels have only been described for one compound heterozygous patient carrying the G943S mutation, and this was indeed found to be within normal range [119]. Although this type of localization defect might still permit cuproenzyme biosynthesis in MD, resulting in alleviation of the clinical phenotype, this might not always be obvious in patients due to an impairment in copper translocation across the mucosal barrier. More likely, this type of localization defect could allow for beneficial effects of copper replacement therapy in these patients. The second type of localization defects is on the opposite side of the spectrum. Several MD- and WD-causing mutations result in constitutive peripheral localization of ATP7A or ATP7B, respectively [109, 113, 117]. Of interest is the MD-causing L873R mutation in ATP7A, targeting a residue located adjacent to the conserved TGE motif. In this case a constitutively peripheral localization of ATP7A as a result of this mutation, was associated with hyperphosphorylation of ATP7A, suggesting that copper-induced relocalization is dependent on the ATPase catalytic cycle (further discussed in box 3) [109]. The third type of localization defect is probably the most common and clinically important. Here, MD- and WD-causing mutations result in mislocalization, or possibly retention, of ATP7A and ATP7B within the endoplasmic reticulum [111, 114, 117, 119, 123, 124, 154-156]. Strikingly, this defect was observed for the two most common WD-causing mutations, H1069Q and R778L [117, 119, 123]. Some controversy exists about the localization of the H1069Q variant, as this has also been reported to localize to aggresomes [124]. ER mislocalization of proteins is often due to misfolding and associated with proteasomal degradation [157]. A well known example of this process in human disease development is the ER associated degradation of ΔF508 CFTR mutant in cystic fibrosis [158]. In analogy, the H1069Q mutation in ATP7B indeed results in an increased proteolysis rate [123, 124]. Whether the increased proteolysis resulting

from the H1069Q mutation is due to defects in the conformation of ATP7B remains unclear, but the solution structure of the N-domain of ATP7B containing the H1069Q mutation showed no folding defects [134].

Post-translational modifications

Most efforts in investigating putative post-translational modifications of ATP7A and ATP7B have focused on the formation of the acylphosphate intermediate during the catalytic cycle. However, recent studies have led authors to suggest that both ATP7A and ATP7B are subject to basal, and copper-induced, phosphorylation that is distinct from the formation of the acylphosphate intermediate [116, 159]. Both basal and copper-induced phosphorylation could still be observed with a catalytically inactive mutant of ATP7B, indicating that these types of phosphorylation are indeed distinct from the formation of the acylphosphate intermediate, and that specific kinases are required [160]. Copper-induced phosphorylation of ATP7B was partially inhibited by an inhibitor of casein kinase II [116]. Phosphoamino acid analysis indicated that both ATP7A and ATP7B are phosphorylated on serine residues [159, 161]. In addition to phosphorylation, modification of ATP7A with N-linked glycan chains has also been observed [137]. Although little is known about the functional effects of ATP7A glycosylation, it is interesting that this type of modification is probably specific to ATP7A as the amino acid sequence of ATP7B does not contain any consensus glycosylation sites at relevant extra-cytoplasmic domains.

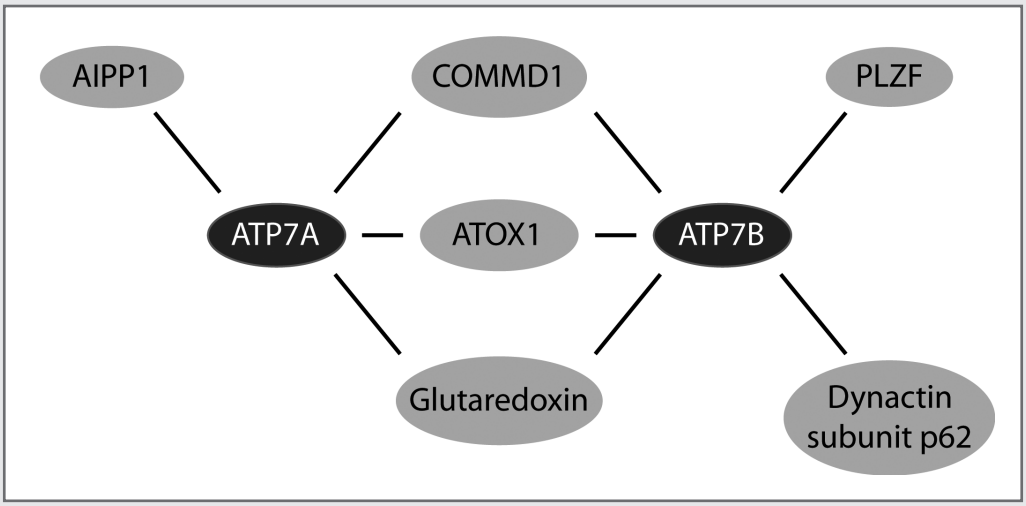
Taken together, studies on post-translational modifications of ATP7A and ATP7B are still in their initial phases, but could shed important new light on our understanding of the regulation of these copper-transporting ATPases. The importance of these modifications is emphasized by the absence of copper induced phosphorylation of ATP7B due to the WD-causing G591D mutation, and absence of ATP7A glycosylation due to the MD-causing G1019D mutation (tables 1 and 2) [111, 116].

Protein-protein interactions

Protein-protein interactions form an essential mechanism through which many proteins exert their functions. Mapping of the protein-protein interactome provides a valuable framework for elucidating the functional organization of the human proteome, and consequently to understanding the molecular pathology of human disease [162]. In recent years, significant process has been made in unraveling the ATP7A and ATP7B interactome (box 4). Here we will elaborate on two of the interacting partners of ATP7A and ATP7B that can be directly linked to the development of copper homeostasis disorders.

Due to its toxic potential, concentrations of free copper inside the cell are extremely low. In fact, it has been estimated that yeast maintains a concentration of less than one free copper ion per cell [163]. As a consequence of such low free-copper concentrations, donor proteins are required for the delivery of substrate copper to copper transporting ATPases. Originally isolated as a suppressor of oxidative toxicity in yeast, Atx1p (and its human ortholog ATOX1)

BOX 4. THE ATP7A AND ATP7B INTERACTOME



Identification of protein-protein interactions is a powerful tool to understand protein function, and how perturbation of protein function results in human disease. Over the last few years a number of interacting partners for ATP7A and ATP7B have been reported, resulting in a rapid elaboration of the ATP7A and ATP7B interactome (see figure) [182, 255-258]. The best characterized interacting partner of ATP7A and ATP7B is ATOX1. Through its role to deliver copper to ATP7A and ATP7B, ATOX1 plays an essential role in the function of ATP7A and ATP7B, which is further discussed in the text. However, additional proteins might be involved in the delivery of copper to ATP7A and ATP7B. Among these is Glutaredoxin, which interacts with both ATP7A and ATP7B in a copper-dependent manner, and has been hypothesized to regulate copper binding by ATP7A and ATP7B [255]. In addition, copper delivery to ATP7A and ATP7B might be regulated by the immunophillin FKBP52. This protein interacts with ATOX1 in a copper-dependent manner, and overexpression of FKBP52 results in increased cellular copper efflux [259]. The exact mechanisms through which FKBP52 attenuates its role in the copper excretion pathway need to be further characterized. COMMD1, the protein defective in CT, interacts with both ATP7A and ATP7B [114, 182] (P. de Bie et al., unpublished observations). The function of these interactions remains to be characterized, but the phenotype resulting from absence of COMMD1 in CT suggests that COMMD1 and ATP7B co-operate to facilitate billiary copper excretion (further discussed in the text). Another recently identified interacting partner for ATP7A is ATPase interacting PDZ protein 1 (AIPP1) [258]. This PDZ domain containing protein binds to the carboxy terminal tail of ATP7A where a putative PDZ binding motif is present. As this motif is required for targeting of ATP7A to the basolateral membrane, it was proposed that AIPP1 has a regulatory role in the copper induced trafficking of ATP7A [150, 258]. Through its amino terminal tail, ATP7B interacts with the dynactin subunit p62 in a copper-dependent manner [256]. As the dynactin protein complex is involved in membrane vesicle movement along microtubules, this interaction suggests that the p62 dynactin subunit facilitates copper-induced trafficking of ATP7B [260]. Further identification and characterization of novel interacting partners of ATP7A and ATP7B will also shed new light on previously unanticipated functions of these proteins, as evidenced by the recently observed interaction between ATP7B and a promyelocytic leukemia zinc finger protein (PLZF) isoform. Characterization of the interaction between the PLZF isoform and ATP7B suggested that ATP7B attenuates activation of the ERK signaling pathway [257]. Further investigation is needed to determine the implications of this observation.

was shown to be required for copper transporting ATPase-mediated cuproenzyme biosynthesis [164-166]. Subsequently, it was demonstrated that ATOX1 interacts with both ATP7A and ATP7B [167, 168]. This interaction is conserved in yeast and bacteria, illustrating its importance [169-172]. An MxCxxC containing MBS homologous to those in ATP7A and ATP7B is present in ATOX1, through which it was shown to bind copper [169, 173]. The interaction of ATOX1 with ATP7A or ATP7B is copper dependent and requires intact MBSs of both ATOX1 and ATP7A or ATP7B [167-169, 174-177]. These data suggest that ATOX1 delivers copper to ATP7A and ATP7B, which in fact has been demonstrated *in vitro* [178-181]. *Atox1* knockout mice display a phenotype similar to that observed in MD patients including symptoms such as growth failure, skin laxity, hypopigmentation and seizures [166]. Consistent with a copper excretion defect caused by defective copper delivery to ATP7A, cultured fibroblasts isolated from *Atox1* knockout mice exhibited an increase in copper retention and content [115, 166]. A time- and dose-dependent impairment of copper-induced relocalization of ATP7A was also observed in these cells [115]. One possible defect underlying the development of MD and WD could thus be an impairment of the interaction of ATOX1 with ATP7A or ATP7B respectively. Indeed, several mutations in the amino terminal tail of ATP7B prevented its interaction with ATOX1 [167]. Two of these mutations, G85V and G591D, affect highly conserved glycine residues in the proximity of the MxCxxC core sequence in MBS 1 and 6 respectively, indicating an important role for this conserved residue in coordination of the ATOX1-ATP7B interaction [167]. However, these mutations also resulted in mislocalization of ATP7B to the ER, which might also underlie the loss of interaction of ATP7B with ATOX1 due to these mutations [114].

Recent studies have shown that COMMD1, the protein defective in CT, interacts with ATP7B and that this interaction is mediated by the amino terminal tail of ATP7B [182]. Transient knock-down of COMMD1 in HEK293 cells resulted in increased cellular copper levels [183]. These data suggest that COMMD1 and ATP7B co-operate in the excretion of copper from the hepatocyte, and that absence of the interaction between these proteins underlies the pathophysiology of CT in Bedlington terriers affected with the *COMMD1* deletion. Strikingly, COMMD1 also interacts with ATP7A, indicating that COMMD1 has its role in general copper homeostasis that is not restricted to the liver (P. de Bie et al., unpublished observation). COMMD1 has recently also been implicated in several other cellular processes, including the NF-κB and HIF1 signaling pathways [85, 184-189]. In these two pathways COMMD1 is thought to exert its regulatory role by regulating the proteasomal degradation of key components of these pathways [184, 186, 187, 190]. With this in mind, COMMD1 might also regulate the proteasomal degradation of ATP7A and ATP7B, thus regulating copper homeostasis. Other examples exist in which cuproenzymes or copper transport proteins, such as the copper chaperone for superoxide dismutase 1 (CCS) and Hephaestin, are regulated by means of proteasomal degradation in response to altering copper levels [191, 192]. The Wilson disease-causing mutations G85V, L492S, G591D, and A604P resulted in increased binding of ATP7B to COMMD1, suggesting that disruption of this interaction could underlie the development of WD in some cases [114]. The G85V and G591D mutations were

also associated with ER mislocalization and increased degradation of ATP7B, supporting the hypothesis that COMMD1 facilitates the degradation of ATP7B [114]. Recently, 10 homologues of COMMD1 have been described that are characterized by a conserved domain, and are also involved in regulation of NF-κB signaling [85, 189]. It would be interesting to investigate if these homologues of COMMD1 also have a functional role in copper homeostasis, possibly through interactions with ATP7B or ATP7A.

GENOTYPE-PHENOTYPE CORRELATIONS IN THE DEVELOPMENT OF MD AND WD

Proper characterization of the defects in MD and WD is required to resolve the clinical heterogeneity that is observed in both disorders. Either environmental or genetic variations might underlie this clinical heterogeneity. For example, development of ICC, ETIC and ICT is associated with a high copper intake [5, 6, 8, 63]. Although such correlation has not been observed for WD, it is possible that high copper intake might exacerbate the symptoms, while dietary components having low copper but high zinc concentrations might have a beneficial effect. Genetic variations in genes other than *ATP7A* and *ATP7B* might modulate the clinical expression of MD and WD. In various other disorders with a Mendelian mode of inheritance, it was shown that modifier genes can modulate penetrance, dominance modification, expressivity and pleiotrophy [193]. This is well exemplified by genetic modification of the severity and expressivity of cystic fibrosis by polymorphisms in various genes, including *Mannose binding lectin* (*MBL*) and *Transforming growth factor* β (*TGF*-β) (reviewed in [194, 195]). *COMMD1* has been proposed to be a modifier gene for the clinical presentation of WD as heterozygosity for a silent missense mutation in *COMMD1* was found possibly associated with an earlier onset of the disorder in patients with known *ATP7B* mutations [80]. In other cohorts of WD patients, no association between variations in COMMD1 and the clinical expression of WD was observed [78, 81, 82]. However, these cohorts were not classified to include WD patients with identical mutations in *ATP7B*, which should be performed to reliably assess the possible role of COMMD1 as a modifier gene for the clinical presentation of WD. Variations in the prion-protein and Apolipoprotein E coding genes have been proposed to modify the presentation of WD, although for the latter this has not been consistently observed [196-199].

Notwithstanding the role modifier genes and environmental factors play, it is evident from the functional data discussed above that different mutations in *ATP7A* or *ATP7B* can potentially result in different functional effects. Therefore it appears likely that also the type of mutation, or the residue(s) affected, modulate the clinical expression of MD or WD. For example, functional redundancy among the six amino terminal MBSs explains the relatively low amount of MD- or WD-causing missense mutations within the amino terminal tails of ATP7A and ATP7B. In addition, it was recently observed that translation reinitiation after adeletion in the amino terminal coding region of the ATP7A transcript produces a truncated protein containing only the fifth and sixth MBSs. Interestingly, this truncated protein was still partial functional, and the patient in which this phenomenon was opbserved displayed a remarkably mild MD phenotype

[200]. A suggestive correlation between the severity of the phenotype and the extent to which missense mutations impair the function of Atp7a was recently observed in three types of Mottled mice [201]. Many studies were focused on establishing genotype-phenotype correlations in both MD and WD. Severe mutations such as nonsense and frame-shift causing insertions/deletions are generally believed to completely disrupt protein function, and would therefore be expected to yield a more severe clinical phenotype. Indeed, for WD it was shown that severe mutations lead to an earlier onset of the disease, but a correlation with the type of presentation (neurological vs. hepatic) was not observed [202-205]. Similarly, it has been suggested that severe mutations in *ATP7A* result in classical MD, whereas mild MD would be caused by mutations that still permit residual activity of ATP7A [206]. On the other hand, OHS is often associated with splice site mutations. Although these mutations are predicted to dramatically affect ATP7A mRNA splicing, expression of the normal transcript is often still detectable, indicating that minor amounts of normally spliced ATP7A expressed in these patients are sufficient to allow for an alleviation of the phenotype [18, 40, 207-209]. In addition, it was suggested in a recent case report that in two brothers with MD carrying the same mutation in *ATP7A*, differences in severity of the disease correlated with the amount of ATP7A expressed in these patients [107].

Genotype-phenotype correlation analyses for missense mutations are less clear. Approximately 30% of all cases with MD result from *de novo* mutations, as a result of which mutations are often very rare, thus prohibiting proper genotype-phenotype correlation analysis using large numbers of patients. Unfortunately, such studies in WD are also hampered by the large number of mutations detected; most mutations are very rare, and therefore most WD patients are compound heterozygous. In a large number of studies no genotype-phenotype correlation could be observed. As most of these studies relied on small patient cohorts, combining data of several independent studies in a meta-analysis could be usefull to shed more clarity on this issue, as was done for the common H1069Q mutation [210]. In several independent cohorts, a correlation between ATP7B H1069Q homozygosity and a neurological presentation of WD was observed [210-215]. In addition, this genotype has been associated with a significantly later age at onset [122, 203, 204, 210, 212-218]. In several other studies one or both of these observations could not be statistically confirmed [122, 203, 211, 216-223]. However, a meta-analysis of all genetic studies devoted to H1069Q genotype and phenotype prior to 2004 indicated that overall, H1069Q homozygosity is indeed associated with a late neurological presentation of WD [210]. A similar correlation with a later age at onset in WD was observed for patients homozygous for the R969Q mutation [204]. In some cohorts, homozygosity for the R778L mutation was suggested to correlate with an early, hepatic presentation of WD [224, 225]. It has been postulated that alternative splicing of *ATP7B* in the brain, resulting in absence of exon 8 (harboring the R778 residue), underlies this suggested correlation [225, 226]. However, also here other studies could not confirm the correlation of the homozygous R778L genotype with hepatic presentation, although a tendency towards this observation was sometimes present [227-229].

CONCLUDING REMARKS

Mutations in the structurally and functionally highly homologous copper transporting ATPases ATP7A and ATP7B underlie both copper deficiency and overload diseases. Tremendous progress has been made in the characterization of the function of these proteins, and how this is impaired in MD and WD. From the data presented in tables 2 and 3, it is evident that different mutations in ATP7A or ATP7B result in a variety of defects in the molecular functions of these proteins. Consistent with this notion, to some extent a correlation of the genotype with the heterogeneous phenotypes has been observed in these disorders. However, it is also very likely that some of the different mutations in ATP7A or ATP7B result in overlapping functional impairments. This overlap could form a potential bias in genotype-phenotype correlation studies, in which correlations might be missed due to such an overlap in test and control patients. To overcome this potential bias, genotype-phenotype correlations should be performed using patient groups classified based primarily on the functional effects of the disease-causing mutations. This approach would also permit the performance of correlation studies with relatively mild, or rare, mutations. At present this approach is unfortunately not yet possible, as only a limited number of MD- or WD-associated variants of ATP7A and ATP7B have been functionally characterized (tables 2 and 3). Furthermore, a thorough characterization of all molecular mechanisms that participate in

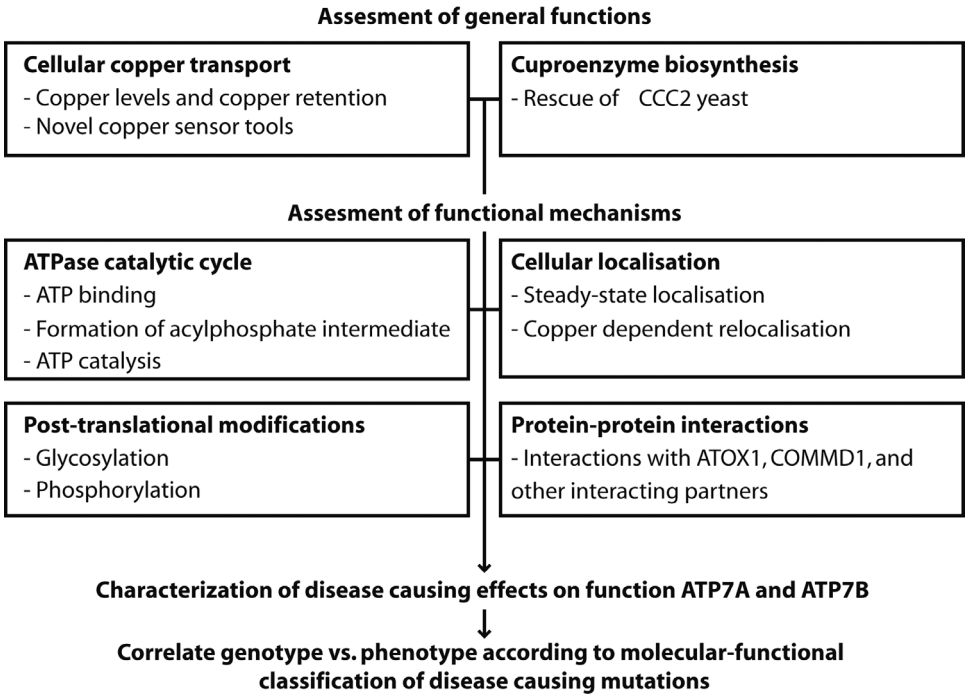


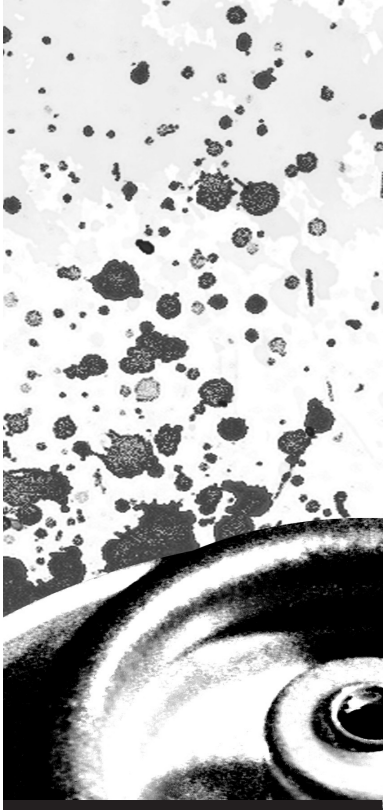
Figure 3. Proposed flow chart to analyze of MD and WD causing mutations in ATP7A and ATP7B and to correlate the molecular defects to disease phenotype

MD and WD causing mutations can have profound effects on the functions of ATP7A and ATP7B. Thorough analysis of the functional and mechanistic defects resulting from these mutations will result in enhanced understanding of MD and WD pathophysiology and the clinical heterogeneity observed in patients.

the copper transport functions of ATP7A and ATP7B would be required, as these mechanisms are highly interdependent (as schematically suggested in figure 4). Such characterization of mutations in ATP7A and ATP7B should be an important focus of future studies on the functional genetics of MD and WD, and will result in valuable insights into the molecular pathogenesis of MD and WD.

ACKNOWLEDGEMENTS

We thank members of the Leo Klomp and Cisca Wijmenga laboratories for helpful discussions. The work in these two laboratories is funded by the Netherlands Organization for Scientific Research (Zon-MW, grant 40-00812-98-03106), the Dutch Digestive Diseases Foundation (MLDS, grant WS 02-34), the Wilhelmina Children's Hospital (WKZ) Fund (grant 901-04-219). The authors declare they have no competing interests.



CHAPTER 2

The many faces of the copper metabolism protein MURR1/COMMD1

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ABSTRACT

Copper is an essential transition metal but it is toxic in excess and its metabolism therefore needs to be tightly regulated. Defects in the regulation of copper can lead to various disorders characterized either by copper deficiency or copper excess. Recently we characterized the *COMMD1* (previously *MURR1*) gene as the defective gene in canine copper toxicosis. The molecular functions of COMMD1 remain unknown, but significant progress has been made in identifying the cellular processes in which COMMD1 participates through the identification of proteins interacting with COMMD1. This review discusses how COMMD1 not only functions as a regulator of copper homeostasis, but also of sodium transport and the NF-κB signaling pathway. We outline the possible mechanisms through which COMMD1 exerts these newly identified functions.

COPPER AND COPPER REQUIREMENT

Copper is an essential trace element for all living organisms as it functions as a co-factor for a number of cuproenzymes. Copper's ability to exist in two oxidation states enables it to be an important co-factor for various enzymes that require redox chemistry for their function. These enzymes include Cu/Zn superoxide dismutase, which protects the cell against free radicals, and the cytochrome C oxidase complex, which is an essential component of the mitochondrial respiratory chain. In contrast to its necessity, its ability to shift between oxidation states also renders copper highly toxic when in excess, due to its potential to facilitate the generation of reactive oxygen species. A tight regulation of copper homeostasis is therefore required. Under normal homeostatic conditions in mammals, the uptake of copper through the intestine ensures essential needs are met while excretion through the bile prevents toxicity [230]. Various genetic diseases of copper metabolism are characterized by either depletion or accumulation of copper, underlining the importance of a tight regulation (Table 1). Menkes disease [3] is characterized by a general copper deficiency due to malabsorption of copper from the diet and results in early retardation in growth, peculiar ('kinky') hair, and focal cerebral and cerebellar degeneration. The disease is caused by mutations in the *ATP7A* gene [25-27], which encodes a copper translocating P-type ATPase. Wilson disease is a copper overload disorder [4], caused by mutations in the *ATP7B* gene [53-55]. In Wilson disease copper accumulates in the liver and brain, causing extensive hepatic and neurological abnormalities. Other, non-Wilsonian, forms of hepatic copper overload syndromes have been described [62, 261] including Indian childhood cirrhosis (ICC) [5], endemic Tyrolean infantile cirrhosis (ETIC) [6], and sporadic cases occurring worldwide grouped together as idiopathic copper toxicosis (ICT) [7, 8]. These non-Wilsonian copper overload disorders are fatal at an early age due to liver failure as a consequence of chronic liver cirrhosis. Many of these disorders can be classified as ecogenetic disorders, in the sense that both an excessive copper intake and a genetic defect underlie their pathology [5, 6, 63].

Table 1. Lists of human and animal disorders characterized by a defective copper homeostasis

	Defective gene	OMIM	References
Human copper deficiency or overload disorders			
Menkes Disease	*ATP7A*	309400	[25-27]
Occipital horn syndrome	*ATP7A*	304150	[18]
Wilson Disease	*ATP7B*	277900	[53-55]
Indian childhood cirrhosis	?	215600	[5, 305]
Endemic Tyrolean infantile cirrhosis	?	215600	[6]
Idiopathic copper toxicosis	?	215600	[7, 8]
Animal models for copper deficiency or overload			
Canine copper toxicosis	*COMMD1*	607238	[76]
Mottled mouse	*Atp7a*	309400	[34, 39]
Brindled mouse	*Atp7a*	309400	[35]
LEC rat	*Atp7b*	277900	[66]
Toxic milk mouse	*Atp7b*	277900	[70]
North Ronaldsay sheep	?		[306]

COPPER TOXICOSIS IN BEDLINGTON TERRIERS

One particular form of copper overload in dogs has long been considered an excellent model for studying hepatic copper overload and thereby gaining insight into the etiology of non-Wilsonian copper overload disorders. Canine copper toxicosis is an autosomal recessive disorder with a high frequency in Bedlington terriers [72]. Biliary copper excretion is markedly reduced in affected dogs [262] and as a result, copper accumulates in lysosomes of hepatocytes, eventually leading to liver cirrhosis and chronic hepatitis [263]. Linkage analysis studies showed that the copper toxicosis locus was closely associated with the C04107 microsatellite marker [264]. Mapping of the C04107 marker to canine chromosome 10q26, a region syntenic to human chromosome 2p13-p16, excluded known copper-related candidate genes such as *ATP7B* and *ATOX1* based on their chromosomal localization [73, 74, 265, 266]. To positionally clone the copper toxicosis gene a physical map of the copper toxicosis region was constructed using a bacterial artificial clone contig and radiation hybrid mapping [76, 267]. Homozygosity and linkage disequilibrium mapping further confined the copper toxicosis region to approximately 500 kb, containing 16 putative coding sequences, of which 6 were mapped genes [268]. A homozygous 39.7 kb genomic deletion encompassing exon 2 of the *COMMD1* (previously *MURR1*) gene was subsequently identified by positional cloning as the genetic cause of canine copper toxicosis [76]. The *COMMD1* gene was initially described for its proximity to the imprinted *U2af1-rs1* gene in the mouse genome [269]; its canine ortholog consists of three exons and encodes a 188 amino acid protein with a predicted M_r of 23 kDa [75, 76]. Although the mutant allele is predicted to lead to an in-frame deletion in the COMMD1 transcript, resulting in a truncated protein of 94 amino acids, no full length nor truncated COMMD1 protein is detectable in liver homogenates of

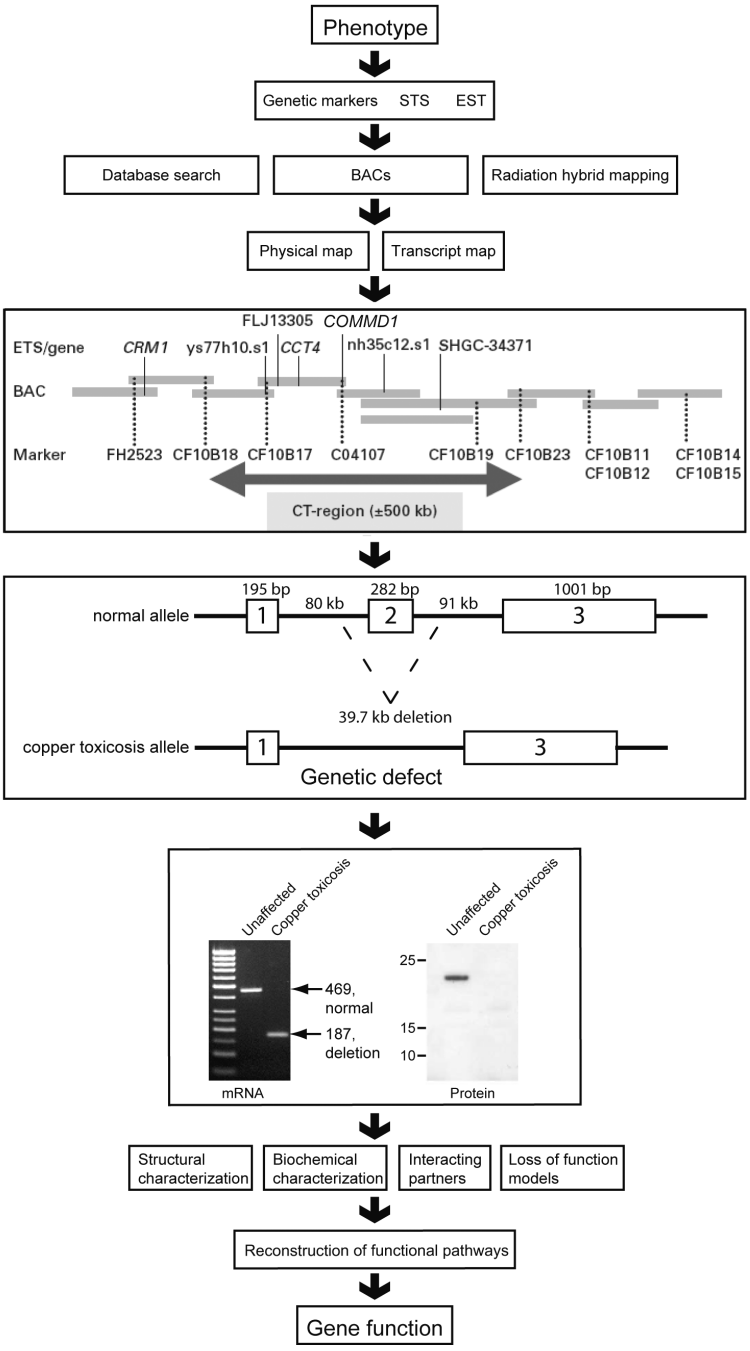

Figure 1. Schematic overview of positional cloning strategy employed in cloning the COMMD1 gene.
Genetic mapping was used to map the disease gene onto the dog genome [264]. The map location was determined by comparative mapping [74]. Radiation hybrid mapping was used to construct a physical map [267]. A bacterial artificial chromosome (BAC) contig was constructed and new polymorphic markers were isolated from the BAC clones to narrow down the canine copper toxicosis (CT) gene region further by genetic mapping. Haplotype sharing revealed a region of approximately 500 kb shared by all affected animals so genes from this region were subjected to mutation analysis by sequencing. The COMMD1 gene was found to be mutated in affected Bedlington terriers, which was associated with a short RNA product [76]. The mutation results in a loss of function, since no protein can be detected in the livers of affected dogs [75]. Further functional characterization of COMMD1 will be based on characterizing biochemical features, protein-protein interactions involving COMMD1, and loss of function models. Abbreviations used: STS, sequence tagged site; ETS, expression tagged site.

Bedlington terriers affected with copper toxicosis [75], suggesting that a complete loss of function of COMMD1 underlies the canine copper toxicosis pathology (summarized in figure 1). Although the C04107 marker is located in intron 1 of the *COMMD1* gene at approximately 13.5 kb from the proximal breakpoint of the *COMMD1* deletion (Forman et al, personal communication), several different haplotypes comprising the copper toxicosis locus have been described implicating a new level of complexity in the disease [83, 84, 264, 270, 271]. One notable group includes affected dogs that do not contain the *COMMD1* exon 2 deletion, nor any other mutation in the *COMMD1* coding region [83, 84]. This may suggest that as yet unidentified mutations in regulatory elements of the *COMMD1* gene underlie the disorder in these dogs. Alternatively, these dogs could have mutations in entirely different genes, for which the other COMMD proteins (discussed below) are potentially interesting candidates.

COMMD1 mRNA is expressed abundantly in liver, but is also readily detected in other tissues [76]. Consistent with this observation, an ubiquitous expression pattern of COMMD1 protein has been detected in murine tissues and various human cell lines using a polyclonal antiserum raised against COMMD1 [75]. Although COMMD1 is strongly conserved throughout evolution, it seems to be confined to higher eukaryotes (i.e. bile-containing organisms). The COMMD1 sequence contains no apparent copper binding motifs, but, it does contain a novel conserved domain known as the copper metabolism gene MURR1 (COMM) domain [85]. The COMM domain is a leucine rich domain consisting of roughly 85 amino acids, and is present in 9 other human genes (*COMMD2-10*). Identification of the COMM domain led to reannotation of MURR1 to COMMD1. Strikingly, all ten COMMD proteins can be found in complex with COMMD1[85], although the exact composition of these COMMD1-COMMD complexes *in vivo* still needs to be determined. The exact molecular function of COMMD1 remains unknown, but significant progress has recently been made in identifying several cellular processes in which COMMD1 participates, through the identification of proteins interacting with COMMD1.

COMMD1 AS A REGULATOR OF COPPER HOMEOSTASIS

The liver plays a key role in the excretion of copper from the human body (figure 2). Within the liver, copper excretion is critically dependent on the function of ATP7B, a copper translocating P-type ATPase that is structurally and functionally homologous to ATP7A. ATP7B contains 6 tandemly repeated copper binding sites in its amino terminus. On entering the cell through the copper transporter 1 (CTR1) [232] copper is delivered to the Golgi compartment by the copper chaperone ATOX1 [164]. ATOX1 is able to bind copper and undergoes a transient copper-dependent association with ATP7B [167, 168, 177]. Under physiological conditions ATP7B resides in the *trans* Golgi network. When copper levels rise however, ATP7B translocates to a diffuse vesicular compartment [105] from which copper is secreted from the cell in an unknown manner. A similar copper-dependent trafficking has been observed for ATP7A, which translocates to a vesicular compartment and the plasma membrane upon elevation of copper levels [138]. Molecular mechanisms of the copper-dependent intracellular trafficking of ATP7B and ATP7A

are largely unknown, but trafficking of ATP7A is markedly impaired in cells isolated from *ATOX*$^{-/-}$ mice [115]. It has also been shown that mutations that impair the interaction of ATP7B with ATOX1 are associated with Wilson disease [167], suggesting that ATOX1 plays an essential role in this mechanism.

COMMD1 also plays a critical role in copper excretion, as can be deduced from the hepatic copper overload and reduced biliary excretion phenotype in Bedlington terriers with a *COMMD1* deletion. Recent studies have shown that COMMD1 is able to bind to the N-terminal copper binding region of ATP7B but not that of ATP7A [182]. These data suggest that COMMD1 and ATP7B co-operate in the excretion of copper from the hepatocyte, and that abolishment of the interaction between these proteins underlies the pathophysiology of canine copper toxicosis. Copper transport to the Golgi compartment is unaffected in canine copper toxicosis as the dogs exhibit normal serum ceruloplasmin levels, suggesting that COMMD1 possibly participates in the ATP7B-mediated transport of copper from the trans-Golgi to the bile canaliculus. Since the dogs show massive copper accumulation in lysosomes of the hepatocyte, a possible role for COMMD1 might be to facilitate de-granulation of lysosomal contents into the bile (Figure 2). This model is consistent with the observation that COMMD1 localizes to a vesicular compartment showing partial overlap with the transferrin receptor and CD63, which are markers for early endosomes

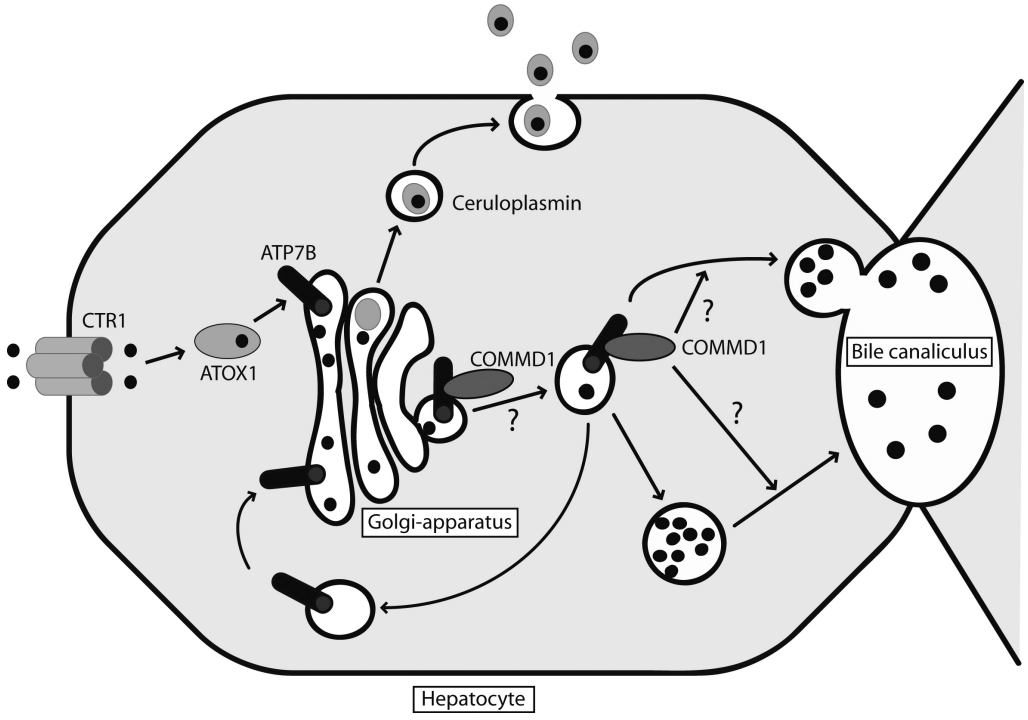

Figure 2. Overview of the ATP7B-mediated copper export pathway in the hepatocyte.
Upon entering the cell through the copper transporter CTR1, copper is delivered to ATP7B in the golgi apparatus by the copper chaperone ATOX1. In the golgi apparatus copper is incorporated in various cuproenzymes including ceruloplasmin. When copper levels in the cell rise, ATP7B redistributes to a vescicular compartment. Upon relocalization of ATP7B copper is excreted from the hepatocyte through the bile via an unknown mechanism that probably involves COMMD1.

and lysosomes, respectively [75]. Presently, no mutations in human *COMMD1* in patients with Wilson disease have been described, although heterozygosity for a silent missense mutation in *COMMD1* is possibly associated with an earlier onset of the disorder in patients with known *ATP7B* mutations [80]. In addition, no mutations in COMMD1 were detected in a cohort of non-Wilsonian copper overload patients containing 12 ICC, one ETIC and 10 ICT patients [61], excluding canine copper toxicosis as a genetic model for these disorders. Transient knock-down of COMMD1 in HEK293 leads to increased cellular copper levels [183], supporting the view of COMMD1 as a regulator of copper homeostasis. Further studies of ATP7B trafficking and functioning in COMMD1-deficient systems might enhance our understanding of COMMD1 functioning in the ATP7B-mediated copper excretion pathway.

Recent studies imply that cellular COMMD1 levels are regulated by the X-linked inhibitor of apoptosis (XIAP). As an E3 ubiquitin ligase, XIAP ubiquitinates COMMD1, thereby targeting it for proteasomal degradation [183]. Interestingly, it was observed that fibroblasts and liver tissue of *XIAP*$^{-/-}$ mice display a reduced copper content [183], indicating that XIAP regulates copper homeostasis, possibly by mediating COMMD1 levels. Several proteins that are either involved in copper homeostasis or that require copper for their function are regulated at their protein levels in response to altered intracellular copper levels: these include hephaestin [192], CCS [191], and the copper transporter CTR1 [272-274]. The existence of copper-dependent internalization and degradation of CTR1 is, however, still under debate [275, 276]. It remains to be investigated if copper regulates XIAP activity, thereby providing a mechanism for copper-regulated COMMD1 degradation. However, it has already been shown that COMMD1 levels remain constant under changing copper levels [75], suggesting that copper dependence of XIAP mediated COMMD1 ubiquitination would be unlikely.

COMMD1 AS A REGULATOR OF SODIUM UPTAKE

The amiloride-sensitive epithelial sodium channel (EnaC) constitutes the rate-limiting step for sodium reabsorption in epithelial cells that line the distal part of the renal tubule, the distal colon, the duct of several exocrine glands, and the lung [277]. ENaC consists of three similar subunits, of which initially the α-, β- and the γ-ENaC subunits have been characterized [277, 278]. An additional δ-ENaC subunit was later identified that co-operates with the β- and the γ-ENaC subunits to attain an ameloride-induced sodium current [279]. COMMD1 has been reported to interact with the C-terminus of δ-ENaC [188], and has also been detected in complex with β- and γENaC, but not with αENaC. Coexpression of COMMD1 with δ-, β- and γENaC inhibits the ameloride-induced sodium current, which depends on the C-terminus of δENaC as demonstrated by deletion mapping. An inhibitory effect of COMMD1 on the αβγENaC was also observed, but to a lesser extent [188]. How COMMD1 inhibits αβγENaC activity is unclear, but it has been postulated that this occurs through binding to the β- or γENaC subunits, although a direct interaction between COMMD1 and these subunits independent of δENaC has not been demonstrated. These findings shed a new and unexpected light on the cellular functions of COMMD1.

For correct functioning of αβγENaC, it trafficks from the Golgi compartment to the cell membrane after glycosylation on all three subunits [280, 281]. It has been suggested that on the cell membrane αβγENaC stability is regulated by Nedd4-mediated ubiquitination and clathrin-mediated endocytosis [281]. Relatively little is known about the exact mechanisms of ENaC trafficking and the possibility of δβγENaC trafficking still needs to be investigated. Nevertheless, based on the interactions of COMMD1 with both ATP7B and δβγEnaC, and the fact that both transporters are possibly regulated in their activity by intracellular trafficking, we could speculate that COMMD1 plays a key role in regulating this process. Studies determining the role of COMMD1 on subcellular localization and trafficking of both ATP7B and δβγENaC need to be performed to investigate this hypothesis.

Copper uptake in fish gill has been shown to be inhibited by elevated sodium levels [282] and vice versa [283, 284], linking sodium transport to copper transport. The exact link between copper and sodium transport needs further characterization, but has been postulated to involve ENaC [285]. Considering the critical role of COMMD1 in copper metabolism we could speculate that regulation of ENaC activity by COMMD1 might provide the link between sodium and copper transport.

COMMD1 AS A REGULATOR OF NF-κB SIGNALING

The nuclear factor kappa-B (NF-κB) complex plays an important role in the transcriptional regulation of a wide array of genes, the majority of which are involved in immune and stress responses [286]. In unstimulated cells different NF-κB proteins exist as either homo- or heterodimers bound to NF-κB inhibitor (IκB) proteins [287]. NF-κB proteins are characterized by the presence of the Rel homology domain that is responsible for their dimerization, their interaction with IκB proteins, and their DNA binding [288-291]. IκB proteins act as inhibitors of NF-κB signaling by binding to the NF-κB dimers and thereby masking the nuclear localization sequence of the NF-κB subunit RelA, as a result of which the NF-κB complex is retained in the cytoplasm [292-294]. In the classical pathway of NF-κB activation, IκB proteins are phosphorylated by activated IκB kinases (IKK), which targets IκB proteins for ubiquitination and subsequent proteasomal degradation [295-297]. After IκB degradation the nuclear localization signal of NF-κB is unmasked, resulting in nuclear translocation and transcriptional activation of κB target genes.

The identification of COMMD1 as interacting partner for XIAP, an activator of NF-κB signaling, led to investigations indicating that COMMD1, as well as other COMMD proteins, can be a potent repressor of NF-κB activation induced by various stimuli in several different cell lines [85, 184]. COMMD1 was shown to interact with several NF-κB subunits as well as with IκBα, indicating that COMMD1 directly participates in the NF-κB pathway [85, 184].

Ubiquitination of IκBα upon activation of the classical NF-κB pathway is mediated by the SCF ubiquitin ligase complex [298-302]. The SCF complex consists of the core subunits Cullin1, RBX1 and Skp1. The latter acts to recruit F-box containing proteins that determine target specificity of the SCF complex [303]. siRNA mediated knock down of COMMD1 leads to decreased

IκBα levels, suggesting that COMMD1 has a protective effect on IκBα. An interaction between COMMD1 and the SCF subunit Cullin1 has been detected [184], which led to the hypothesis that COMMD1 protects IκBα from proteasomal degradation by preventing ubiquitination of IκBα by the SCF complex [184, 190]. However, as this hypothesis suggests, due to increased IκBα levels, NF-κB would be retained in the cytoplasm upon overexpression of COMMD1, no impairment of nuclear translocation of NF-κB upon stimulation with TNF is observed [85]. This suggests that other mechanisms of NF-κB inhibition by COMMD1 are at play, with one possibility being that COMMD1 regulates binding of NF-κB to its target promoter sites [85]. Further research is needed to further determine the molecular events resulting in NF-κB inhibition by COMMD1. Some remaining questions include how the interactions between COMMD proteins play a role in this process, as well as whether other effector proteins are required and whether there are post-translational modifications of players involved in this pathway.

NF-κB has long been recognized as a key transcription factor facilitating the replication of HIV-1 by initiating transcription of the viral genome through κB sites on the HIV long terminal repeat [304].This link between HIV replication and NF-κB activity led to investigations that revealed that COMMD1 overexpression represses HIV-1 replication in resting $CD4^+$ lymphocytes, whereas HIV-1 replication is stimulated in these cells by siRNA-mediated knockdown of COMMD1 [184]. These data reveal an unexpected putative role for COMMD1 in protection against HIV infection.

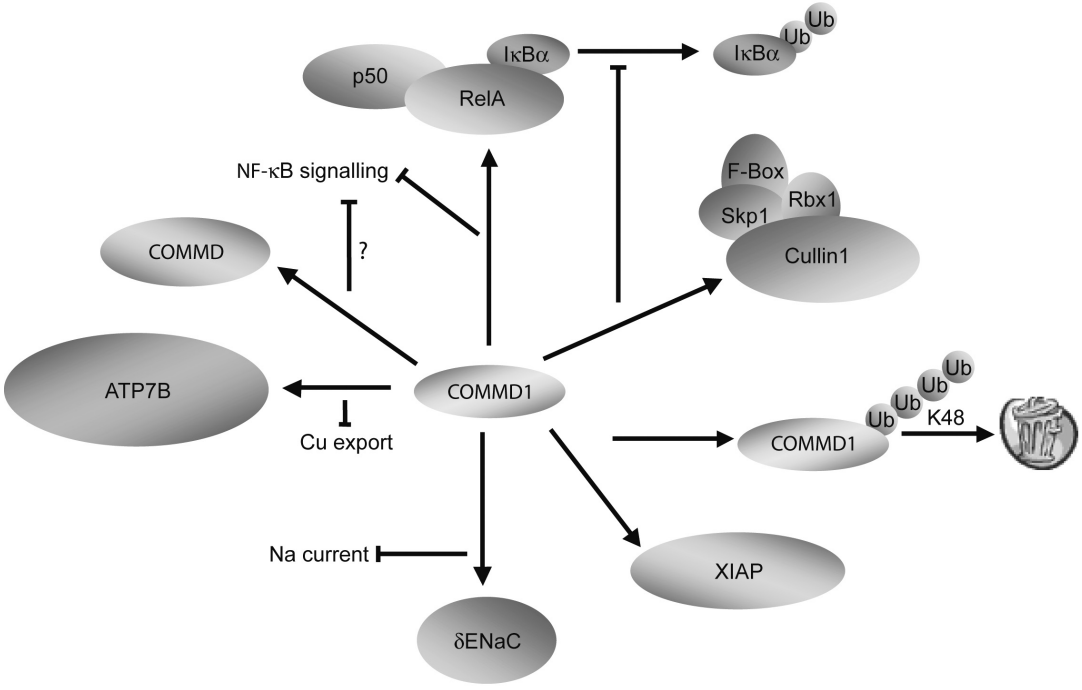

Figure 3. Schematic overview of different COMMD1 protein-protein interactions and their functional implications.
Known protein-protein interactions involving COMMD1 are summarized in this figure. Through identification of its molecular partners, COMMD1 can be placed in several biological pathways. Exact biological mechanisms however are still lacking, and further research is required.

CONCLUDING REMARKS

The cloning of COMMD1 presents the first example of using a purebred dog population from private owners to identify a disease gene. Although no mutations in the *COMMD1* gene have been found to underlie human genetic diseases, its cloning reveals a possible new pathway in copper homeostasis. Further exploration of this pathway may lead to new insights into copper homeostasis and eventually to the identification of new candidate genes for copper overload disorders of unknown etiology. However, based on the data presented here, COMMD1 appears to be involved in multiple cellular processes (summarized in figure 3), which is consistent with the ubiquitous expression pattern of COMMD1. Although the pathology of liver cirrhosis, one of the pathophysiological features of canine copper toxicosis, is known to coincide with NF-κB activation, no other signs of an uncontrolled NF-κB signaling pathway nor defects in sodium homeostasis are observed in affected Bedlington terriers. One might speculate that functional redundancy by other COMMD proteins alleviates the phenotype one might expect in affected dogs. It will be interesting to investigate if the other COMMD proteins share more functions with COMMD1 than their ability to inhibit NF-κB activation. The exact molecular functions of COMMD1 are unknown, but most probably involve specific COMMD1 protein-protein interactions. A future approach in investigating the functions of COMMD1 will therefore be to continue identifying and characterizing novel protein-protein interactions that involve COMMD1. Other approaches that should prove useful are the generation and characterization of COMMD1-deficient cell lines and knock-out mice.

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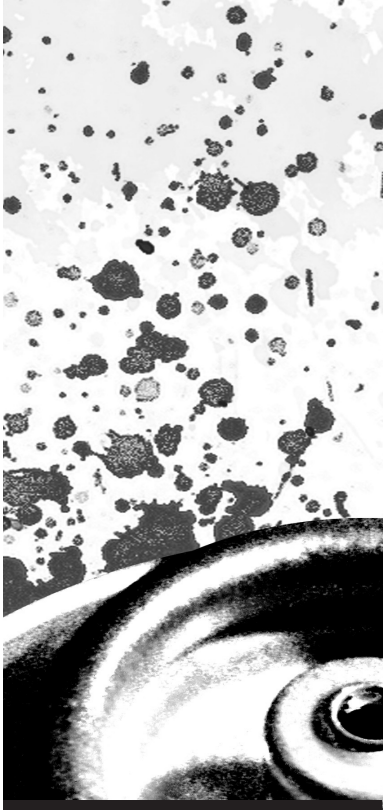

CHAPTER 3

Characterization of COMMD protein-protein interactions in NF-κB signalling

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ABSTRACT

COMMD proteins constitute a recently identified family of NF-κB inhibiting proteins, characterized by the presence of the COMM domain. Here, we report detailed investigation of the role of this protein family, and specifically the role of the COMM domain, in NF-κB signalling through characterization of protein-protein interactions involving COMMD proteins. The small, ubiquitously expressed COMMD6 consists primarily of the COMM domain. Therefore COMMD1 and COMMD6 were further analyzed as prototype members of the COMMD protein family. Using specific antisera, interaction between endogenous COMMD1 and COMMD6 is described. This interaction was verified by independent techniques, appeared to be direct and could be detected throughout the whole cell, including the nucleus. Both proteins inhibit TNF-induced NF-κB activation in a non-synergistic manner. Mutation of the amino acid residues W24 and P41 in the COMM domain of COMMD6 completely abolished the inhibitory effect of COMMD6 on TNF induced NF-κB activation, but this was not accompanied by loss of interaction with COMMD1, COMMD6 or the NF-κB subunit RelA. In contrast to COMMD1, COMMD6 does not bind to IκBα, indicating that both proteins inhibit NF-κB in an overlapping, but not completely similar manner. Taken together, these data support the significance of COMMD protein-protein interactions and provide new mechanistic insight into the function of this protein family in NF-κB signalling.

INTRODUCTION

COMMD proteins constitute a recently described protein family initially identified as interacting partners of COMMD1 (previously known as MURR1), the prototype member of this protein family [85]. In total 10 COMMD proteins, characterized by the presence of the COMM (copper metabolism gene MURR1) domain, are known, of which COMMD1 remains best characterized. Initially, COMMD1 was implicated as a regulator of copper homeostasis by the observation that complete absence of COMMD1 protein due to a genomic deletion encompassing exon 2 of *COMMD1* causes copper toxicosis in Bedlington terriers, a severe hepatic copper overload disease in dogs [75, 76, 267]. Within the liver, copper excretion is mediated by ATP7B [230], a copper translocating P-type ATPase mutated in Wilson disease, a hereditary copper overload disorder in man with pathophysiological similarities to copper toxicosis in Bedlington terriers [53-56]. COMMD1 directly interacts with the N-terminal copper binding domain of ATP7B [182]. Furthermore, transient knock-down of COMMD1 in HEK293 cells leads to increased cellular copper levels [183]. Taken together, these studies suggest that COMMD1 functions as a regulator of ATP7B-mediated hepatic copper excretion.

More recently however, it was demonstrated that COMMD1 is a potent inhibitor of NF-κB mediated transcription, and consequentially can inhibit HIV-1 replication in $CD4^+$ T-lymphocytes [85, 184]. Interestingly, all other COMMD proteins inhibit TNF-induced NF-κB activation to a similar extent as COMMD1 [85]. Although the molecular mechanisms through which COMMD proteins inhibit the NF-κB response have not yet been fully characterized, protein-protein interactions between COMMD proteins and several NF-κB subunits have been detected [85, 184]. The notion

that COMMD1 interacts with IκBα, and that overexpression of COMMD1 results in decreased ubiquitination of IκBα, led to the hypothesis that COMMD1 inhibits NF-κB by regulating IκBα levels in the cell [184, 190]. Additionally, it has also been observed that COMMD1 influences binding of NF-κB to its target promoter sequence [85], indicating that multiple mechanisms may be at play. Taken together, these studies implicate that COMMD proteins constitute a novel family of regulators of NF-κB activity.

In the present study, COMMD protein-protein interactions were further characterized. As COMMD6 lacks the variable extended amino terminus observed in all other COMMD proteins, this protein consists almost solely of the COMM domain, making it an excellent prototype member to study the functions of this novel domain. Thus, COMMD1 and COMMD6 were chosen as prototype members of this family. We show that COMMD protein-protein interactions occur endogenously, and that they are direct and can be detected throughout the whole cell, including the nucleus. In addition, we show that the COMM domain plays an essential role in both COMMD protein-protein interactions and in NF-κB inhibition by COMMD proteins.

MATERIALS AND METHODS

Constructs

Full-length and partial coding sequences of *COMMD1* and *COMMD6* were amplified from human or canine liver cDNA and cloned in pCRII vector (Invitrogen, Breda, the Netherlands). For yeast two-hybrid assays, full-length human *COMMD1* cDNA was subcloned in pDNR3 (Clontech, BD Biosciences, San Jose, CA, USA) and subsequently subcloned in pLP-GBKT7 and pLP-GADT7 using the creator cloning kit (Clontech). Partial coding sequences of human or canine COMMD1, and full length *COMMD6*, were subcloned in pGBT9 or pGAD-GH (Clontech). For production of recombinant COMMD6, *COMMD6* coding sequence was subcloned in pQE-30 (Qiagen, Venlo, the Netherlands). For expression studies *COMMD1* and *COMMD6* were subcloned in pEBB [307] or pZeoSV (Invitrogen) containing sequences encoding Flag, HA, GST, YFP-N or YFP-C epitopes as indicated for each experiment. A Flag tagged COMMD6 isoform a expression plasmid was generated by inserting the extra coding sequence in pEBB-COMMD6-Flag using the Quickchange site directed mutagenesis method (Stratagene, La Jolla, CA, USA). Mutations in the pEBB-COMMD6-Flag expression plasmid were also generated with the Quickchange site directed mutagenesis method. pCDNA3.1-Flag-INCA1 has been kindly provided by Dr E. Kalkhoven (Department of Metabolic and Endocrine Diseases, University Medical Centre Utrecht, Utrecht, the Netherlands). The coding sequence for a fusion protein between ubiquitin and Smac/Diablo (derived from [308]) was cloned into pEBB-YFP-C to obtain pEBB-Ub-Smac/Diablo-YFP-C. 2κB-luciferase reporter, IκBα S.D., IκBα-HA and COMMD-GST expression plasmids have been described previously [85, 309-311]. The sequence of all constructs was verified by automated sequencing.

RT-PCR

A human cDNA panel was obtained from Clontech. Fragments specific for *COMMD1*

(sense and antisense primer sequences: 5'-ATGGCGGCGGGCGAGCTTG-3' and 5'-TCAGTTAGGCTGGCTGATCAGTG-3' respectively) *COMMD6* (sense and antisense primer sequences: 5'-ATGGAGGCGTCCAGCGAGCC-3' and 5'-TCACACCGTTTCAATAACTGCAGC-3' respectively) and *COMMD6* isoform a (sense and antisense primer sequences: 5'- -3' and 5'-AGAAGCAGAAAGGAGACTGGAGG-3' respectively) were amplified by PCR, separated by 1.5% agarose gel electrophoresis and visualized using the GelDoc EQ from Bio-Rad (Bio-Rad, Hercules, CA, USA). RACE PCR was performed using the SMART RACE cDNA amplification kit according to the manufacturers instructions (Clontech).

Generation and purification of COMMD6 antiserum

Recombinant HIS6-COMMD6 fusion protein was purified from Escherichia coli strain M15(pREP4) transformed with pQE-30-COMMD6 according the instructions in the Qiaexpressionist manual (Qiagen). Protein purity was validated by SDS-PAGE, followed by Coomassie Brilliant Blue staining, or detection with alkaline-phosphatase conjugated Ni-NTA upon western blotting. Antiserum to COMMD6 was obtained by serial immunization of rabbits with purified recombinant His6-COMMD6 (Eurogentec, Seraing, Belgium). For affinity purification, His6-COMMD6 was coupled to CnBr-activated sepharose 4B according to the manufacturers instructions (Amersham Biosciences, Uppsala, Sweden). Crude COMMD6 antiserum was allowed to hybridize to the His6-COMMD6 coupled sepharose beads for 4 hours at 4°C under constant rotation. The beads were subsequently washed three times with PBS, 1% triton X-100, once with 10 mM tris-HCl pH 7.5, and once with 10 mM tris-HCl pH 7.5, 0.5 M NaCl. Anti-COMMD6 antibodies were eluted from the beads with 0.1 M glycine-HCl pH 2.5 for 30 minutes, after which the pH was immediately normalized to pH 7.5 using 1 M tris-HCl pH 8.0

Cell culture and transfections

Human embryonic kidney HEK293 cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and L-glutamine and penicillin/streptomycin. Calcium phosphate precipitation was used to transfect HEK293 cells as previously described [307].

Yeast two-hybrid assay, Immunoprecipitation, GST pull down assays and immunoblotting

YGH1 yeast were transformed with yeast expression plasmids according to the SBEG method [312]. Yeast two-hybrid protein interactions were assessed according to the instructions in the Matchmaker Gal4 two-hybrid system 3 manual (Clontech). For GSH-sepharose precipitations, HEK293 cells were lysed in Triton-lysis buffer (1% Triton X-100, 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol), and for immunoprecipitations HEK293 cells were lysed in BSA supplemented Ripa lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5% BSA). Both lysis buffers were supplemented with 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (Roche, Basel, Switzerland) and 10 mM DTT. Immunoprecipitations, and precipitations with GSH-sepharose, were performed

as previously described [183]. Protein detection was performed by immunoblotting for COMMD1 [75], COMMD6, Flag, GST (Santa Cruz, Santa Cruz, CA, USA), HA (Sigma), or RelA (Santa Cruz) as described previously [75, 183]. For *in vitro* transcription-translation, proteins were synthesized by TNT T7-coupled reticulocyte lysate (Promega, Leiden, the Netherlands) and labelled with Tran[^{35}S] (ICN Biomedicals, Costa Mesa, CA, USA) according to the manufacturers instructions using pZeo-COMMD1-HA, pZeo-COMMD6-Flag and pCDNA3.1-Flag-INCA1 as templates. Precipitations with anti-COMMD1, anti-COMMD6, or anti–Flag were performed as previously described [183] and samples were separated by SDS-PAGE. Radiolabeled proteins were visualized by fluorography.

Bimolecular fluorescence complementation studies

HEK293 cells seeded in cover glass chambers were transfected with constructs encoding COMMD1 fused to the N- or C-terminal half of YFP and with COMMD6 fused to the C- or N-terminal half of YFP respectively. Nuclear counterstaining was performed by adding Hoechst 33342 to phenol-red-free culture media to a final concentration of 5 μg/ml and incubating cells for 30 minutes at 37°C. Images were obtained from living cells utilizing a Zeiss Axiovert 100M confocal microscope equipped with a Zeiss LSM 510 Meta spectrometer.

Luciferase reporter assays

For luciferase assays, cells were seeded in 6-well plates in triplicate for each treatment group. TNF (Roche) treatments consisted of 500 units/ml for 12 hours. Luciferase activity was measured as described previously [313] using the Luciferase Assay System (Promega) and a TR717 Applied Biosystems microplate luminometer.

RESULTS

The COMMD6 gene is highly conserved and ubiquitously expressed

The *COMMD6* gene is located on chromosome 13 of the human genome and encodes a putative 85 amino acid residue protein with a predicted Mr of 8 kDa (schematically depicted in Fig. 1A). An additional human *COMMD6* transcript, termed *COMMD6* isoform a, has been annotated in the NCBI database. Whereas *COMMD6* is transcribed from four exons, the *COMMD6* isoform a transcript contains the sequence encoded by one extra exon located between exon 3 and 4, yielding an in-frame insertion of 12 additional amino acids (Fig. 1A). RT-PCR expression profiling of RNA preparations obtained from different human and mouse tissues indicated that both *COMMD6* transcript isoforms are ubiquitously expressed throughout all tissues investigated, similarly as observed for COMMD1 and the other COMMD genes (Fig. 1B and data not shown) [75, 76, 85].

Alignment of sequences of several COMMD6 orthologues is depicted in figure 1C; this analysis revealed the interesting observation that COMMD6 proteins in higher vertebrates lack an extended amino terminus found in lower organisms. Repeated attempts to identify putative 5' extensions of the human *COMMD6* transcript, both by *in silico* approaches and 5'RACE were unsuccessful (data not shown). COMMD6 is the only member of the COMMD protein family in which structural diversity between vertebrate and non-vertebrate COMMD is observed. As COMMD6 in higher vertebrates consists primarily of the COMM domain, this indicates that the COMM domain is important, and maybe even sufficient, for COMMD6 function.

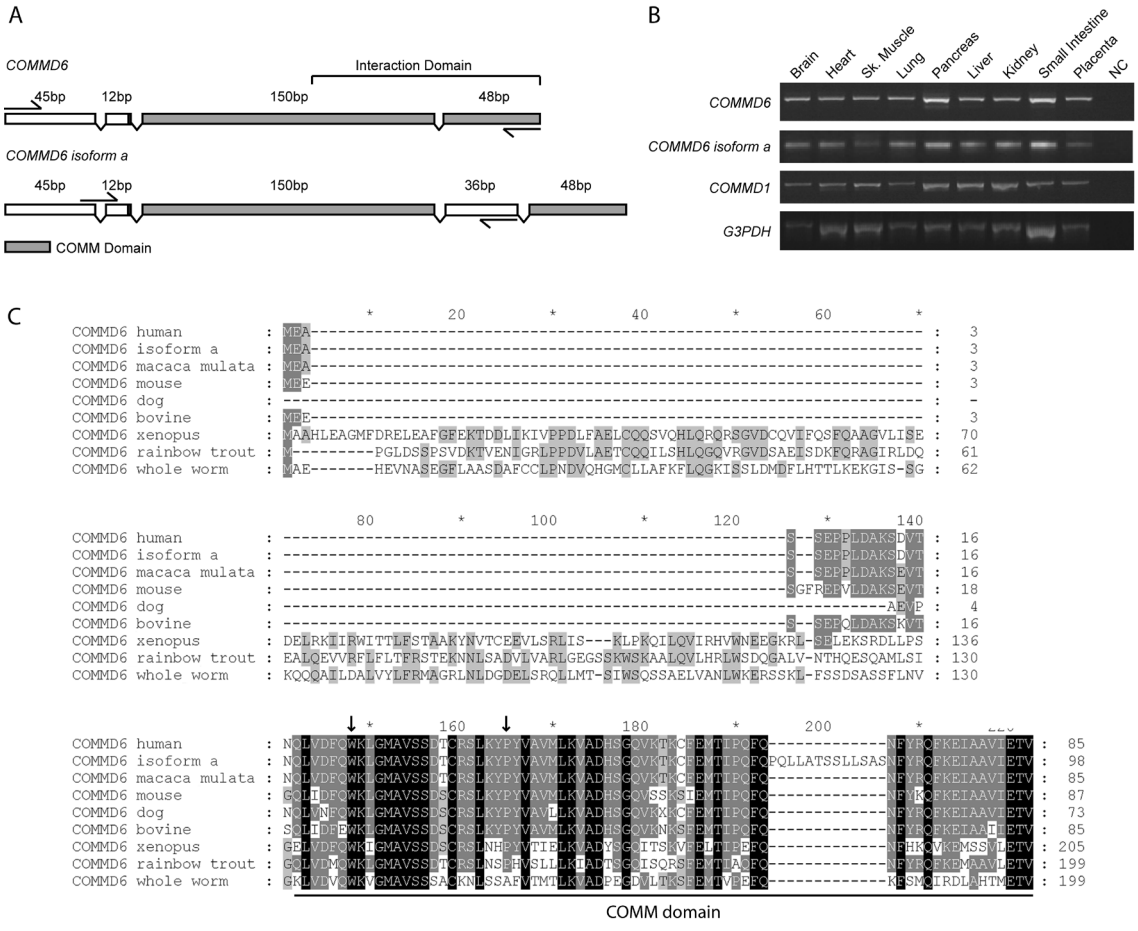

Figure 1. Genetic description of COMMD6
A: schematic representation of *COMMD6* and the *COMMD6* isoform a transcripts. Boxes indicate individual exons; exon sizes are indicated in basepairs. Sequences encoding the COMM domain are indicated in grey. B: RT-PCR analysis of the expression of COMMD6, COMMD6 isoform a and COMMD1 in different human tissues. G3PDH RT-PCR was performed as positive control, as a negative control (NC) H_2O was used as template. C: Alignment of COMMD6 amino acid sequences of different species. The COMM domain is underlined. Black, dark grey or light grey background colours indicate a 100%, 50% or 20% degree of conservation, respectively. Arrows indicate residues mutated for experiments described in figures 5 and 6.

Endogenous COMMD1 and COMMD6 interact in mammalian cells

Previously it has been shown that most COMMD proteins, including COMMD6, associate with COMMD1. However these data were all based on studies using overexpressed COMMD proteins. To determine if endogenous COMMD1 and COMMD6 can be detected in complex with each other, antiserum against COMMD6 was generated. As COMMD1 and COMMD6 share a high degree of similarity in their COMM domains, it was first necessary to exclude that antibodies directed against COMMD1 cross-react with COMMD6 and *vice versa*. Both by western blot analysis (data not shown) and by immunoprecipitation of radiolabeled, *in vitro* synthesized COMMD proteins (Fig. 2A), it was established that the anti-COMMD antisera reacted specifically with their cognate antigens. Immunoprecipitation using cell lysates of HEK293 cells revealed that COMMD1 and COMMD6 interacted with each other using antisera raised against either COMMD1 or COMMD6, whereas neither protein could be detected in immunoprecipitates using preimmune serum or antibodies directed against GST (Fig. 2B). To determine if COMMD1 can interact with both COMMD6 isoforms, COMMD1-GST fusion protein was expressed in HEK293 cells together with COMMD6-Flag or COMMD6 isoform a-Flag fusion proteins. Glutathione sepharose precipitation

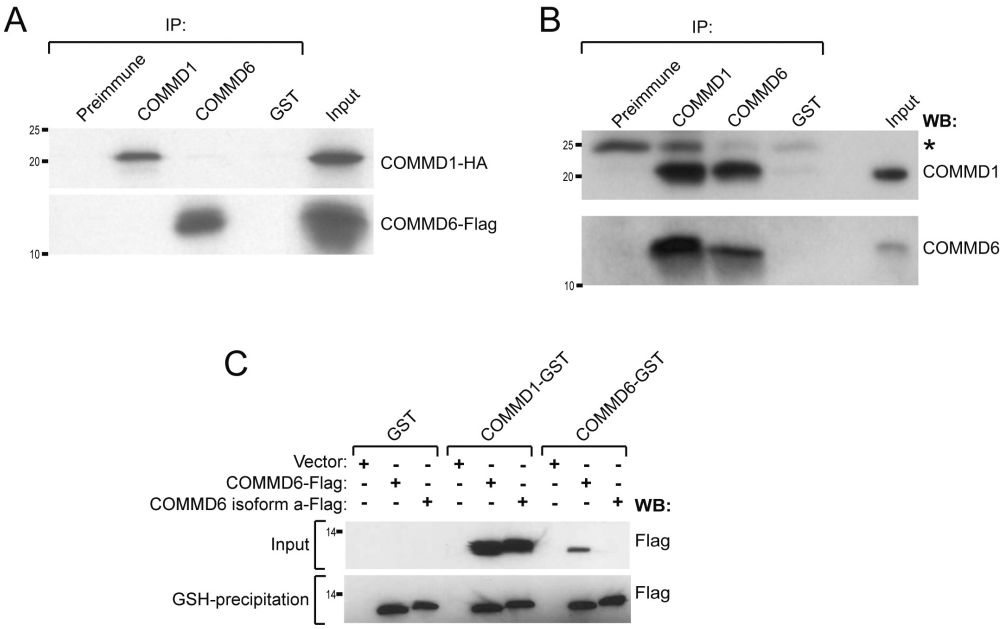

Figure 2. Detection of endogenous COMMD1 and COMMD6 containing complexes

A: Constructs encoding COMMD1-HA (upper panel) or COMMD6-Flag (lower panel) were used in coupled *in vitro* transcription-translation reactions in the presence of radiolabelled amino acids. Labelled proteins were analyzed directly (Input) or immunoprecipitated (IP) using preimmune rabbit serum, anti-COMMD1 antiserum, affinity purified anti-COMMD6 antiserum or anti-GST antibody. Proteins were separated by SDS-PAGE and visualized by fluorography. Apparent molecular size markers are indicated in kDa on the left. B: Cell lysates of untransfected HEK293 cells were directly analyzed (Input) or used for immunoprecipitation (IP) using preimmune rabbit serum, anti-COMMD1 antiserum, anti-COMMD6 antiserum or anti-GST antibody. Precipitates were rinsed and separated by SDS-PAGE and immunoblotted using antibodies as indicated. IgG light chain is indicated by *. Apparent molecular size markers are indicated in kDa on the left. C: HEK293 cells were transfected with cDNA constructs encoding COMMD6-Flag, COMMD6 isoform a-Flag, GST, COMMD1-GST or COMMD6-GST as indicated. Cell lysates were made which were used for glutathione-sepharose precipitation. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

revealed that COMMD1 can interact with both COMMD6 isoforms, whereas COMMD6 can only homodimerize with COMMD6, and does not bind COMMD6 isoform a (Fig. 2C).

From these and previous [85] experiments, it remained unknown if interactions between COMMD proteins are direct or dependent on the presence of other proteins. To investigate whether a direct interaction between COMMD1 and COMMD6 exists, yeast two hybrid analysis was applied, and co-immunoprecipitation studies were performed using *in vitro* synthesized, [^{35}S]Methionine-labelled COMMD1 and COMMD6. As depicted in figures 3A and 3B, these approaches independently revealed a direct and specific interaction between these two proteins. Homodimerization of COMMD1 or COMMD6 was not detected by yeast two-hybrid methodology (data not shown). Deletion of exon 3 of COMMD1 abolished interaction between COMMD1 and COMMD6 in our yeast two-hybrid setting, whereas the carboxy terminal 39 amino acids of COMMD6 are sufficient for interaction with COMMD1 (Fig. 3C and data not shown). This indicates that the direct interaction between COMMD1 and COMMD6 is mediated by the carboxy terminal part of the COMM domain.

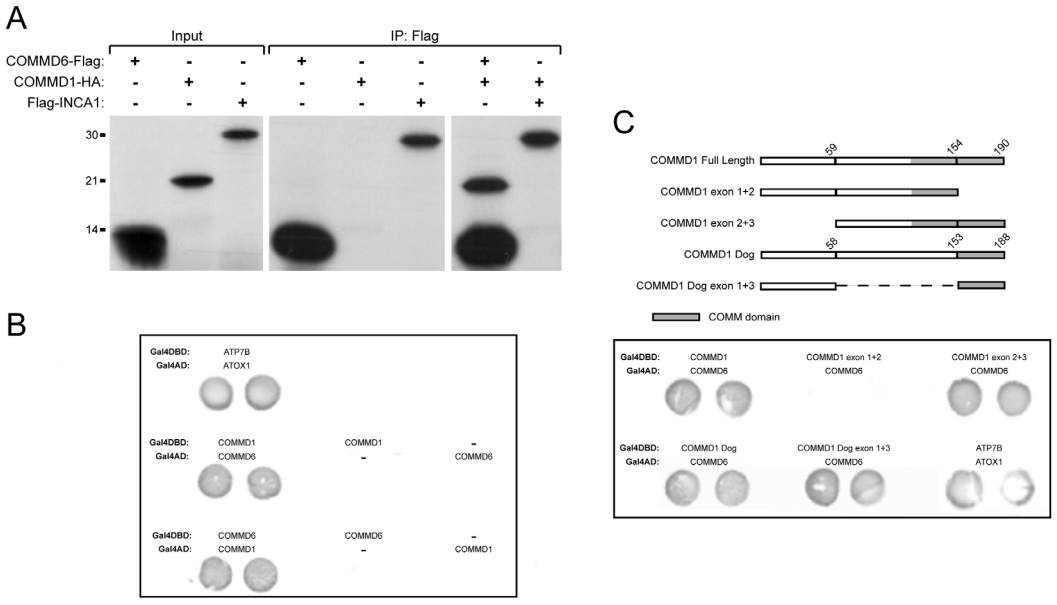

Figure 3. The interaction between COMMD1 and COMMD6 is direct and mediated through the COMM domain
A: Constructs encoding the indicated proteins were used in coupled *in vitro* transcription-translation reactions in the presence of radiolabelled amino acids. Labelled proteins were analyzed directly (Input; left panel) or immunoprecipitated using anti-Flag antibody (IP: Flag; right panel). Proteins were separated by SDS-PAGE and visualized by fluorography. Apparent molecular size markers are indicated in kDa on the left. B: *YGH1* yeast were transformed with constructs encoding COMMD1 and COMMD6, fused to Gal4DBD or Gal4AD as indicated. All constructs were also transfected together with empty vectors as negative controls. Strains were grown in duplicate on selective media and subjected to a filter lift test for β-Galactosidase activity. The interaction between ATOX1 and ATP7B was used as positive control for β-Galactosidase activity. C: Different COMMD1 deletion constructs based on COMMD1 exon boundaries are schematically depicted. Numbers indicate predicted amino acid residues. All constructs were fused to the coding region of the Gal4DBD. *YGH1* yeast were transformed with the COMMD1-GAL4DBD deletion constructs and COMMD6 Gal4AD as indicated. Strains were grown in duplicate on selective media and subjected to a filter lift test for β-Galactosidase activity. The interaction between ATOX1 and ATP7B was used as positive control for β-Galactosidase activity.

To visualize the interaction between COMMD1 and COMMD6 in living cells, bimolecular fluorescence methodology was applied. In this approach, fusion proteins of COMMD1 and COMMD6 with the N- or C-terminal halves of YFP (N-YFP or C-YFP) were expressed in HEK293 cells. The two complementary portions of YFP are brought into close proximity only when the proteins they are fused to interact, which subsequently allows for a fluorescent signal [314]. As depicted in figure 4, this approach readily revealed interaction between COMMD1 and COMMD6 (panels C and E). This interaction was specific, as no signal was observed when the COMMD1 or COMMD6 fusion proteins were expressed with solely the complementary YFP fragments (panels A and B), or with the complementary YFP fragments fused to the non-interacting protein Smac/Diablo (panel D). The fluorescent signal, which shows the cellular location of interaction between COMMD1 and COMMD6, was distributed throughout the whole cell, with most of the signal in the cytoplasm and to a lesser extent in the nucleus. In most cells some perinuclear aggregation of the signal was observed, the nature of which is unknown (Fig. 4, panel C).

COMMD6 inhibits TNF induced NF-κB activation through conserved residues in the COMM domain

The COMMD protein family has recently been implicated as a family of NF-κB inhibitors [85]. To investigate if different COMMD proteins inhibit NF-κB in a synergistic manner, HEK293 cells were transfected with a 2κB luciferase reporter construct together with expression vectors encoding COMMD1, COMMD6 or a combination of both. A super dominant form of IκBα was used as a positive control. In accordance with the data previously described, COMMD1 and COMMD6 inhibit NF-κB with the same efficiency [85]. No synergism or additive effect was observed when COMMD1 and COMMD6 were expressed together, whereas expression of either protein together with a super dominant form of IκBα (IκBα S.D.) resulted in complete inhibition of NF-κB activation (Fig. 5A). A reduction in COMMD1 and COMMD6 expression levels was observed upon co-expression with IκBα S.D. However, a similar reduction of other proteins expressed from the same vector backbone, but not of endogenous COMMD1was also observed (data not shown). These phenomena are therefore most likely explained by the stimulatory effect of NF-κB on the exogenous promoter in the expression system, similarly as has been described before for other commonly used expression system promoters [286].

Human COMMD6 consists primarily of the COMM domain. Within the COMM domain two amino acid residues, W24 and P41 are absolutely conserved in all human COMMD proteins (Fig. 1C). Mutation of either residue in COMMD6 individually to alanine did not affect NF-κB reporter activity, although a slight increase in basal activation was observed for COMMD6-P41A. Strikingly however, when both residues were mutated simultaneously (COMMD6-W24A-P41A) the repression of TNF-induced NF-κB signalling was completely abolished. In fact, a dose dependent increase in both basal and TNF-induced luciferase activity was observed, indicating that the COMMD6-W24A-P41A double mutant behaved as a super dominant activator of NF-κB (Fig. 5B and data not shown). Taken together, these data suggest that COMMD6, like COMMD1, is a potent endogenous repressor of NF-κB signalling, and that specific residues in the COMM domain are essential for this inhibitory effect.

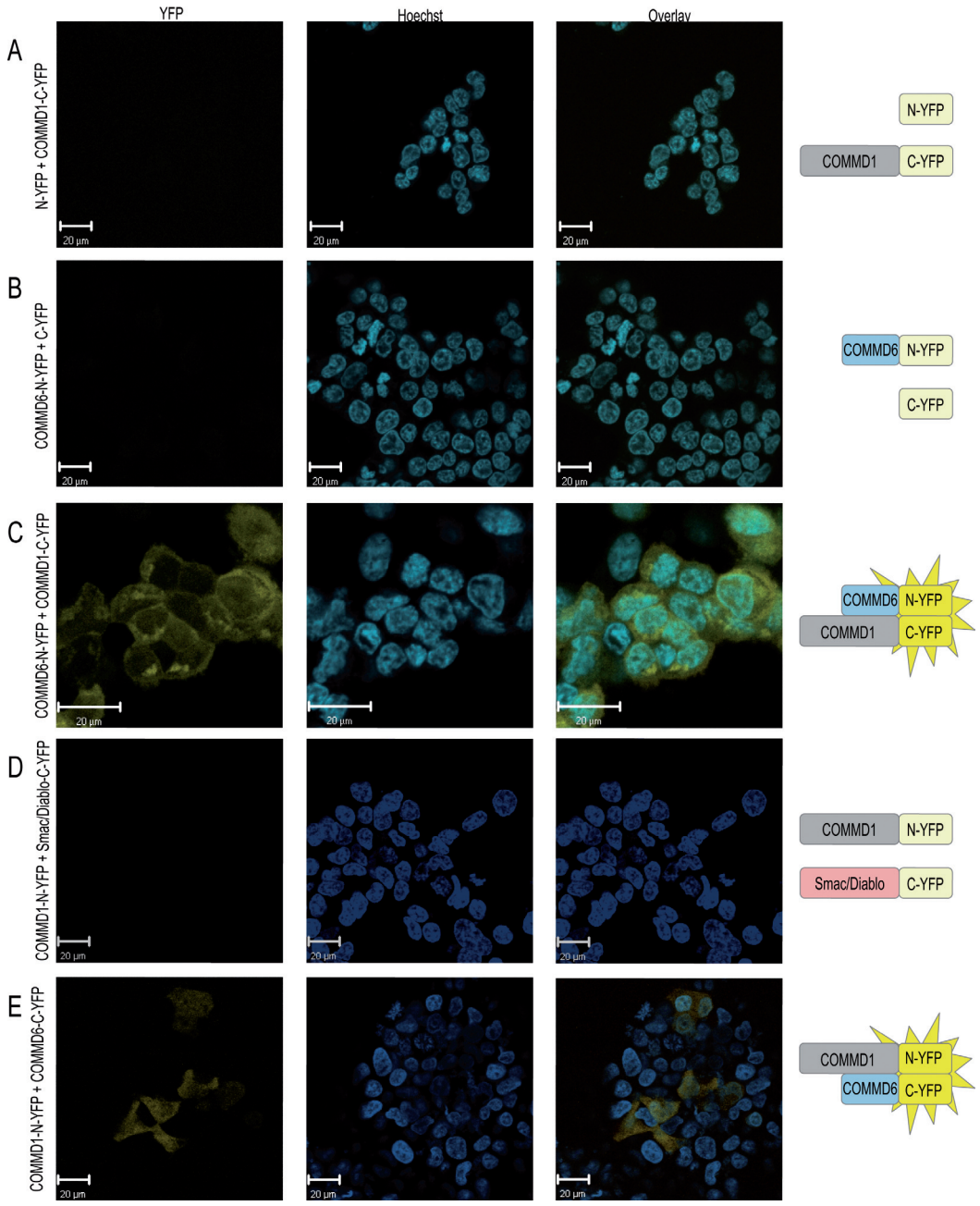

Figure 4. Bimolecular fluorescence complementation with COMMD1 and COMMD6
The principle of bimolecular fluorescence complementation is schematically depicted. HEK293 cells were transfected with expression vectors encoding COMMD1-C-YFP and N-YFP (panel A), COMMD6-N-YFP and C-YFP (second panel B), COMMD1-C-YFP and COMMD6-N-YFP (panel C), COMMD1-N-YFP and Smac/Diablo-C-YFP (panel D) or COMMD1-N-YFP and COMMD6-C-YFP (panel E). After overnight transfection, nuclear counter stain was performed by incubating cells with Hoechst prior to acquiring confocal images. The YFP images, Hoechst images and overlay images are shown as indicated. Magnification bars (20 µm) are presented in each image.

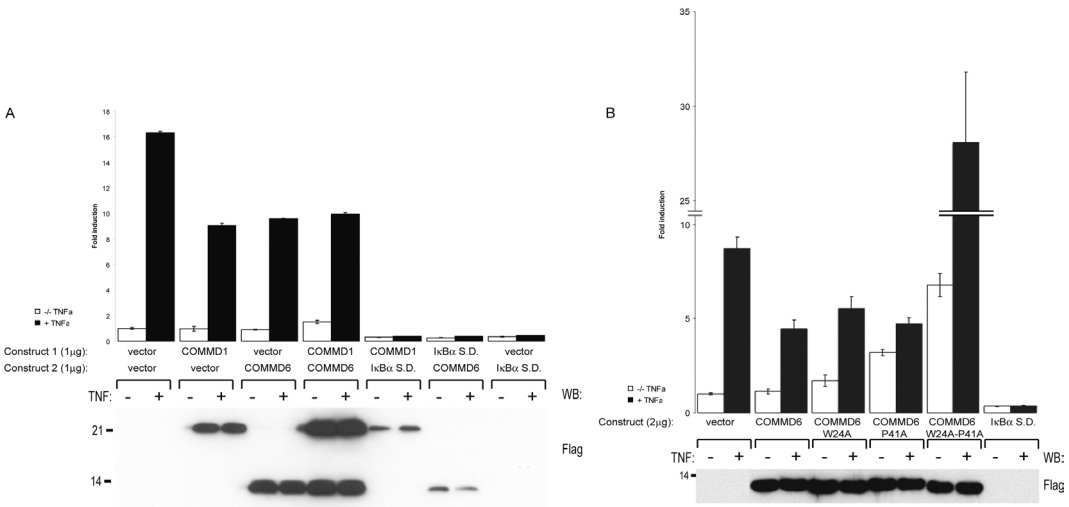

Figure 5. COMMD6 inhibits NF-κB signalling through residues in the COMM domain
HEK293 cells were transfected with empty vector (A, B), or expression vectors encoding COMMD1-Flag (A), COMMD6-Flag (A, B), COMMD6-Flag mutants W24A, P41A, W24A-P41A (B) or IκBα-S.D. (A, B) along with a 2κB-luciferase reporter plasmid as indicated. Cells were incubated with or without TNF, as indicated by white and black bars respectively, and lysed after 12 hours. Upper panels: Luciferase activities in the lysates were measured and expressed as fold induction relative to unstimulated empty vector-transfected cells. Results are representative of three independent experiments performed in triplicate. Error bars represent standard deviations. Lower panels: Immunoblotting of cell lysates using anti Flag antibodies was performed confirm expression of COMMD1-Flag and COMMD6-Flag. Apparent molecular size markers are indicated in kDa on the left.

The W24A and P41A mutations in COMMD6 do not influence binding of COMMD6 to COMMD1 or to itself

Based on the observation that COMMD1 and its interacting partner COMMD6 both acted as repressors of NF-κB reporter activity, we hypothesized that the observed NF-κB repression by COMMD6 is mediated through its interaction with COMMD1. This hypothesis predicts that the super dominant activation by COMMD6-W24A-P41A is associated with a failure of this COMMD6 mutant protein to interact with COMMD1. To test this possibility, glutathione sepharose precipitates of COMMD1-GST were analyzed for the presence of COMMD6 proteins. The results revealed that wild-type COMMD6 and all three COMMD6 mutants (COMMD6-W24A, COMMD6-P41A, COMMD6-W24A-P41A) bound to COMMD1 (Fig. 6A). Homomultimerization of COMMD6 was also unaffected, as not only wild-type COMMD6 but also the double mutant could be detected in complex with wild-type COMMD6-GST (Fig. 6B).

To assess if association of COMMD6 with NFκB in the cell was affected by the two introduced mutations, the ability of COMMD6 and the COMMD6 mutants to bind to the NF-κB subunit RelA was determined. RelA was readily detected in complex with both COMMD1 and COMMD6. All three COMMD6 mutants were able to bind to RelA at least as strong as wild-type COMMD6 (Fig. 6C).

COMMD1 may exert its NF-κB inhibiting effects through binding of IκBα, thereby preventing the proteasomal degradation of IκBα [16]. To investigate if COMMD6 inhibits NF-κB by participating in this mechanism, the possibility that COMMD6 and the COMMD6 mutants

could bind to IκBα was determined. Glutathione sepharose precipitation revealed that wild-type COMMD6 and the three COMMD6 mutants failed to bind HA-IκBα, whereas COMMD1 abundantly interacted with HA-IκBα under the same conditions (Fig. 6D). These data indicate that COMMD1 and COMMD6 have overlapping, but not completely similar functions in the NF-κB pathway.

DISCUSSION

COMMD proteins constitute a novel family of NF-κB inhibiting proteins. As protein-protein interactions seem to be an important mechanism for these proteins to exert their function (reviewed in), we here further characterized the interaction between COMMD proteins using COMMD1 and COMMD6 as prototype members. We show for the first time that COMMD protein-protein interactions occur endogenously. Moreover, these interactions are direct and dependent on the COMM domain. The interaction between COMMD1 and COMMD6 occurs throughout

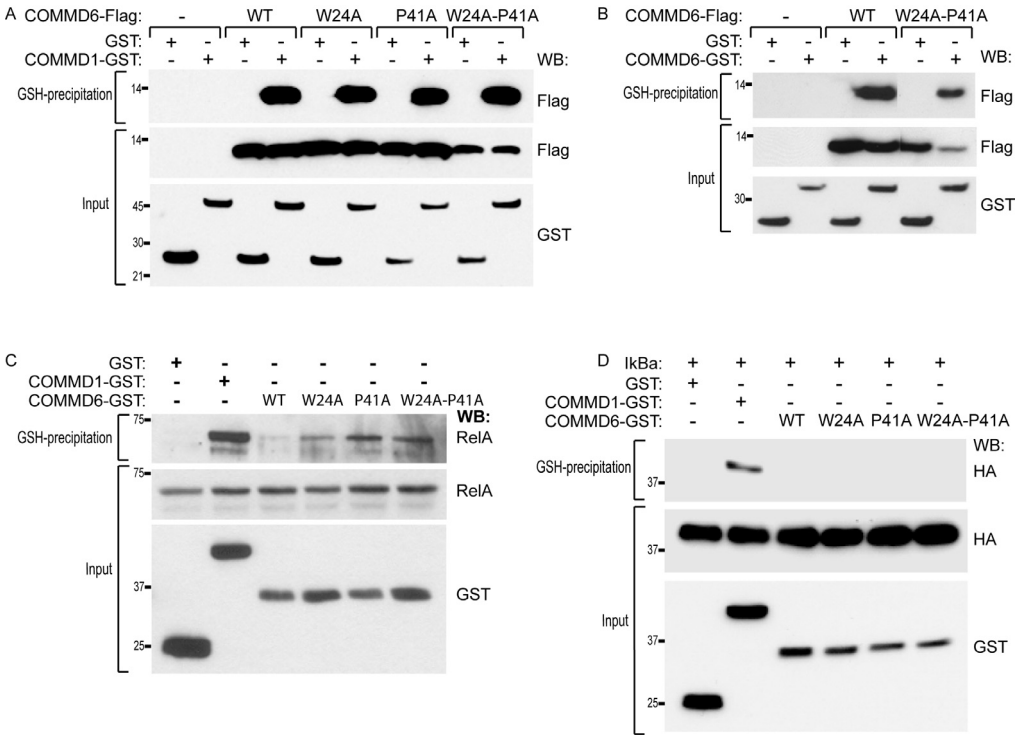

Figure 6. COMMD6-Flag W24A-P41A mutant interacts with COMMD1, COMMD6 and RelA, but not with IκBα
A and B: Glutathione-sepharose precipitation using cell lysates of HEK293 cells expressing the COMMD6-Flag wild-type and point mutants and COMMD1-GST (A) or COMMD6-GST (B). Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left. C and D: Glutathione-sepharose precipitation performed on the cell lysates of HEK293 cells expressing COMMD1 and wild-type and mutant COMMD6 as GST fusion proteins that were either untransfected (C) or transfected with an expression vector encoding HA-IκBα (D). Precipitates were washed and separated by SDS-PAGE and immunoblotted for RelA (C), HA (D), or GST (C+D) as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

the living cell, including the nucleus. In addition, by providing a dominant negative mutant of COMMD6, unable to repress NF-κB activation, we show for the first time that the function of these proteins is dependent on conserved residues in the COMM domain.

COMMD6 is predicted to be a ubiquitously expressed, small (8 kDa), soluble protein, which could potentially function as a homo-multimer. *COMMD6* sequences appear to be highly conserved during evolution, although in humans a *COMMD6* splice variant is expressed that has not been observed in other organisms, and the predicted amino termini of COMMD6 proteins in higher organisms are significantly smaller than those observed in lower organisms. In fact, COMMD6 in higher vertebrates consists primarily of the COMM domain, indicating that the COMM domain is important, and maybe even sufficient, for COMMD6 function. This makes COMMD6 an excellent prototype member of the COMMD protein family to study the function and mechanism of action of the COMM domain. Using antisera directed against COMMD6, we were able to demonstrate that COMMD1-COMMD6 interactions occur endogenously in HEK293 cells. Both COMMD1 and COMMD6 interact with almost all COMMD proteins (data not shown, [85]). It remains unclear what the exact composition is of the detected COMMD complexes, but direct interactions between COMMD proteins could indeed occur as evidenced from the direct interaction between COMMD1 and COMMD6 demonstrated in the present study. In addition, homomultimerization of COMMD proteins can occur, as complexes containing multiple COMMD1 or COMMD6 molecules could be detected by GSH-sepharose precipitation analysis (Fig. 2C and [85]). Homomultimerization of COMMD1 or COMMD6 could not be detected with yeast two-hybrid methodology. This could either indicate that that homomultimerization of COMMD proteins is not direct and thus only occurs in higher-order complexes, or that COMMD homomultimerization occurs with a relatively lower affinity than COMMD heteromultimerization, since the yeast two-hybrid system is known to be only suitable for the detection of high-affinity interactions [174, 315]. The interaction between COMMD1 and COMMD6 was abolished by deletion of exon 3 of COMMD1. This independently confirms that interaction between COMMD proteins is mediated by the COMM domain, and that the carboxy terminal part of the COMM domain could be sufficient for interaction (Fig. 3C, [85]). Using bimolecular fluorescence complementation methodology, the subcellular distribution of COMMD1 and COMMD6 containing complexes was determined. The signal was distributed throughout the cell, including the nucleus. Some perinucleur aggregation of the signal was observed, the nature of which remains elusive. Considering the irreversible nature of the bimolecular fluorescence complementation interaction, it cannot be excluded that this aggregation resulted from artificial accumulation of interacting complexes or from protein overexpression. However, endogenous COMMD1 and COMMD1 tagged with fluorescent proteins were previously also detected as a punctate staining pattern without overlap with lysosomes or mitochondria [75, 183], suggesting that this localization might be biologically relevant.

Within the cell, different NF-κB proteins exist as homo- or heterodimers that are capable of binding DNA at particular sequences known as κB-sites and subsequently activate transcription [287]. IκB proteins act as inhibitors of NF-κB signalling by binding to the NF-κB dimers and preventing their nuclear translocation [293]. COMMD1 and both isoforms of COMMD6 inhibited NF-κB to a similar extent (Fig 5A, data not shown). Co-expression of both COMMD1 and COMMD6 did not exacerbate the inhibition of NF-κB mediated transcription, suggesting that both proteins exert their effect through the same pathway. Consistent with this observation, both COMMD1 and COMMD6 are associated with the RelA subunit of NF-κB. Previously, it has been suggested that COMMD1 regulates the proteasomal degradation of IκBα [184, 190], but whereas COMMD1 is associated with IκBα, no binding of COMMD6 to IκBα was observed. The most straightforward explanation of these data is that the COMM domain of COMMD1 acts to recruit COMMD6 to the NF-κB complex after TNF-induced dissociation of IκBα. This would eventually result in inhibition of NF-κB mediated transcriptional activation. Recently published data suggest that this inhibition takes place in the nucleus [85]. Consistent with this observation, the observed bimolecular fluorescence signal reflecting the interaction between COMMD1 and COMMD6 was also partly detectable in the nucleus. Mutation of the W24 and P41 residues in COMMD6 completely abolished all its ability to inhibit NF-κB signalling. This mutant behaved as a dominant inducer of NF-κB signalling in a dose dependent manner, while retaining its ability to bind COMMD1, COMMD6 and RelA. Overexpression of COMMD6-W24A-P41A might therefore sequester COMMD1, rendering COMMD1 unavailable for interaction with endogenous COMMD6 or possibly other COMMD proteins, leading to disinhibition of NF-κB signalling. These data suggest that COMMD6 is an endogenous inhibitor of NF-κB, and show for the first time that specific conserved residues in the COMM domain are essential for inhibition of NF-κB mediated transcription by COMMD proteins.

Taken together, the recent identification of the COMMD family of proteins potentially provides a complete new avenue to study the molecular mechanisms of NF-κB activation and inhibition. This could be of great interest since COMMD1 restricts HIV-1 replication in $CD4^+$ T-lymphocytes through its NF-κB inhibiting activity [184]. Thus, the COMMD protein family could potentially play an important role in the pathogenesis of HIV-1 infections. Previous studies based on RNAi-mediated inhibition of COMMD expression revealed that COMMD1 and COMMD6 are endogenous inhibitors of NF-κB [85, 184]. Consistent with these observations, the dominant negative COMMD6-W24A-P41A mutant induces NF-κB activation, even when no exogenous stimulus was added. As all COMMD proteins are ubiquitously expressed, their activities would need to be regulated to avoid complete inhibition of NF-κB signalling. The inhibition of TNF-induced NF-κB activation involves recruitment of COMMD1 to chromatin [85]. The mechanisms that induce and terminate this recruitment might be critical in the regulation of the activity of all COMMD proteins. In addition, it has been previously shown that COMMD1 is a substrate for ubiquitination by XIAP, a potent activator of NF-κB signalling [183]. The exact signals leading to the ubiquitination and subsequent degradation of COMMD1 are unknown, but these could

potentially provide a mechanism to regulate the NF-κB inhibiting properties of COMMD1, and possibly other COMMD proteins. Further research on the mechanisms of NF-κB inhibition by COMMD proteins, and the regulation hereof, is still required, for which the COMMD6-W24A-P41A double mutant will be a valuable tool. A COMMD1 knock-out mouse model will also provide an excellent model to study the regulation of both NF-κB activation and copper homeostasis by COMMD1 in vivo. Finally, given the variety of cellular processes in which COMMD1 is involved , one might speculate that other COMMD proteins also have more functions outside the NF-κB pathway. It would be particularly interesting if these proteins would also have a function in copper metabolism. This could possibly lead to the identification of novel candidate genes for hereditary disorders of copper metabolism because the underlying genetic mutations for many of such disorders remain still unknown [61].

ACKNOWLEDGEMENTS

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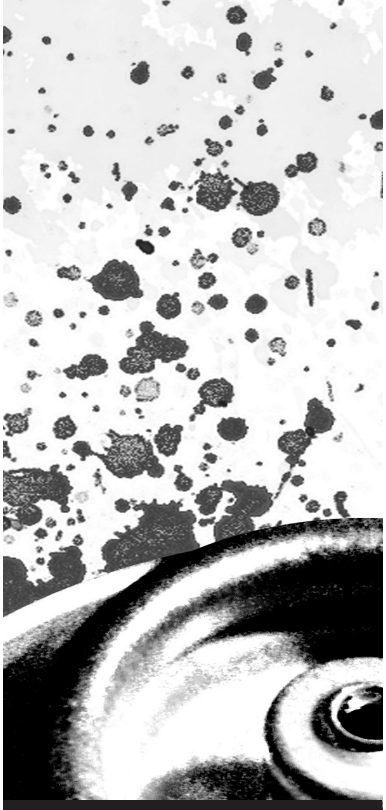

CHAPTER 4

Protein-protein interactions with ATP7B implicate COMMD genes as candidate genes for human disorders of hepatic copper overload

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Submitted

ABSTRACT

The pediatric diseases Indian childhood cirrhosis (ICC), endemic Tyrolean infantile cirrhosis (ETIC) and idiopathic copper toxicosis (ICT) are characterized by severe hepatic dysfunction due to accumulation of excess copper. These disorders are caused by a combination of excessive dietary copper intake and a genetic predisposition that has yet to be unraveled. Wilson disease, caused by mutations in *ATP7B*, is a copper overload disorder that shares pathophysiological similarities with ICC, ETIC and ICT. Recently, COMMD1 was identified as an interacting partner of the copper transporting P-type ATPase ATP7B. Disruption of *COMMD1* causes copper toxicosis in Bedlington terriers, indicating that COMMD1 plays an essential role in hepatic copper excretion, most likely through its interaction with ATP7B. COMMD1 has nine human homologous characterized by the conserved COMM domain. We aimed to investigate the potential role of these nine family members of COMMD1 in the regulation and deregulation of human copper homeostasis. COMMD2, COMMD8 and COMMD10 were identified as novel interacting partners for ATP7B, indicating that these proteins have a regulatory role in hepatic copper excretion. The genes encoding for these COMMD proteins were investigated as candidate genes for ICC, ETIC, and ICT. Several single nucleotide polymorphisms were detected in *COMMD2* and *COMMD10* that are potentially associated with disease development.

INTRODUCTION

The trace element copper requires a delicate homeostatic balance to assure that the needs for normal cellular processes are met, whereas toxicity due to accumulation of excess copper needs to be prevented. Copper is required for numerous cellular processes, including mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, connective tissue formation, pigmentation, peptide amidation and iron metabolism [1]. Copper deficiency, as observed in the pediatric disorder Menkes disease, leads to severe developmental delay, and death usually occurs before three years of age [13]. In amounts that exceed cellular needs, copper is highly toxic due to its potential to facilitate the production of reactive oxygen species by means of Fenton chemistry [2]. Because the liver plays a central role in the excretion of copper, copper overload is usually characterized by extensive liver damage, as can be observed in Wilson disease, an autosomal recessive disorder caused by mutations in the *ATP7B* gene [53-55]. Other, non-Wilsonian, forms of hepatic copper overload syndromes have been described [62, 261], including Indian childhood cirrhosis (ICC) [5], endemic Tyrolean infantile cirrhosis (ETIC) [6], and a class of sporadic cases occurring worldwide and grouped together as idiopathic copper toxicosis (ICT) [7, 8]. These non-Wilsonian copper overload disorders have a uniformly low age of onset (before two years of age) and are fatal before five years of age due to liver failure as a consequence of decompensated liver cirrhosis. In contrast to Wilson disease, which manifests independent of excess copper intake, phenotypic expression of ICC, ETIC and some cases of ICT appear to be associated with both an excessive copper intake and an underlying genetic defect [5, 6, 8, 63].

Recently, COMMD1 was implicated as a novel regulator of hepatic copper excretion as

a deficiency of this protein causes copper toxicosis in Bedlington terriers [75, 76, 268], a canine disorder that shares pathophysiological features with Wilson disease, ICC, ETIC and ICT [72]. Subsequent identification of COMMD1 as an interacting partner of the copper transporting P-type ATPase encoded by *ATP7B* further supports the role of COMMD1 in copper homeostasis [114, 182], and suggests that these two proteins cooperate to facilitate excretion of copper from the hepatocyte into the bile canaliculus. Although *COMMD1* has been postulated as a candidate gene for ICC, ETIC and ICT, and as a modifier gene for the clinical presentation of Wilson disease, no disease associated mutations in *COMMD1* have been detected in patients with these disorders [61, 78-81].

COMMD1 is the prototype member of the recently identified COMMD protein family, consisting of 10 proteins that are characterized by the presence of the COMM domain [85, 189]. All COMMD proteins bind to COMMD1, and have been implicated as regulators of the transcription factor NF-κB [85, 184]. Based on these structural and functional similarities among members of the COMMD protein family, we hypothesized that COMMD proteins share more properties with COMMD1, and thus could also play a role in copper homeostasis. In this study, we initially investigated the role of COMMD proteins in human copper homeostasis through the study of protein-protein interactions of COMMD proteins with ATP7B. As our data directly implicate several *COMMD* genes as putative regulators of copper homeostasis, these were further studied as candidate genes for ICC, ETIC and ICT.

MATERIALS AND METHODS

Constructs

COMMD-GST constructs were described previously [85, 183]. Generation of pEBB-ATP7B-Flag, pEBB-ATP7B(1-650)-Flag, and plasmids encoding short hairpin RNAs (shRNAs) that target COMMD1 or ATOX1 will be described elsewhere [114].

Cell culture and transfections

Human embryonic kidney HEK293T and Phoenix packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and L-glutamine and penicillin/streptomycin. Calcium phosphate precipitation was used to transfect HEK293T cells as previously described [307]. To produce amphotropic retrovirus supernatants, Phoenix packaging cell were transfected with empty pRETRO-SUPER, or pRETRO-SUPER constructs targeting COMMD1 or ATOX1, using Fugene-6 (Roche, Basle, Switzerland) according to the manufacturers instructions. On adding 4 μg/ml polybrene, HEK293T cells were infected with the retrovirus supernatants and grown on media supplemented with 1 μg/ml puromycin (Sigma, Steinheim, Germany) to allow for monoclonal selection.

Immunoprecipitations, GST pull-down assays, and immunoblotting

Cells were lysed in lysis buffer (1% Triton X-100, 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol) supplemented with 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (Roche, Almere, the Netherlands) and 1 mM DTT. Precipitations with GSH-sepharose were performed as previously described [183]. Protein detection was performed by immunoblotting for Flag (Sigma), GST (Santa Cruz, Santa Cruz, CA, USA), SCHAD, COMMD1 [275] or ATOX1 [167]as described previously [75].

Patients

In total 18 patients with non-Wilsonian hepatic copper toxicosis were included in this study: 12 ICC, one Austrian ETIC and five unrelated German infants with ICT. The clinical details of these 18 study patients have been described previously [61]. WD was excluded by sequencing all 21 exons of the ATP7B gene including the exon-intron boundaries [61].

Mutation analysis of COMMD2, COMMD8 and COMMD10

DNA from all the patients was isolated from peripheral blood as previously described by Miller et al [316]. Each exon, including the intron boundaries, of *COMMD2*, *COMMD8* and *COMMD10* was amplified by PCR using primers denoted in table 1. PCR fragments were generated using Amplitaq Gold (Applied Biosystems, Foster City, CA, USA) in a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA) according to the manufacturers instructions. Sequence reactions were performed on the PCR products using an ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The sequence products were purified using Sephadex G-50 superfine (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and the MAHV N45 purification plate (Millipore, Bedford, MA, USA). After purification, the samples were analyzed on an ABI Prism 3700DNA Analyzer (Applied Biosystems). The sequences obtained were compared to sequences deposited in the NCBI and Ensembl databases.

RESULTS

Several COMMD proteins interact with the copper transporter ATP7B

Recently, nine COMMD proteins were identified as structural and functional homologues of COMMD1 [85, 189]. To address the putative role of the entire COMMD protein family in copper metabolism, we tested whether COMMD proteins other than COMMD1 interact with ATP7B. All COMMD family members were expressed as GST fusion proteins in HEK293T cells together with ATP7B-Flag or empty expression vector as a negative control. Immunoblot analysis of glutathione precipitates revealed that ATP7B-Flag not only interacted with COMMD1-GST, but also with COMMD2-GST, COMMD8-GST and COMMD10-GST (Figure 1). Incubation of HEK293T cells with $CuSO_4$ or the copper chelator BCS did not affect the interaction between COMMD2-GST, COMMD8-GST or COMMD10-GST with ATP7B-Flag, indicating that these interactions or not dependent on cellular copper levels (data not shown).

COMMD2, COMMD8 and COMMD10 bind to ATP7B in a manner independent of COMMD1 and ATOX1

The interaction of COMMD1 with ATP7B is mediated through the copper binding amino-terminal tail of ATP7B [114, 182]. To determine if COMMD2, COMMD8 and COMMD10 bind to the same region of ATP7B, full length ATP7B or the amino-terminal tail of ATP7B (ATP7B(1-650)), were expressed as Flag epitope tagged proteins in HEK293T cells together with either GST or COMMD1, COMMD2, COMMD8 and COMMD10 as GST fusion proteins. COMMD1-GST abundantly interacted with both full length ATP7B-Flag and with ATP7B(1-650)-Flag, whereas COMMD2-GST, COMMD8-GST and COMMD10-GST only interacted with full length ATP7B-Flag, indicating that different regions of ATP7B are required for its interactions with different COMMD proteins (Figure 2).

Table 1. Primer pairs used to amplify each exon of COMMD2, COMMD8 and COMMD10 for mutation analysis

Amplified region	Sense primer (5′→ 3′)	Antisense primer (5′ → 3′)
COMMD2, exon 1-2	GTGTAACGCGTTAGTTCGTAG	CAGGGAATGGCTCGCAACC
COMMD2, exon 3	GAATGACAAGGAGCAGGAGC	CACAGGTACCTGCCAGGAC
COMMD2, exon 4	GAATGGTGGTCAGTGGTTAAG	ACCGCATCTTAGTTAAGGTGG
COMMD2, exon 5a[1]	GAAGTCTGTAATTCATCTGAAAC	ACTCTGATAGATGCGTGCCAC
COMMD2, exon 5b[1]	CATGAGGTCAGGAGATTGAGAC	CTGTGGCTATTAACACTGTGG
COMMD8, exon 1	GGCGAGTCGTAGCTTTAGAC	CTTCCTCCTCCCTTCCCTAG
COMMD8, exon 2	CTTAAGCTGGTCTTGCAGATAAC	GTTGTTACTGATGATAGCCCTTG
COMMD8, exon 3	GTGCAAAAGGAGAGTGGAATTG	TACTTATGCCCAAGTTAATTCAAG
COMMD8, exon 4	GTACTGCTAACTAACCTCTGATG	GGGTGACAGAGTGAGACTCC
COMMD8, exon 5a[1]	CAGCATCAGTCTTGTATAACCTG	GAAAGACATGGAATCTCCTGAG
COMMD8, exon 5b[1]	GCTTAAAGAATTGTGTGAATAGC	CGTATAACTACTGAGTTATTTGG
COMMD10, exon 1	CAC GAT TGG AGT GTC GGG AG	GCAGCAAGGACAGCCTTGTG
COMMD10, exon 2	GAAATGTACCCGTTGCCCTG	CACAGAACCAGTGTCTCTAAG
COMMD10, exon 3	CATGCACATCAGTTTCTTTTCAC	CATTCACAGCTTCCATCTTGC
COMMD10, exon 4	GAATCTTCCAGCCCTTGGTC	GCCTGACTCTACCTCTCAAC
COMMD10, exon 5	CCTGCTGAGTGGAGATTTGC	CCTTGCAAGATAAGCTATTTCTG
COMMD10, exon 6	ACTCAGAGGACAATCTTATGTC	GGAAGTTTAAAGAACTTAGTTCC
COMMD10, exon 7a[1, 2]	CACTTGATATGAGTTTCGTTCC	GTCATAGTGTTGCTTTCTTGCC
COMMD10, exon 7aa[1, 2]	GCTAGAGACTATACAAGCACAG	GGCAAGAAAGCAACACTATGAC
COMMD10, exon 7b		TCATGGCATAAAATAATCTATAATG

[1] The last exon of each gene was amplified and sequenced in two parts to ensure full coverage including the 3′ untranslated region.
[2] Due to a thymidine repeat, extra primer pairs were required to ensure that a reliable sequence was obtained from the first part of exon 7 of COMMD10.

Next, we assessed whether absence of COMMD1 would lead to impaired binding of COMMD2, COMMD8 and COMMD10 to ATP7B. HEK293T cells were stably transfected with plasmids encoding short hairpin RNAs (shRNAs) directed against COMMD1, resulting in efficient knockdown of endogenous COMMD1 (~10 % residual expression). COMMD1 knockdown did not prevent interaction of COMMD2-GST, COMMD8-GST or COMMD10-GST with ATP7B-Flag, indicating that these COMMD proteins interact with ATP7B independent of COMMD1 (Figure 4). As ATOX1 is essential for ATP7B function and copper-dependent trafficking, the effects of ATOX1 knockdown on COMMD-ATP7B interactions were also determined. An efficient knockdown of ATOX1 (~5% residual expression) did not affect the interactions between COMMD2-GST, COMMD8-GST, or COMMD10-GST with ATP7B-Flag. These data suggest that COMMD2, COMMD8 and COMMD10 bind to other regions of ATP7B than COMMD1, and do so independent of COMMD1 or ATOX1.

Sequence analysis of COMMD2, COMMD8 and COMMD10 in ICC, ETIC and ICT patients

The interactions demonstrated between COMMD2, COMMD8, and COMMD10 with the copper transporter ATP7B suggest that the genes encoding these COMMD proteins are good candidate genes for copper overload disorders with unknown etiology such as ICC, ETIC and ICT. Nucleotide sequences of the exons and flanking intronic sequences of *COMMD2*, *COMMD8* and *COMMD10* were determined in a cohort of 18 patients (12 ICC, 1 ETIC and 5 ICT patients). The results of the sequence analyses are summarized in table 2. Several sequence variations were detected in *COMMD2* and *COMMD10*, all previously described as single nucleotide polymorphisms (SNP) in the dbSNP and HapMap databases. Seven SNPs were detected in total, of which six were in coding sequences and one in a non-coding sequence. Two of the SNPs detected in *COMMD2* do not result in amino acid changes. These synonymous SNPs include rs11549572 coding for leucine at position 165, and rs7633238 coding for asparagine at position 189. The third SNP in *COMMD2* (rs9843784) results in a predicted amino acid change, coding for either isoleucine or leucine at position 137. Twelve of the 18 patients were homozygous for the isoleucine-coding variant of this SNP and six patients were heterozygous for this SNP, whereas none were found to be homozygous

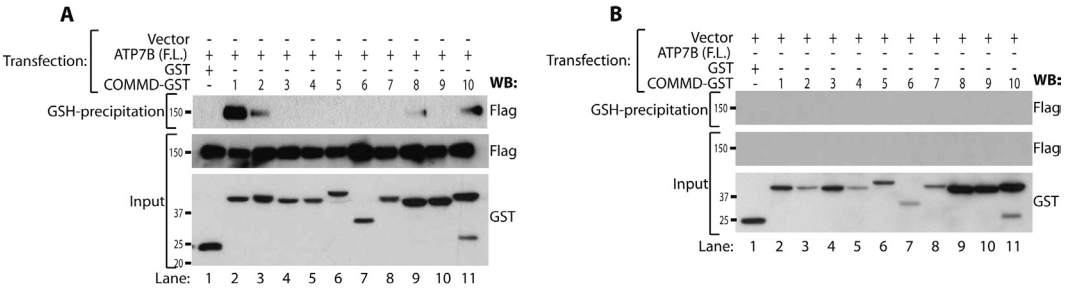


Figure 1. COMMD2, COMMD8 and COMMD10 interact with ATP7B

Glutathione-sepharose precipitation using cell lysates of HEK293T cells transfected with cDNA constructs encoding GST, or each of the COMMD proteins fused to GST together with ATP7B-Flag expression vector (A) or empty expression vector (B). Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

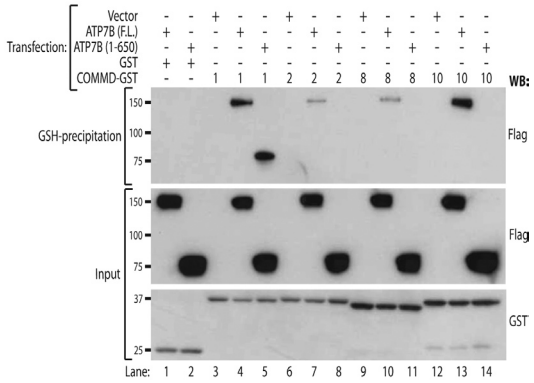


Figure 2. COMMD2, COMMD8 and COMMD10 bind to other regions of ATP7B than COMMD1
Glutathione-sepharose precipitation using cell lysates of HEK293T cells expressing full length ATP7B (ATP7B(F.L.)-Flag) or ATP7B(1-650)-Flag together with COMMD1, COMMD2, COMMD8 and COMMD10 as GST fusion proteins. GST expressing cells were used as negative control. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

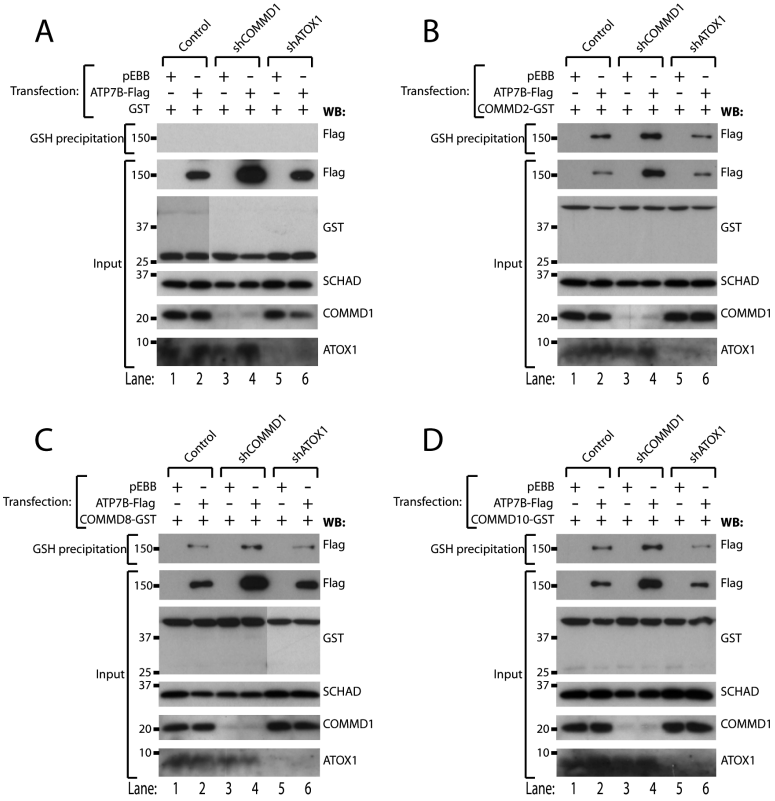


Figure 3. The interaction of COMMD2, COMMD8 and COMMD10 with ATP7B is independent of COMMD1 and ATOX1
HEK293T cells with a stable knockdown of COMMD1 (shCOMMD1) or ATOX1 (shATOX1) were transfected with cDNA constructs encoding ATP7B-Flag together with GST (A) or, COMMD2 (B), COMMD8 (C) and COMMD10 (D) as GST fusion proteins. HEK293T cells stably transfected with an empty shRNA vector was used a negative control (Control). Lysates were used for glutathione-sepharose precipitation. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Equal loading was confirmed by immunoblotting for SCHAD. Apparent molecular size markers are indicated in kDa on the left.

for the leucine-coding variant. In *COMMD10*, one non-coding SNP in intron 2 (rs10065554) was detected. Two synonymous coding SNPs in *COMMD10* were also detected: rs1129494 coding for threonine at position 121, and rs2048220 coding for threonine at position 145. One amino acid changing SNP was detected in *COMMD10* (rs1129495), encoding for either isoleucine or serine at position 137. Seventeen of 18 patients were homozygous for the isoleucine-coding variant, whereas one was heterozygous for this SNP.

DISCUSSION

COMMD1 is the prototype of the recently identified COMMD protein family, and plays a critical role in copper excretion, as can be deduced from the hepatic copper overload phenotype in Bedlington terriers with a *COMMD1* deletion [76, 85]. COMMD proteins appear to depend on protein-protein interactions for their functions (reviewed in [185]). COMMD1 interacts with the copper-binding amino terminal domain of the copper transport protein ATP7B [182]. Consistent with its role in the NF-κB and HIF-1 pathways [184, 186, 187], COMMD1 expression is associated with increased degradation of ATP7B, through which it potentially regulates cellular copper excretion [114]. In this study, COMMD2, COMMD8 and COMMD10 were identified as novel interacting partners of ATP7B. Other COMMD proteins did not associate with ATP7B, indicating that the interactions observed in this study are very specific. This is supported by the observation that COMMD2, COMMD8 and COMMD10 bind to ATP7B independently of COMMD1. As the deletion of COMMD1 detected in Bedlington terriers is sufficient for the development of hepatic copper accumulation, it seems unlikely that COMMD1, COMMD2, COMMD8 and COMMD10 have a completely redundant role in copper metabolism. This is also supported by our observation that of these four COMMD proteins only COMMD1 binds to the amino terminal region of ATP7B. Taken together, these data suggest that COMMD1, COMMD2, COMMD8, and COMMD10 play a role in regulating copper homeostasis, but that different mechanisms might be involved.

The identification of *COMMD1* as the gene causing copper toxicosis in Bedlington terriers initially led to the postulation of *COMMD1* as a candidate gene for human copper overload disorders [76]. However, no disease-causing mutations in *COMMD1* were detected in several cohorts of patients suffering from Wilson disease or copper overload disorders of unknown etiology [61, 78-81]. Based on the interactions of their protein products with ATP7B, we investigated *COMMD2*, *COMMD8* and *COMMD10* as candidate genes for copper overload disorders of unknown etiology. Sequencing of all exons including intron boundaries revealed no disease-causing mutations in *COMMD2*, *COMMD8* or *COMMD10*. However, replacement of untinned copper cooking utensils has reduced the amount of dietary copper intake leading to a virtual eradication of ICC and ETIC [5, 6, 317]. Therefore, it is likely that the underlying genetic causes are either mild mutations or common polymorphisms, rather than more severe mutations that would disrupt gene function completely. This hypothesis is supported by previous studies indicating that the disease pathophysiology of various other liver disorders associated with exposure to environmental factors are influenced by common gene polymorphisms [318]. One

Table 2. Results of sequence analysis of *COMMD2*, *COMMD8* and *COMMD10* in non-Wilsonian copper toxicosis patients.

Patient	Reference	Diagnosis	Age at onset	Origin	Mutation analysis								
					COMMD2			COMMD8	COMMD10				
					rs9843784 ORF 337[3] AA 113[4]	rs11549572 ORF 495 AA 165	rs7633238 ORF 567 AA 189		rs10065554 IVS2-30	rs129494 ORF 336 AA 112	rs129495 ORF 383 AA 128	rs2048220 ORF 408 AA 136
1	[327]	ICC	Infancy	India	T/G (Ile/Leu)	G/G (Leu)	G/G (Asn)	w.t.[2]	T/T (Intronic)	G/G (Thr)	T/T (Ile)	C/C (Thr)
2[1]	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/G (Asn)	w.t.	T/C (Intronic)	G/T (Thr)	T/T (Ile)	C/T (Thr)
3	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/G (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)
4	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/G (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)
5	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	G/G (Asn))	w.t.	T/C (Intronic)	G/T (Thr)	T/T (Ile)	C/T (Thr)
6	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/G (Asn)	w.t.	T/T (Intronic)	G/G (Thr)	T/T (Ile)	C/C (Thr)
7	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/A (Asn)	w.t.	T/C (Intronic)	G/T (Thr)	T/T (Ile)	C/T (Thr)
8	[327]	ICC	Infancy	India	T/G (Ile/Leu)	G/G (Leu)	G/G (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)
9	[327]	ICC	Infancy	India	T/T (Ile)	G/A (Leu)	A/G (Asn)	w.t.	T/T (Intronic)	G/G (Thr)	T/T (Ile)	C/C (Thr)
10[1]	[327]	ICC	Infancy	India	T/G (Ile/Leu)	G/G (Leu)	A/G (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)
11[1]	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	A/G (Asn)	w.t.	C/C (Intronic)	G/T (Thr)	T/T (Ile)	C/T (Thr)
12	[327]	ICC	Infancy	India	T/T (Ile)	G/G (Leu)	G/G (Asn)	w.t.	T/C (Intronic)	G/T (Thr)	T/G (Ile/Ser)	C/T (Thr)
13	[6]	ETIC	8 months	Austria/Tyrol	T/T (Ile)	G/G (Leu)	A/A (Asn)	w.t.	T/T (Intronic)	G/G (Thr)	T/T (Ile)	C/C (Thr)
14	[63]	ICT	17 months	Germany	T/G (Ile/Leu)	G/G (Leu)	G/G (Asn)	w.t.	T/C (Intronic)	G/T (Thr)	T/T (Ile)	C/T (Thr)
15	[63]	ICT	7 months	Germany	T/T (Ile)	G/G (Leu)	A/A (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)
16	[328]	ICT	9 months	Germany	T/G (Ile/Leu)	G/G (Leu)	A/G (Asn)	w.t.	T/T (Intronic)	G/G (Thr)	T/T (Ile)	C/C (Thr)
17	[328]	ICT	13 months	Germany	T/G (Ile/Leu)	G/G (Leu)	A/G (Asn)	w.t.	T/C (Intronic)	G/G (Thr)	T/T (Ile)	C/T (Thr)
18	[328]	ICT	10 months	Germany	T/T (Ile)	G/G (Leu)	G/G (Asn)	w.t.	C/C (Intronic)	T/T (Thr)	T/T (Ile)	T/T (Thr)

[1] patients born from consanguinous patients. [2] W.t. indicates no observed sequence variations. [3] ORF indicates the position of the sequence variation in the transcript starting from the A of the starting codon. [4] AA indicates the position of the amino acid encoded by the codon harboring the sequence variation.

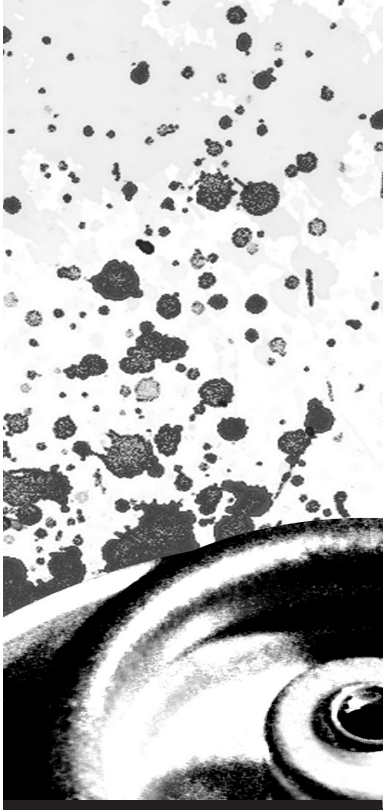
example of such a disorder is alcohol-induced liver disease. In addition to a direct relationship between alcohol intake and the degree of liver injury, there is a genetic predisposition for this disorder [319]. Common polymorphisms in the *Tumor necrosis factor* (*TNF*), *Interleukin-1*β (*Il-1*β), *Interleukin-10* (*Il-10*), *CD14 endotoxin receptor* (*CD14*), *Manganese superoxide dismutase* (*MnSOD*), and *Glutathione peroxidase-1* (*GPx1*) genes are associated with the severity of alcohol-induced liver disease [320-326]. It might therefore be possible that the SNPs detected in *COMMD2* and *COMMD10* in this study predispose patients for copper accumulation and contribute to copper overload disease development, but only in combination with an excessive copper intake. In this light, SNP rs9843784, which changes amino acid residue 113 from an isoleucine to a leucine in COMMD2, is of particular interest. Our observation that all patients carry at least one isoleucine coding allele suggests that this genotype is associated with disease development. However, statistical interpretation of the involvement of the SNPs detected in *COMMD2* and *COMMD10* in the pathogenesis of ICC, ETIC and ICT is hampered by the small sample size and the absence of appropriate control population frequencies for these SNPs, particularly with respect to environmental copper exposure.

As an additional implication from this study, the SNPs in *COMMD2* and *COMMD10* might have a modifying role in the clinical presentation of Wilson disease. Wilson disease is highly variable with respect to age of onset and presence or severity of hepatic and neurological symptoms, even in patients with the same mutations in *ATP7B* [46, 48]. In various other disorders with a Mendelian mode of inheritance modifier genes can modulate penetrance, dominance modification, expressivity and pleiotrophy [193]. This is well exemplified by genetic modification of the severity and expressivity of cystic fibrosis by polymorphisms in various genes, including *Mannose binding lectin* (*MBL*) and *Transforming growth factor* β (*TGF*-β) (reviewed in [194, 195]). *COMMD1* has indeed been proposed as a modifier gene for the clinical presentation of Wilson disease as heterozygosity for a silent missense mutation in *COMMD1* was possibly associated with an earlier onset of the disorder in patients with known *ATP7B* mutations [80]. To determine if polymorphisms in *COMMD2* and *COMMD10* have a modifying role in Wilson disease, SNP association studies should be performed in a cohort of Wilson disease patients with the same mutation in *ATP7B*, but with different phenotypes.

In conclusion, we have presented biochemical evidence that implicates several members of the COMMD family of proteins in the regulation of copper homeostasis. In addition, we have identified several genetic polymorphisms that are potentially associated with non-Wilsonian copper overload disorders. Further studies on the molecular functions of these proteins will provide valuable insight into human pathologies of copper homeostasis.

ACKNOWLEDGEMENTS

This work has been funded by the Dutch Digestive Diseases Foundation grant WS 02-34. We are indebted to Dr. Anand Pandit and Dr. Ashish Bavdekar (KME Hospital Pune, India) for providing the DNA samples of the ICC patients. We thank Eric Kalkhoven and Jackie Senior for critically reading the manuscript, and members of the Klomp and Wijmenga laboratories for helpful discussions.



CHAPTER 5

Several COMMD proteins interact with ATP7A, but do not regulate ATP7A-mediated copper excretion

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Submitted

ABSTRACT

The copper transporting P-type ATPases ATP7A and ATP7B play critical roles in copper homeostasis. Mutations in *ATP7A* cause Menkes disease, the copper deficiency disorder, while mutations in *ATP7B* result in Wilson disease, the copper overload disorder. COMMD1 has recently been identified as interacting partner of ATP7B. This interaction appears essential for the maintenance of copper homeostasis as evidenced by the copper overload phenotype in COMMD1-deficient Bedlington terriers. Recently, nine human homologues of COMMD1 have been identified, of which three interact with ATP7B implicating the family of COMMD proteins in the regulation of copper homeostasis. Here, we further address the role of the COMMD protein family in copper metabolism, by investigating the potential of these proteins to interact with ATP7A. Our results identify several COMMD proteins, including COMMD1 as novel interacting partners of ATP7A. Although COMMD1 did not appreciably regulate copper-induced trafficking of ATP7A, nor ATP7A-mediated copper excretion, these results suggest a broader role for COMMD1 in copper homeostasis than initially proposed.

INTRODUCTION

Copper is an essential trace metal required for a variety of biological processes, including mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, connective tissue formation, pigmentation, peptide amidation and iron metabolism [1]. Nevertheless, amounts exceeding the cellular needs of this metal are toxic, and therefore a tight homeostatic balance of cellular copper metabolism exists, in which the copper transporting ATPases ATP7A and ATP7B play crucial roles. Menkes disease (MD), caused by mutations in *ATP7A* [25, 26], is a severe X-linked disorder associated with copper deficiency. Clinical features of MD are a direct consequence of dysfunction of several copper-dependent enzymes (cuproenzymes), and mainly include severe mental retardation, neurodegeneration, growth retardation, hypothermia, laxity of skin and joints, hypopigmentation, and peculiar 'kinky or steely' hair [13-15]. At the other end of the spectrum of the copper dysbalance is Wilson disease (WD), a copper overload disorder caused by mutations in *ATP7B* [53-56]. Clinical features of WD result from toxic accumulation of copper primarily in the liver and the brain, and therefore include hepatic abnormalities (cirrhosis and chronic hepatitis, culminating in progressive liver failure), neurological defects (Parkinsonian features, seizures), and psychiatric symptoms (personality changes, depression, psychosis) [45, 46].

Although mutations in ATP7A and ATP7B result in opposing disorders in copper homeostasis, both proteins are highly homologous in structure and function. Both ATP7A and ATP7B facilitate cellular copper excretion, by virtue of their ability to transport copper across cellular membranes at the cost of ATP hydrolysis. Within their amino terminal regions, both proteins contain six copper binding sequences, through which they bind copper by a transient copper-dependent interaction with the copper chaperone ATOX1 [167, 179]. Under basal conditions, ATP7A and ATP7B reside in the *trans* Golgi network (TGN), where they facilitate the incorporation of copper into the lumen of the biosynthetic pathway for subsequent incorporation

in copper-dependent enzymes. When the copper concentration in the cell exceeds a certain threshold, both ATP7A and ATP7B reversibly translocate to the cell periphery [105, 142, 144]. A key difference in the expression and localization of these proteins forms the basis of the distinct functions of ATP7A and ATP7B. Consistent with its role in excretion of copper from the hepatocyte into the bile, ATP7B traffics to a dispersed vesicular compartment in the vicinity of the canalicular membrane [46, 142, 144, 147, 154]. In contrast, ATP7A is expressed on the basolateral membrane of different cell types including enterocytes, consistent with its role to translocate copper across the intestinal barrier, and facilitating uptake of copper from the diet [150-153].

Recently, COMMD1 was identified as interacting partner of ATP7B [114, 182]. Deficiency of COMMD1 causes copper toxicosis in Bedlington terriers [75, 76, 268], a canine disorder that shares pathophysiological features with WD [72]. These observations suggest that COMMD1 regulates hepatic copper excretion through its interaction with ATP7B. COMMD1 is the prototype member of the recently identified COMMD protein family, consisting of 10 proteins that are characterized by the presence of the COMM domain [85, 189]. All COMMD proteins bind to COMMD1, and have been implicated as regulators of the transcription factor NF-κB [85, 184]. Based on these structural and functional similarities among members of the COMMD protein family, we hypothesized that multiple COMMD proteins could play a role in copper homeostasis. Consistent with this hypothesis, we recently identified three COMMD proteins other than COMMD1 as interacting partners of ATP7B (P. de Bie et al., submitted). To further characterize the potential function of COMMD proteins in copper metabolism, we studied if COMMD proteins interact with ATP7A.

MATERIALS AND METHODS

Constructs

COMMD-GST constructs were described previously [85, 183, 309]. pCMB-ATP7A-MYC was kindly provided by Dr. M. Petris (Department of Biochemistry, University of Missouri, Columbia, MO, USA). Separate domains of ATP7A consisting of amino acid residues 1-654, 795-944, 1008-1368, or 1411-1500 were amplified by PCR using pCMB-ATP7A-MYC as template, and cloned in pCRII (Invitrogen, Breda, the Netherlands). Subsequently, these fragments were subcloned in pEBB-Flag to generate ATP7A(1-654)-Flag, ATP7A(795-944)-Flag, ATP7A(1008-1368)-Flag, and ATP7A(1411-1500)-Flag. To generate the pGL3-E1b-TATA-4MRE construct (MRE-luciferase reporter), four metal responsive elements (MRE) from the murine metallothionein Ia promotor were amplified by PCR from the 4xMRE(d) construct [329, 330], and subsequently subcloned into the E1b-TATA pGL3 vector (kindly provided by Dr. Eric Kalkhoven, University Medical Centre Utrecht, the Netherlands). All constructs were verified by sequence analysis.

Cell culture and transfections

Human embryonic kidney HEK293T, HeLa, and Phoenix packaging cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and L-glutamine and penicillin/streptomycin. In some experiments, cells were incubated with media supplemented with $CuCl_2$, $CuSO_4$, or bathocuproinedisulfonic acid (BCS) (Sigma) for the indicated time periods. Calcium phosphate precipitation was used to transfect HEK293T cells as previously described [307]. To produce amphotropic retrovirus supernatants, Phoenix packaging cells were transfected with empty pRETRO-SUPER, or pRETRO-SUPER constructs targeting COMMD1 using Fugene-6. On adding 4 μg/ml polybrene, HeLa cells were infected with the retrovirus supernatants and grown on media supplemented with 1 μg/ml puromycin (Sigma, Steinheim, Germany) to allow for monoclonal selection.

Immunoprecipitations, GST pull-down assays, and immunoblotting

Cells were lysed in lysis buffer (1% Triton X-100, 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol; pH 7.9) supplemented with 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (Roche) and 1 mM DTT. For copper or BCS treated cells, lysis buffer was also supplemented with 1mM $CuSO_4$ and 5mM DTT instead of 1mM DTT, or with 1mM BCS, respectively, as previously described [167]. Precipitations with GSH-sepharose were performed as previously described [183]. Protein detection was performed by immunoblotting using anti-Flag antibody (Sigma), anti-GST antibody (Santa Cruz, CA, USA), anti-ATP7A antibody (Santa Cruz), anti-short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) polyclonal antiserum or anti-COMMD1 polyclonal antiserum [275] as described previously [75].

Confocal immunofluoresence microscopy

For fluorescence microscopy, HeLa cells were seeded and grown on cover slips (Marienfeld, Bad Mergentheim, Germany). Fixation was performed with 4% paraformaldehyde as previously described [75]. Subsequently, cells were incubated with antibodies to ATP7A (kindly provided by Dr. A. Monaco, Wellcome Trust Centre for Human Genetics, University of Oxford, UK). Antibodies were subsequently visualized by incubation with Alexa Fluor 488-conjugated donkey anti-rabbit-IgG (H+L) (Invitrogen). The cover slips were mounted with FluorSave reagent (Calbiochem), and images were obtained utilizing a Zeiss Axiovert 100M confocal microscope equipped with a Zeiss LSM 510 Meta spectrometer.

MRE-Luciferase Reporter Assay

For Luciferase reporter assays HEK 293T cells were transfected with MRE-Luciferase reporter, and the metal-insensitive Renilla luciferase encoding pRL-TK Vector (Promega Benelux BV, Leiden, the Netherlands) to correct for differences in transfection efficiencies. COMMD1-Flag and ATP7A-MYC constructs were co-transfected as indicated in the figure legends. After overnight transfection, cells were rinsed and incubated for 24 hours in medium containing $CuCl_2$ as indicated per

experiment. Cells were harvested in passive lysisbuffer according to the manufacturer's protocol (Promega) and assayed by luminometry (Berthold Technologies, Vilvoorde, Belgium) for Firefly luciferase activity and Renilla luciferase activity according to the manufacturer's protocol (Promega; Dual-Luciferase Reporter Assay System). The relative light units (RLU) were calculated by dividing Firefly luciferase by Renilla luciferase values.

RESULTS

Several COMMD proteins interact with COMMD1

Previously, we have identified COMMD1, COMMD2, COMMD8 and COMMD10 as interacting partners of ATP7B [182] (P. de Bie et al., submitted). To further address the role of the COMMD protein family in copper metabolism, we tested whether COMMD proteins interact with ATP7A. All COMMD family members were expressed as GST fusion proteins in HEK293T cells together with ATP7A-MYC. ATOX1-GST was used as a positive control. Strikingly, whereas previous studies have failed to demonstrate an interaction between COMMD1 and ATP7A [182], immunoblot analysis of glutathione precipitates revealed that ATP7A-MYC interacts with COMMD1-GST. In addition, ATP7A-MYC was detected in complex with COMMD2-, COMMD3-, COMMD4-, COMMD5-, COMMD8- and COMMD10-GST fusion proteins (Figure 1).

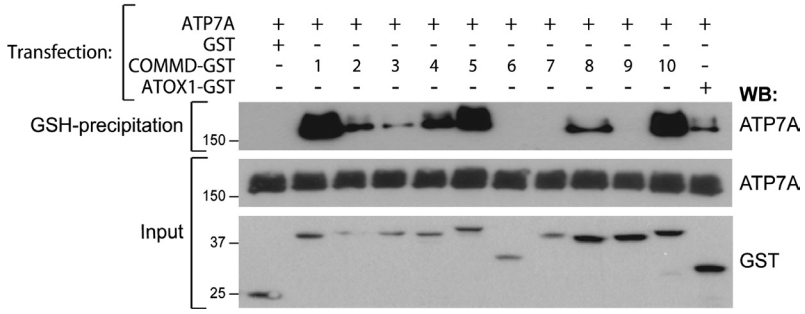

Figure 1. Interaction of several COMMD proteins with ATP7A
Glutathione-sepharose precipitation using cell lysates of HEK293T cells transfected with cDNA constructs encoding GST, or each of the COMMD proteins fused to GST together with ATP7A-MYC expression vector. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

ATP7A interacts with COMMD1 through its amino-terminus in a manner independent of copper

ATP7A consists of several distinct functional domains (Figure 2A). These domains are required for copper binding, ATP binding, phosphorylation, de-phosphorylation, and localization of ATP7A, respectively. To characterize the potential function of the interaction of COMMD1 with ATP7A, we investigated which of these domains mediate this interaction. Separate truncated fragments of ATP7A were expressed as Flag epitope tagged proteins in HEK293T cells together with either GST or a COMMD1-GST fusion protein. Expression of the region between transmembrane domains six and seven (ATP7A(1008-1368)-Flag), and the carboxy terminal tail of ATP7A (ATP7A(1411-1500)-Flag) was observed upon long exposure (data not shown). Analysis of glutathione sepharose

precipitates from lysates of these cells showed a specific interaction of both the amino terminal region of ATP7A-Flag (ATP7A(1-654)-Flag) and the region between transmembrane domains five and six of ATP7A-Flag (ATP7A(1008-1368)-Flag) with COMMD1-GST, indicating that either of these regions are sufficient for the interaction of ATP7A with COMMD1 (Figure 2B). The amino terminus of ATP7A harbors six metal-binding sequences known to bind copper. This region is essential for several functional aspects of ATP7A, including copper-induced trafficking of ATP7A towards the cell periphery and ATP7A-mediated copper transport activity. In addition, the copper chaperone ATOX1 interacts with the amino-terminal region of ATP7A in a copper-dependent manner [168, 174]. We therefore assessed if the interaction of COMMD1 with ATP7A is sensitive to copper. HEK293T cells expressing ATP7A-MYC together with GST, COMMD1-GST or ATOX1-GST were grown in media supplemented with $CuSO_4$, or the copper chelator bathocuproinedisulfonic acid (BCS). Whereas BCS incubation efficiently prevented interaction of ATP7A-MYC with ATOX1-GST (Figure 2C), no reproducible effects of either $CuSO_4$ or BCS incubation on the interaction of COMMD1-GST with ATP7A-MYC were observed (Figure 2C). These data suggest that COMMD1 binds to the amino terminus of ATP7A, independently of cellular copper levels.

COMMD1 does not regulate copper-induced trafficking of ATP7A

A key mechanism underlying copper transport mediated by both ATP7A and ATP7B consists of copper-induced trafficking of these proteins. Under copper limited conditions, both ATP7A and ATP7B reside in the TGN, but in conditions of copper excess, both proteins relocate to a dispersed vesicular compartment in the periphery of the cell. In addition, ATP7A is also expressed at the cell membrane upon copper overload. This process is highly dependent on the six metal-binding sequences in the amino terminal region of ATP7A, and the nucleotide binding and phosphorylation domains within the region between transmembrane domains six and seven of ATP7A. As COMMD1 interacts with both these regions, we hypothesized that COMMD1 has a regulatory role in copper-dependent trafficking of ATP7A. To address this hypothesis, the localization of ATP7A in response to copper was determined in COMMD1-deficient and control HeLa cells. Incubation with BCS resulted in localization of ATP7A in dense perinuclear region consistent with its previous reported localization within the TGN. Treatment with 200 μM $CuSO_4$ for 2 hours resulted in relocalization of ATP7A towards the cell periphery and plasma membrane (Figure 3). This relocalization was not impaired in COMMD1-deficient cells, also not when cells were treated with 200 μM $CuSO_4$ for different time intervals, or with different concentrations of $CuSO_4$ (data not shown). Similarly, knockdown of COMMD1 did not interfere with the copper-dependent relocalization of ATP7B [114]. Taken together, these data suggest that COMMD1 does not have an essential regulatory role in the copper-induced relocalization of the copper transporting ATPases ATP7A and ATP7B.

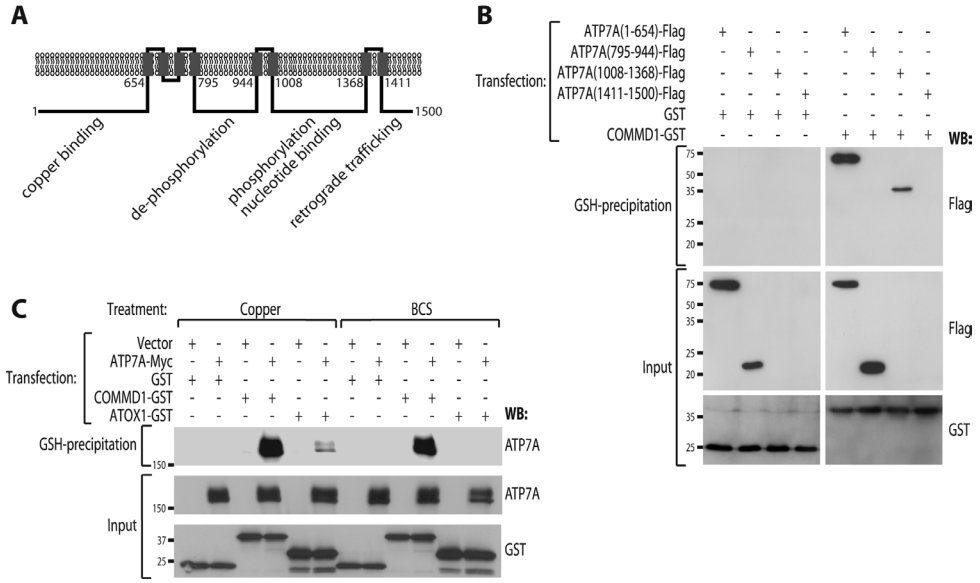

Figure 2. COMMD1 interacts with the amino terminus of ATP7B in a copper independent manner
A. Schematic representation of the distribution of distinct functional domains in ATP7A. The amino terminal tail of ATP7A consisting of amino acid residues 1-654 contains six repetitive copper binding domains. The region between transmembrane domains four and five (amino acid residues 795-944) harbors the actuator domain, involved in de-phosphorylation of ATP7A. The region between transmembrane domains six and seven (amino acid residues 1008-1368) is required for ATP binding and phosphorylation of ATP7A. The carboxy terminal tail contains a double leucine motif that is essential for retrograde trafficking of ATP7A from the cell periphery to the TGN. B. Glutathione-sepharose precipitation using cell lysates of HEK293T cells expressing ATP7A(1-654)-Flag, ATP7A(795-944)-Flag, ATP7A(1008-1368)-Flag, or ATP7A(1411-1500)-Flag together with GST or COMMD1-GST. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left. C. HEK293T cells were transfected with cDNA constructs encoding full length ATP7A-MYC, GST, COMMD1-GST or ATOX1-GST as indicated. Cells were treated overnight with either 200 μM $CuCl_2$ or BCS (bathocuproinedisulfonic acid). Lysates were made in lysis buffer supplemented with $CuCl_2$ or BCS and used for glutathione-sepharose precipitation. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. Apparent molecular size markers are indicated in kDa on the left.

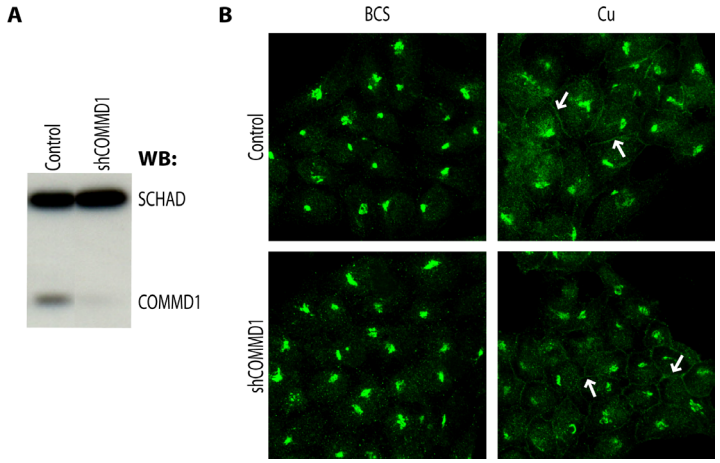

Figure 3. COMMD1 does not regulate copper-induced trafficking of ATP7A
A. Western blot analysis of COMMD1 expression in cell lysates of HeLa cells after a stable knockdown of COMMD1 (shCOMMD1). HeLa cells stably transfected with an empty shRNA vector were used as a negative control (Control). SCHAD was probed as loading control. Apparent molecular size markers are indicated in kDa on the left. B. HeLa control or shCOMMD1 cells were incubated with 50 μM BCS or 200 μM $CuSO_4$. Cells were subsequently analyzed by indirect confocal immunofluorescence using antibodies against ATP7A visualized by Alexa488 conjugated donkey anti-rabbit IgG. Arrows indicate staining consistent with a plasma membrane localization.

ATP7A prevents cytoplasmic copper accumulation independent of COMMD1

Recently, a novel transcription-based intracellular copper sensor was developed in our laboratory. This sensor consists of four metal response elements (MRE) from the murine metallothionein Ia promotor cloned upstream of the Firefly luciferase open reading frame. This MRE-luciferase reporter construct is transactivated in a copper-dependent manner by the metal responsive transcription factor-1 (MTF-1) and can thus be employed to specifically detect alterations in cellular copper levels without interfering with the endogenous mechanisms of copper homeostasis [102]. Activation of the sensor is caused by elevated cytosolic copper concentrations bioavailable for the activation of MTF-1. This is illustrated in Figure 4A; incubation of HEK293T cells expressing the MRE-luciferase reporter with increasing concentrations of copper resulted in an almost linear increase of reporter activity. At 150 μM copper chloride the reporter was induced almost 7-fold (Fig. 4A), but at even higher copper concentrations reporter activation was saturated (data not shown). To assess if this copper sensor can be employed to measure cytoplasmic copper export mediated by ATP7A, HEK293T cells were transfected with the MRE-luciferase reporter in combination with an ATP7A-MYC encoding construct. Consistent with its function to export copper from the cell, overexpression of ATP7A-MYC dramatically inhibited the copper-induced transactivation of the MRE-luciferase reporter (Figure 4A). Next, we assessed if COMMD1 affects the inhibition of the copper-induced transactivation of the MRE-luciferase reporter by ATP7A. COMMD1-Flag and MRE-luciferase reporter were expressed in increasing amounts in the absence or presence of ATP7A-MYC. Cells were subsequently treated with 100 μM copper chloride. In the absence of exogenous ATP7A-MYC, COMMD1-Flag expression resulted in a slight, but not significant, reduction of copper-induced MRE-luciferase transactivation. These results indicate that under these experimental conditions, overexpression of COMMD1 did not significantly reduce cytoplasmic copper content. COMMD1-Flag expression did also not affect ATP7A-MYC mediated inhibition of the copper-induced transactivation of the MRE-luciferase reporter (Figure 4B). These results suggest that COMMD1 does not regulate ATP7A mediated copper transport.

DISCUSSION

Protein-protein interactions form an essential mechanism through which many proteins exert their functions. Mapping of the protein-protein interactome provides a valuable framework to elucidate the functional organization of the human proteome, and consequentially to understand the molecular pathology of human disease [162]. This is well illustrated by the recent identification of a number of interacting partners of ATP7A and/or ATP7B, including COMMD1, which has provided valuable insights into the molecular pathogenesis of copper homeostasis disorders [114, 182, 255-258] (P. de Bie et al., submitted). The interaction between COMMD1 and ATP7A suggests that the function of COMMD1 in copper homeostasis is not limited to the hepatocyte as initially proposed based on the hepatic copper overload phenotype in COMMD1-deficient Bedlington terriers. However, using the MRE-luciferase reporter no clear effects of COMMD1 overexpression

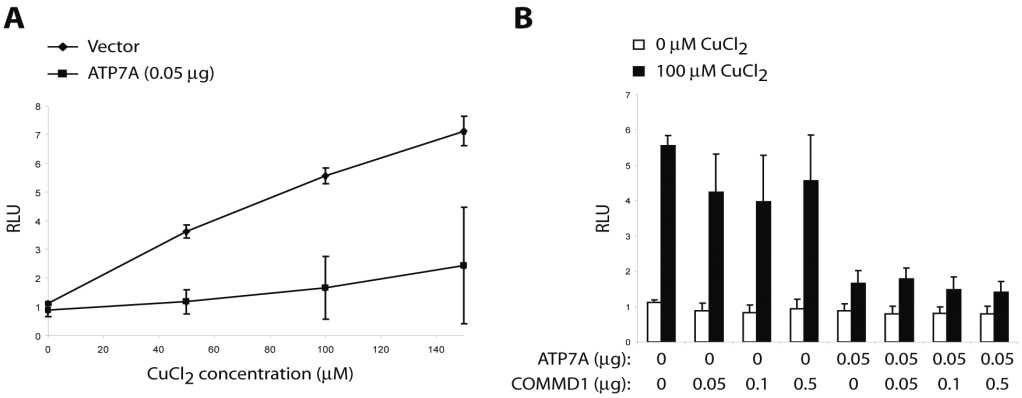

Figure 4. COMMD1 does not regulate copper ATP7A-mediated copper export
A. HEK 293T cells were transfected with MRE-Luciferase reporter in combination with two different amounts of ATP7A-MYC expression vector or empty vector as negative control. One day after transfection, cells were incubated for 24 hours with increasing amounts of $CuCl_2$. MRE-Luciferase reporter activities were measured and relative light units (RLU) were calculated after normalization for Renilla Luciferase activity. **B.** HEK 293T cells were transfected with MRE-Luciferase reporter in combination with ATP7A-MYC expression vector or empty vector, in combination with increasing amounts of COMMD1-Flag expression vector. One day after transfection, cells were incubated for 24 hours with or without 100 μM $CuCl_2$. MRE-Luciferase reporter activities were measured and relative light units (RLU) were calculated after normalization for Renilla Luciferase activity.

on ATP7A-mediated copper export could be demonstrated. In addition, expression of COMMD1 did not affect copper-induced relocalization of ATP7A. Taken together, these data suggest that COMMD1 does not directly regulate copper transport by ATP7A. Recent studies have implicated COMMD1 in a number of cellular processes including copper- and sodium-transport, and the NF-κB and HIF-1 signaling pathways, indicating that COMMD1 has a pleiotropic function. It has been proposed that COMMD1 acts as a negative regulator of the stability of a number of proteins in the NF-κB and HIF1 pathways [184, 186, 187]. In addition, we have observed that overexpression of COMMD1 resulted in an increase in proteolytic turnover of newly synthesized ATP7B [114]. Further studies are currently being undertaken to investigate if COMMD1 and other COMMD proteins have a modulatory role in the stability of ATP7A.

The deletion in *COMMD1* underlying the copper toxicosis phenotype in Bedlington terriers results in complete absence of COMMD1 [75]. Therefore loss of interaction between ATP7B and COMMD1 in affected Bedlington terriers could lead to progressive hepatic accumulation of copper. One of the most challenging questions arising from the present study is how the interaction between COMMD1 and ATP7A would relate to the phenotype observed in Bedlington terriers with a COMMD1 mutation. ATP7A mediates transfer of copper across the mucosal barrier, thereby facilitating uptake of copper from the diet. The copper overload phenotype in COMMD1-deficient Bedlington terriers appears inconsistent with a putative role for COMMD1 in the ATP7A-mediated dietary copper uptake pathway. This was supported by the observation that intestinal absorption of copper from the diet is normal in COMMD1-deficient Bedlington terriers [331]. The inconsistency between a role for COMMD1 in the copper uptake pathway and the copper toxicosis phenotype can possibly be explained in two ways. First, high copper intake through modern commercial dog food over the years has resulted in hepatic copper concentrations in healthy

dogs that is over 10-fold higher than in humans [331]. This might predispose dogs to develop copper toxicosis, as evidenced by the occurrence of copper associated liver disease in a variety of dog breeds other than Bedlington terriers [64, 332]. However, such high copper exposure might also bypass impairments in copper uptake across the mucosal barrier. This possibility is supported by the observation that the function of some Menkes disease-causing mutations in ATP7A can be corrected *in vitro* by incubation with high copper concentrations [111]. A second explanation for the phenotypic inconsistency consists of functional redundancy by other COMMD proteins. Our previous identification of COMMD2, COMMD8 and COMMD10 as interacting partners of ATP7B (P. de Bie et al., submitted), taken together with the data described in this study indicating that COMMD1, COMMD2, COMMD3, COMMD4, COMMD5, COMMD8 and COMMD10 interact with ATP7A, suggests that the COMMD protein family may have redundant functions in relation to ATP7A. Further studies addressing the functional similarities and differences of COMMD proteins in copper transport pathways need to be undertaken to assess the possibility of functional redundancy. Unfortunately, given the ubiquitous expression of all COMMD family members, such studies will be technically challenging [85, 189].

Taken together, our data further implicate COMMD proteins as a family of putative regulators of copper homeostasis. Further research is required to fully understand the role of these proteins in the ATP7A- and ATP7B-mediated copper excretion pathways, but will yield increased understanding of the molecular mechanisms behind the pathogenesis of disorders of copper homeostasis.

ACKNOWLEDGEMENTS

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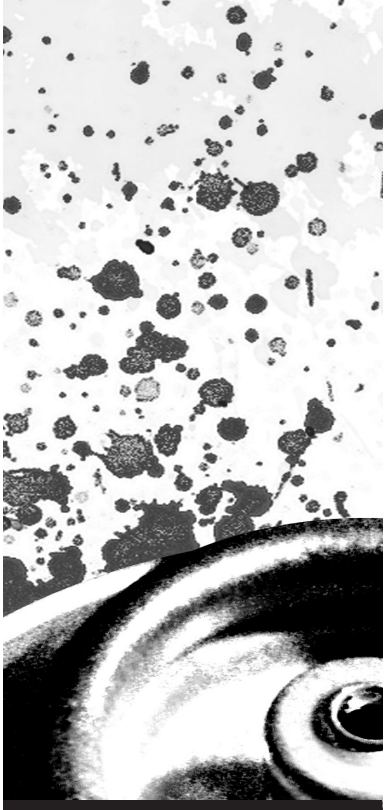

CHAPTER 6

Distinct Wilson-disease mutations in ATP7B are associated with enhanced binding to COMMD1 and reduced stability of ATP7B

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ABSTRACT

Background/Aims: Wilson disease is characterized by hepatic copper overload and caused by mutations in the gene encoding the copper transporting P-type ATPase ATP7B. ATP7B interacts with COMMD1, a protein that is deleted in Bedlington terriers with hereditary copper toxicosis. Here we characterized the implications of the interaction between COMMD1 and ATP7B in relation to the pathogenesis of Wilson disease. *Methods:* GST pull-down experiments, co-immunoprecipitations, immunofluoresensce microscopy, site-directed mutagenesis and biosynthetic labeling experiments were performed to characterize the interaction between COMMD1 and ATP7B and the effects of Wilson disease-causing mutations. *Results*: COMMD1 specifically interacted with the amino-terminal region of ATP7B. This interaction was independent of intracellular copper levels and of the expression of the copper chaperone ATOX1. Four Wilson disease patient derived mutations in this region of ATP7B significantly increased its binding to COMMD1. Two of these mutations also resulted in mislocalization and increased degradation rate of ATP7B. Although COMMD1 did not affect copper-induced trafficking of ATP7B, it markedly decreased the stability of newly synthesized ATP7B. *Conclusions*: Our data implicate COMMD1 in the pathogenesis of Wilson disease and indicate that COMMD1 exerts its regulatory role in copper homeostasis through the regulation of ATP7B stability.

INTRODUCTION

The liver is essential for the maintenance of copper homeostasis as this organ plays a central role in the excretion of copper [230]. Disorders associated with an overload of this essential yet toxic metal are therefore usually characterized by extensive liver damage, as can be observed in the autosomal recessive disorder Wilson disease (WD). The clinical presentation of WD is highly heterogeneous, and usually includes hepatic and/or neurological abnormalities due to toxic accumulation of copper in the liver and the brain [46]. WD is caused by mutations in the *ATP7B* gene, which encodes a copper transporting P-type ATPase [53-55, 59]. ATP7B plays a key role in hepatic copper excretion, by virtue of its ability to transport copper across cellular membranes at the cost of ATP hydrolysis. Within its amino terminal region, ATP7B contains six copper binding sequences, through which it receives copper by a transient copper-dependent interaction with the copper chaperone ATOX1[167, 179]. Under basal conditions, ATP7B resides in the *trans* Golgi network (TGN), where it facilitates the incorporation of copper in cuproenzymes such as ceruloplasmin. When the copper concentration in the cell exceeds a certain threshold, ATP7B translocates to a dispersed vesicular compartment in the periphery of the cell, an event that is generally believed to precede cellular excretion of copper [105].

Recently, COMMD1 was implicated as a novel regulator of hepatic copper excretion as a deficiency of this protein causes copper toxicosis in Bedlington terriers [75, 76], a canine disorder that shares many pathophysiological features with WD [64]. Subsequent identification of COMMD1 as an interacting partner of ATP7B further supports the role of COMMD1 in copper homeostasis [182], and suggests that these two proteins cooperate to facilitate excretion of

copper from the hepatocyte into the bile canaliculus. Although COMMD1 has been postulated as a candidate gene for non-Wilsonian copper overload disorders, and as a modifier gene for the clinical presentation of WD, no disease associated mutations in *COMMD1* have been detected in patients with these disorders, but a silent missense mutation in *COMMD1* was possibly associated with an earlier onset of the disorder in patients with known *ATP7B* mutations [61, 78-81]. Recent studies have revealed that COMMD1 also functions in a variety of other cellular processes [185]. Analysis of a COMMD1 knockout mouse implicated COMMD1 as a negative regulator of hypoxia-inducible factor 1 (HIF-1) [187]. In addition, inhibitory functions for COMMD1 in sodium transport and NF-κB signaling have been identified [85, 184, 188]. The role of COMMD1 in the HIF-1 and NF-κB pathways appear to be mediated through proteolytic regulation of key components of these pathways [184, 186, 187]. Nevertheless, the function of COMMD1 within the regulation of copper homeostasis still remains elusive. To dissect the molecular function of COMMD1 in copper homeostasis, we here further characterized the interaction between COMMD1 and ATP7B and the role of this interaction in the pathogenesis of WD.

MATERIALS AND METHODS

Constructs

COMMD1-GST, COMMD1-HA and IκBα-HA constructs were described previously [85, 183, 309]. pEBB-ATP7B-Flag was generated by subcloning an ATP7B coding cDNA fragment from pEGFP-ATP7B (kindly provided by Dr. H. Roelofsen, University Medical Center Groningen, the Netherlands) into pEBB-Flag. cDNA sequence encoding the first 650 amino acids of ATP7B was amplified with PFU turbo (Stratagene, La Jolla, CA, USA) and subcloned in pEBB-Flag to obtain pEBB-ATP7B(1-650)-Flag. Mutations in pEBB-ATP7B(1-650)-Flag and in pEBB-ATP7B-Flag were introduced using the Quickchange site-directed mutagenesis method (Stratagene). Plasmids encoding short hairpin RNAs (shRNAs) were generated by cloning target sequences against *COMMD1* (GTCTATTGCGTCTGCAGAC) or *ATOX1* (GGTCTGCATTGAATCTGAC) in pRETRO-SUPER as previously described [187].

Cell culture and transfections

HEK293T and HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. In some experiments, cells were incubated with media supplemented with 200 μM $CuSO_4$, 50 μM bathocuproinedisulfonic acid (BCS) (Sigma), 15 μg/ml cycloheximide (Sigma), 3 μM MG132 (Calbiochem, Darmstadt, Germany), or 5 μM lactacystin (Sigma) for the indicated time periods. Standard calcium phosphate precipitation was used to transfect HEK293T cells. HepG2 cells were transfected using Fugene-6 (Roche, Basle, Switzerland). The generation of stable HEK 293T COMMD1 and ATOX1 knockdown cells has been described elsewhere [187].

Biosynthetic labeling of cell lines

After overnight transfection of subconfluent cultures, HEK293T cells were incubated in serum-free medium containing no methionine and cysteine for 30 min, and subsequently metabolically labeled with 50 µCi/ml redivue pro-mix L-[^{35}S]-methionine and L-[^{35}S]-cysteine (GE Healthcare, Diegem, Belgium) for 90 min at 37 °C. Following metabolic labeling, cells were chased with complete medium for different time periods as indicated in each experiment.

Immunoprecipitations, GST pull-down assays, and immunoblotting

Cells were lysed in lysis buffer (1% Triton X-100, 25 mM HEPES; pH 7.9, 100 mM NaCl, 1 mM EDTA, 10% glycerol) supplemented with 1 mM Na_3VO_4, 1 mM PMSF, protease inhibitors (Roche) and 1 mM DTT. For copper or BCS treated cells, lysis buffer was also supplemented with 1mM $CuCl_2$ and 5mM DTT instead of 1mM DTT, or with 1mM BCS, respectively, as previously described [167]. Precipitations with GSH-sepharose, or immunoprecipitations with anti-HA (Sigma), were performed as previously described [183]. Protein detection was performed by immunoblotting for Flag (Sigma), HA, GST (Santa Cruz, Santa Cruz, CA, USA), short-chain L-3-hydroxyacyl-CoA dehydrogenase (SCHAD) [75], COMMD1 [75] or ATOX1 [167] as described previously [75]. To quantify immunoprecipitated proteins, relative densities of protein bands were measured with a Bio-Rad GS-700 imaging densitometer and analyzed with Quantity One software (Bio-Rad, Hercules, CA, USA). Biosynthetically labeled cells were lysed in Ripa lysis buffer (20 mM Tris/HCl, pH 7.4, 0.5% Triton X-100, 150 mM NaCl, 10 mM sodium EDTA, and 0.5% sodium deoxycholic acid), supplemented with 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitors. Cell lysates were subjected to immunoprecipitations using anti-Flag affinity gel (Sigma) after addition of 1.5 volumes buffer A (1% SDS, 1% Triton X-100, 0.5% sodium deoxycholic acid, 0.5% BSA and 1 mM PMSF, in PBS). Immunoprecipitates were washed with buffer A prior to SDS PAGE. Gels were soaked in Amplify (Amersham Pharmacia Biotech) prior to fluorographic exposure.

Confocal immunofluoresence microscopy

For fluorescence microscopy, cells were seeded and grown on cover slips (Marienfeld, Bad Mergentheim, Germany). Cells were fixed with 4% paraformaldehyde, and subsequently incubated with antibodies to ATP7B (kindly provided by Dr. S Lutsenko, Oregon Health & Science University, Portland, OR, USA), COMMD1 (Abnova, Taipei City, Taiwan), p230 (BD Biosciences, Alphen aan den Rijn, the Netherlands) or Calreticulin (Alexis Biochemicals, Lausen, Switzerland). Antibodies were visualized by incubation with Alexa Fluor 488- or Alexa Fluor 568-conjugated donkey anti-mouse, anti-rabbit, or anti-rat IgG (H+L) (Invitrogen, Breda, the Netherlands). Visualization of wild-type or mutant ATP7B-Flag in HepG2 cells was performed by incubation with FITC-conjugated anti-Flag antibodies (Sigma). The cover slips were mounted with FluorSave reagent (Calbiochem), and images were obtained utilizing a Zeiss Axiovert 100M confocal microscope equipped with a Zeiss LSM 510 Meta spectrometer.

RESULTS

COMMD1 and ATP7B are localized in overlapping regions in HEK293T cells

To study the interaction of COMMD1 with ATP7B, ATP7B-Flag was expressed in HEK293T or HepG2 cells together with either GST or a COMMD1-GST fusion protein. Analysis of glutathione sepharose precipitates from lysates of these cells confirmed a specific interaction of ATP7B-Flag with COMMD1-GST in both cell lines (Figures 1A-B). To determine if both proteins are localized in overlapping regions in the cell, double-label immunofluorescence microscopy experiments were performed in HEK293T cells. ATP7B displayed a dense perinuclear distribution, consistent with a localization in the TGN (Figure 1C and F). COMMD1 was localized in cytoplasmic vesicular compartments that were particularly profound in the perinuclear region (Figure 1D). The observed localization of both ATP7B and COMMD1 was consistent with previous reports [75, 105]. Overlay of the images (Figure 1E) revealed that ATP7B and COMMD1 localization partially overlaps, supporting an endogenous interaction between these proteins. Immunoreactivity for COMMD1 was completely absent in HEK293T cells that were stably transduced with plasmids encoding short hairpin RNAs (shRNAs) directed against COMMD1 (Figure 1G), confirming the specificity of the antibody used to detect COMMD1.

COMMD1 interacts with the copper-binding amino terminal region of ATP7B independent of ATOX1 and copper concentration

Next, full length ATP7B-Flag or a truncated variant of ATP7B consisting of the amino-terminal 650 amino acids of the full length protein (ATP7B(1-650)-Flag), were expressed in HEK293T cells together with GST or COMMD1-GST. Analysis of glutathione-sepharose precipitates from lysates of these cells (Figure 2A) indicated that the amino terminal region of ATP7B is sufficient for interaction with COMMD1, as shown previously [182]. Within its amino terminal region, ATP7B contains six copper binding sequences, through which it receives copper by a transient copper-dependent interaction with the copper chaperone ATOX1 [167, 179]. Whereas incubation with the copper chelator BCS efficiently prevented interaction of ATP7B-Flag and ATOX1-GST (Figure 2B, compare lanes 6 and 12), no reproducible effects of $CuSO_4$ or BCS incubation on the interaction of COMMD1-GST with ATP7B-Flag were observed (Figure 2B, compare lanes 4 and 10). These data suggest that COMMD1 binds to the amino terminus of ATP7B, independently of cellular copper levels. To determine if ATOX1 is essential for the interaction of COMMD1 with ATP7B, HEK293T cells were stably transfected with plasmids encoding short hairpin RNAs (shRNAs) directed against *ATOX1*, resulting in an efficient knockdown of ATOX1 (4% residual expression compared to control cells, figure 2C). This knockdown of ATOX1 did not affect the interaction between COMMD1-GST and ATP7B-Flag (Figure 2D). In the reciprocal experiment, efficient knockdown of endogenous COMMD1 (12% residual expression compared to control cells, figure 2C) did not result in impairment of the interaction between ATOX1-GST and ATP7B-Flag (Figure 2D). These data indicate that COMMD1 and ATOX1 bind to the same region of ATP7B, but they do so independent of each other.

COMMD1 does not regulate copper-induced trafficking of ATP7B

To assess if COMMD1 has a regulatory role in copper-dependent trafficking of ATP7B, the localization of ATP7B in response to copper was determined in COMMD1-deficient and control HEK293T cells. Incubation with BCS resulted in localization of ATP7B in dense perinuclear regions overlapping with the TGN marker p230 in both cell lines (Figure 3A-C and G-I). Treatment with 200

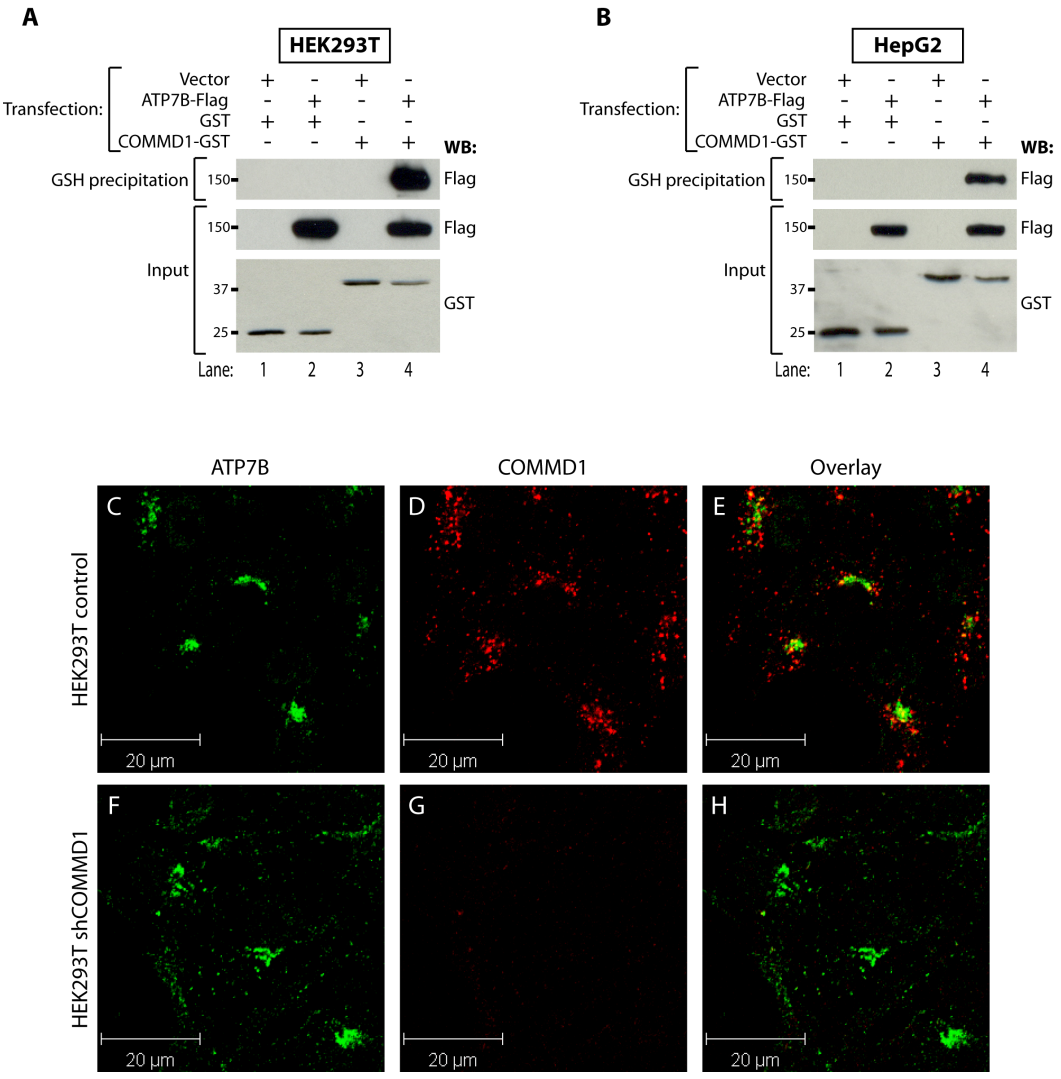


Figure 1. COMMD1 and ATP7B interact and are localized in overlapping regions in the cells
A+B. Glutathione-sepharose precipitation using cell lysates of HEK293T (A), or HepG2 (B) cells expressing ATP7B-Flag together with GST or COMMD1-GST. Precipitates were washed and separated by SDS-PAGE and immunoblotted as indicated. Input indicates direct analysis of cell lysates. C-H. HEK293T cells stably transfected with an empty shRNA vector (control), or HEK293T cells with a stable knockdown of COMMD1 (shCOMMD1) were analyzed by double-label indirect confocal immunofluorescene using antibodies against ATP7B and COMMD1, visualized by Alexa488 conjugated donkey anti-rat and Alexa568 conjugated donkey anti-mouse IgG, respectively. ATP7B localization is presented in images C and F and COMMD1 localization is presented in images D and G. Overlap in staining is depicted in yellow in the overlay images (E and H).

μM $CuSO_4$ for 1 hour resulted in relocalization of ATP7B towards the cell periphery, as appreciated by loss of overlap between ATP7B and p230 staining (Figure 3 D-F). This relocalization was not impaired in COMMD1-deficient cells, also not when cells were treated with 200 μM $CuSO_4$ for

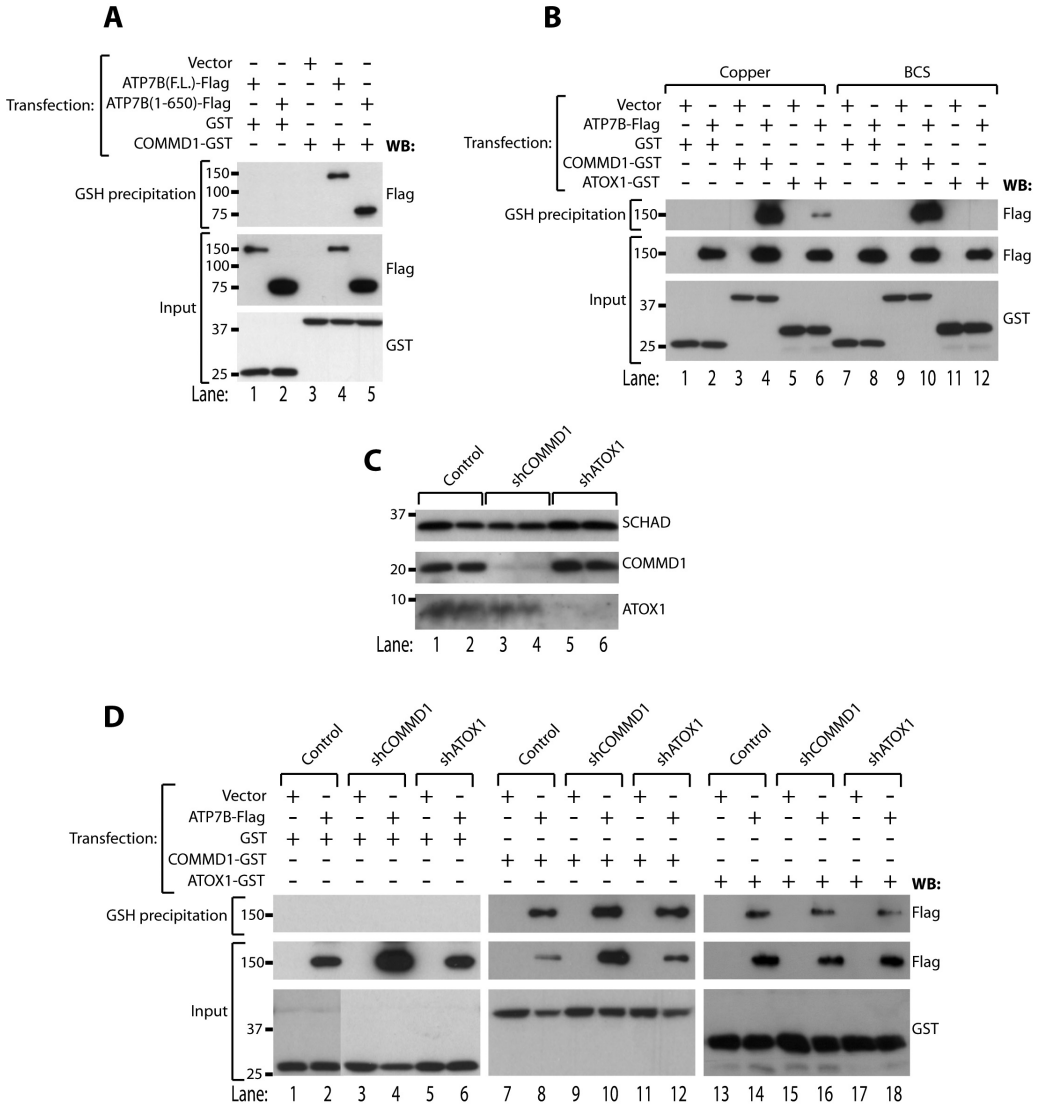


Figure 2. COMMD1 interacts with the amino terminus of ATP7B in a copper- and ATOX1-independent manner
A. Glutathione-sepharose precipitation was performed as described under figure 1A using cell lysates of HEK293T cells expressing ATP7B(F. L.) or ATP7B(1-650) coupled to Flag epitopes together with GST or COMMD1-GST. B. HEK293T cells were transfected with cDNA constructs encoding full length ATP7B-Flag, GST, COMMD1-GST or ATOX1-GST as indicated. Cells were treated overnight with 200 μM $CuSO_4$ or BCS (bathocuproinedisulfonic acid), prior to glutathione-sepharose precipitation as described under figure 1A. C. Western blot analysis of COMMD1 and ATOX1 expression in cell lysates of HEK293T cells after a stable knockdown of COMMD1 (shCOMMD1) or ATOX1 (shATOX1). HEK293T cells stably transfected with an empty shRNA vector were used as a negative control (Control). SCHAD was probed as loading control. D. HEK293T control cells, or HEK293T cells stably expressing shRNAs targeting COMMD1 (shCOMMD1) or ATOX1 (shATOX1) were transfected with cDNA constructs encoding ATP7B-Flag together with GST (D), COMMD1-GST (E), or ATOX1-GST (F). Lysates were used for glutathione-sepharose precipitation, as described under figure 1A.

different time intervals, or with different concentrations of $CuSO_4$ (Figure 3J-L and data not shown). Similarly, overexpression of COMMD1 did not interfere with the copper-dependent relocalization of ATP7B (data not shown). Taken together, these data suggest that COMMD1 does not have an essential regulatory role in the copper-induced relocalization of ATP7B.

Several mutations in ATP7B associated with WD result in increased binding of ATP7B to COMMD1-GST

The interaction between COMMD1 and ATP7B suggests that these two proteins cooperate to maintain copper homeostasis. We therefore hypothesized that alteration of this interaction underlies the development of copper overload in WD. As COMMD1 directly binds to the amino terminus of ATP7B, this hypothesis predicts that WD-causing mutations in this region could interfere with the interaction of ATP7B with COMMD1. To assess this possibility, all the missense mutations causing WD in this region and deposited in the WD mutation database (as of February

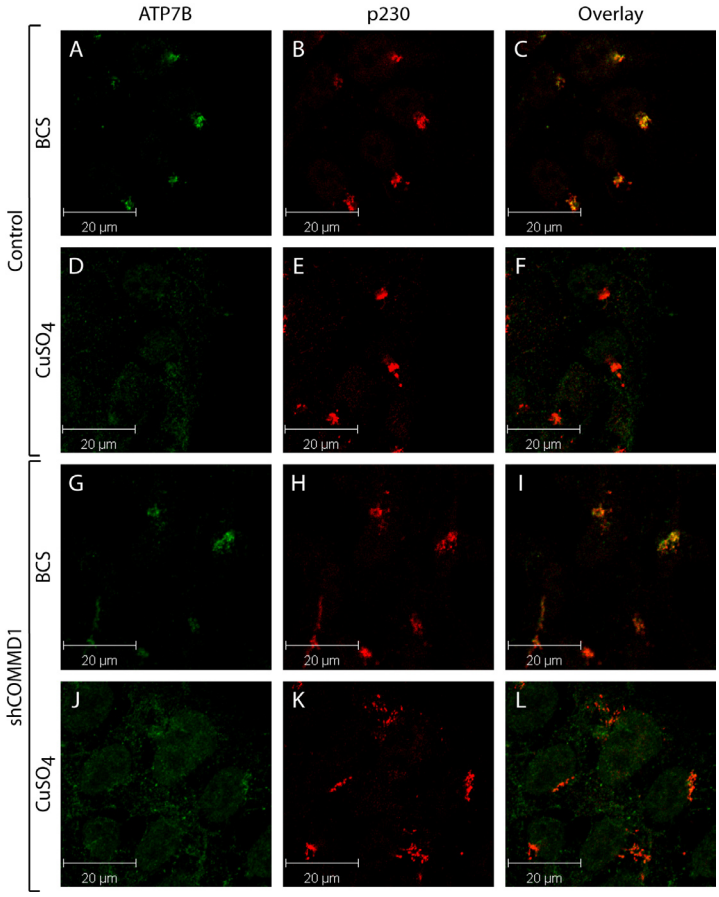


Figure 3. COMMD1 does not regulate copper-induced trafficking of ATP7B
HEK293T cells stably transfected with an empty shRNA vector (Control, A-F), or with shCOMMD1 (G-L) were incubated with 50 µM BCS or 200 µM $CuSO_4$. Cells were subsequently analyzed using antibodies against ATP7B and p230, visualized by Alexa488 conjugated donkey anti-rat and Alexa568 conjugated donkey anti-mouse IgG respectively. ATP7B localization is presented in images A, D, G, and H, and p230 localization is presented in images B, E, H, and K. Overlap in staining is depicted in yellow in the overlay images (C, F, I and L).

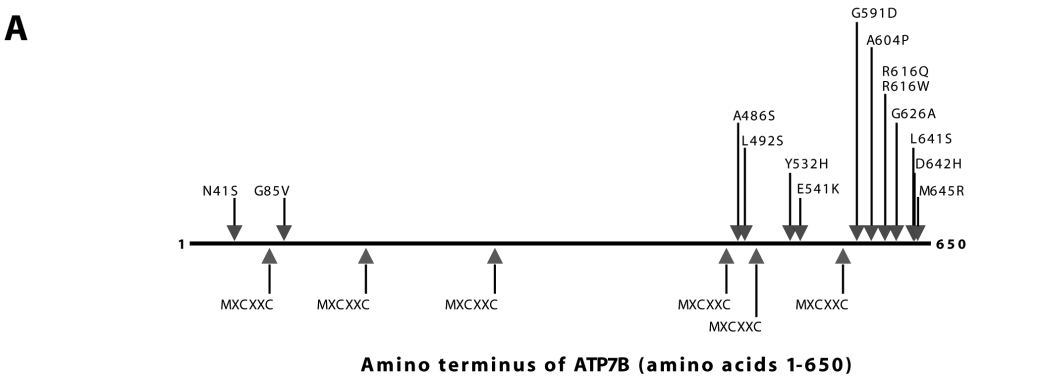
2005) [59] were introduced in ATP7B(1-650)-Flag, yielding a total of 14 mutant cDNAs (Figure 4A). Glutathione precipitation using lysates of HEK293T cells transfected with these mutant cDNAs revealed that all mutants retained their ability to interact with COMMD1-GST (Figure 4B). Quantification of precipitated ATP7B(1-650)-Flag relative to the amount present in the lysate revealed that several mutations; G85V, L492S, G591D, and A604P resulted in a marked increase in the efficiency of ATP7B(1-650)-Flag precipitation by COMMD1-GST (Figure 4C). A slight, but not reproducible, increase of this interaction was also observed for the A486S and Y532H mutations.

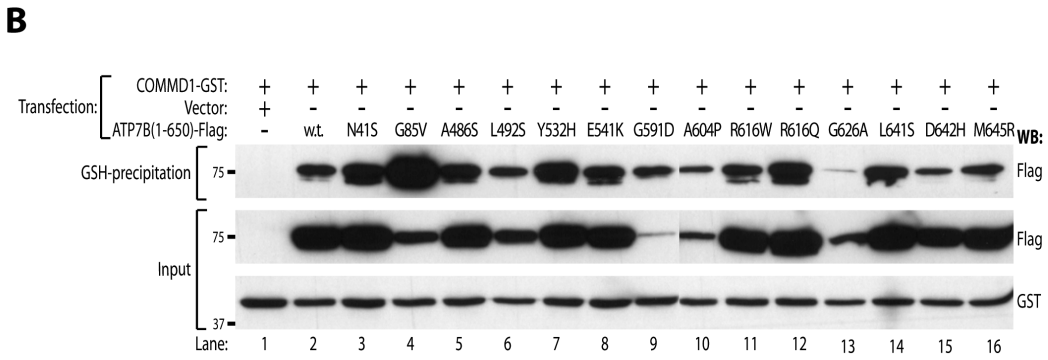
G85 and G591 residues are conserved residues present in all six metal binding regions of ATP7B

Further inspection of the four residues affecting interaction with COMMD1 revealed that the G85 and G591 residues are located in the same conserved position in metal binding regions one and six of ATP7B, respectively (Figure 5A). Interestingly, the G85V mutation most dramatically affected the interaction of ATP7B-Flag with COMMD1-GST, resulting in a relative increase of ATP7B-Flag interacting with COMMD1-GST by factor 28.3. Co-immunoprecipitation of full length ATP7B-Flag harboring either G85V or G591D mutations with COMMD1-HA confirmed the effects of these mutations (Figure 5B). To assess if the other conserved glycine residues in metal binding regions 2-5 of ATP7B equally affect interaction of ATP7B with COMMD1, each of these residues were individually mutated to valines. Glutathione precipitation using lysates of HEK293T cells transfected with these mutant cDNAs revealed that mutation of the glycine residues in metal binding regions two, three and five also resulted in a dramatic increase of ATP7B-Flag interaction with COMMD1-GST (Figure 5C-D). A slight, but not reproducible, increase for this interaction was also observed for mutation of the glycine in metal binding region four. Similar results were obtained when these glycine residues were mutated to aspartic acid (data not shown).

The G85V and G591D mutations lead to defective localization of ATP7B to the endoplasmic reticulum

Next we set out to determine the effects of the G85V and G591D mutations on ATP7B expression and localization. HepG2 cells were transfected with wild-type or mutant ATP7B-Flag. Staining of these cells with FITC-conjugated anti-Flag antibodies revealed an overlapping localization of wild-type ATP7B-Flag with the TGN marker p230 (Figure 6A-C). In contrast, both the G85V and G591D mutant ATP7B-Flag lost co-localization with p230 (Figure 6D-I), and displayed a more dispersed reticular localization pattern overlapping with the ER marker calreticulin (Figure 6M-R). These experiments suggest that the G85V and G591D mutations lead to mislocalization of ATP7B to the ER. Mislocalization of the G85V and G591D mutants could not be rescued by growing the cells at 30 °C, or by treatment with $CuSO_4$. While COMMD1 staining partially overlapped with ATP7B G85V and G591D, expression of these mutants did not result in detectable alteration of overall COMMD1 localization (data not shown).





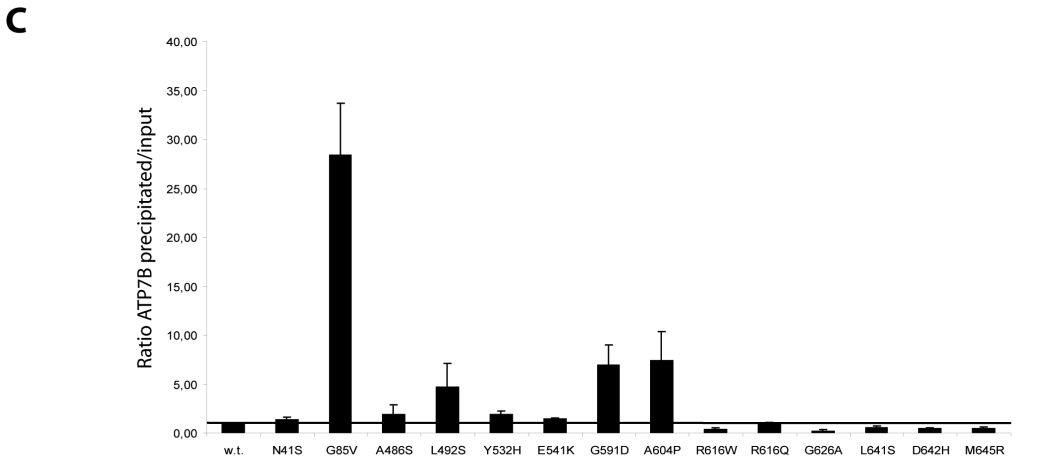


Figure 4. Several WD-causing mutations are associated with increased binding to COMMD1
A. Schematic representation of the amino terminal 650 amino acids of ATP7B. Locations of metal-binding sequences are indicated by lower arrows. Upper arrows indicate locations of WD-causing mutations introduced in ATP7B(1-650)-Flag and ATP7B-Flag for experiments in Figures 4B, 4C and 5B. B. Glutathione-sepharose precipitation using cell lysates of HEK293T cells expressing wild-type or mutant ATP7B(1-650)-Flag and COMMD1-GST, as described under figure 1A. C. Quantification of the ratio of precipitated wild-type and mutant ATP7B(1-650)-Flag relative to input levels. Wild-type ratio was set to 1, and indicated by the black horizontal line. Shown is the average of at least three independent experiments. Error bars indicate standard errors of the mean.

The G85V mutation leads to increased degradation of ATP7B

ER mislocalization of proteins is often associated with increased proteolysis, as has been demonstrated before for the common WD-causing mutation H1069Q in ATP7B [123]. Therefore we sought to determine if the G85V and G591D mutations result in an increase in proteolysis associated with the mislocalization to the ER. The low steady state expression level of the G591D mutant (figure 4B) prohibited reliable analysis of its stability. HEK293T cells were transfected with cDNA constructs encoding wild-type or G85V mutant ATP7B-Flag, and subsequently incubated with protein synthesis inhibitor cycloheximide for different time intervals. Western blot analysis demonstrates that wild-type ATP7B-Flag is relatively stable over a period of 22 hours, but the G85V mutant ATP7B-Flag is clearly degraded within 8 hours after addition of cycloheximide (Figure 7A). As a complementary approach, metabolic pulse-chase labeling was undertaken. This experiment revealed that, although the biosynthesis rates of wild-type and G85V mutant ATP7B-Flag were similar, the G85V mutant is subject to a dramatically increased rate of proteolysis (Figure 7C). Incubation of cells with the proteasomal inhibitors MG132 or lactacystin partially inhibited the

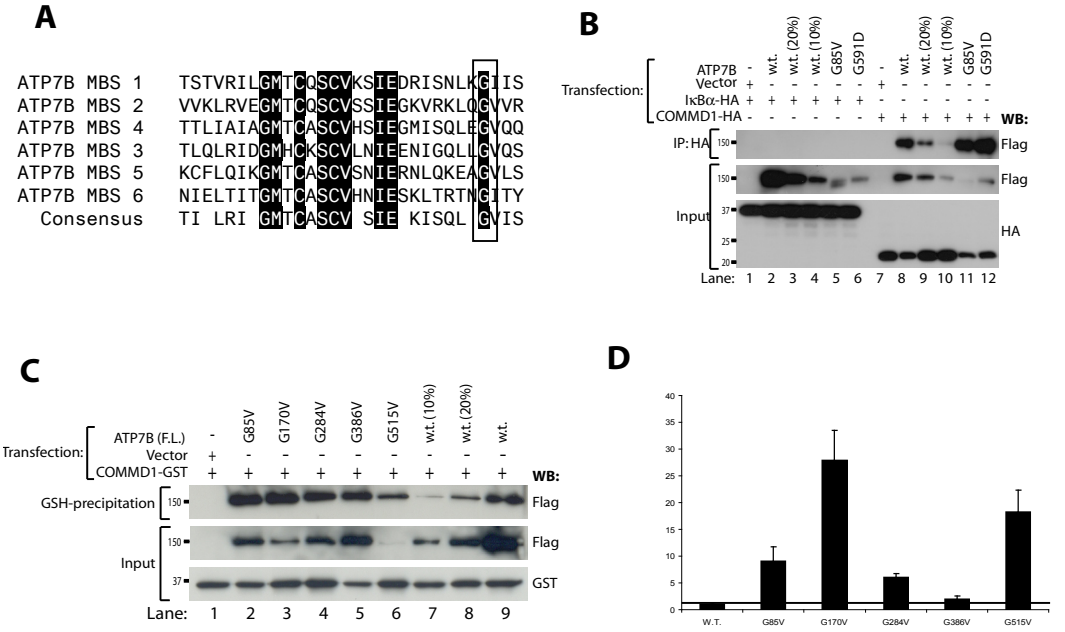


Figure 5. G85 and G591 are conserved residues in all metal binding regions of ATP7B and are important for COMMD1-ATP7B interaction

A. Sequence alignment of all six metal binding regions of ATP7B. The box indicates position of G85 and G591 and conserved glycine residues at the same position in other metal binding regions. B. Full length ATP7B-Flag, ATP7B-G85V-Flag and ATP7B-G591D-Flag were expressed in HEK293T cells together with COMMD1-HA or IκBα-HA. To equalize wild-type ATP7B-Flag expression to mutant ATP7B-Flag expression, HEK293T cells were also transfected with less cDNA (20% and 10%) encoding wild-type ATP7B-Flag. Cells lysates were used for immunoprecipitation with anti-HA. Precipitates were immunoblotted as indicated. Input indicates direct analysis of cell lysates. C. Wild type ATP7B-Flag and glycine to valine mutants of different metal binding regions in ATP7B-Flag were expressed in HEK293T cells together with GST or COMMD1-GST. To equalize wild-type ATP7B-Flag expression to mutant ATP7B-Flag expression, HEK293T cells were also transfected with less cDNA (20% and 10%) encoding wild-type ATP7B-Flag. Cell lysates were used for glutathione-sepharose precipitation as described under figure 1A. D. Quantification of the ratio of precipitated wild-type and mutant ATP7B(1-650)-Flag relative to input levels presented in C. Wild-type ratio was set to 1, and indicated by the black line. The average of three independent experiments is shown except for the G170V (n=2). Error bars indicate standard errors of the mean.

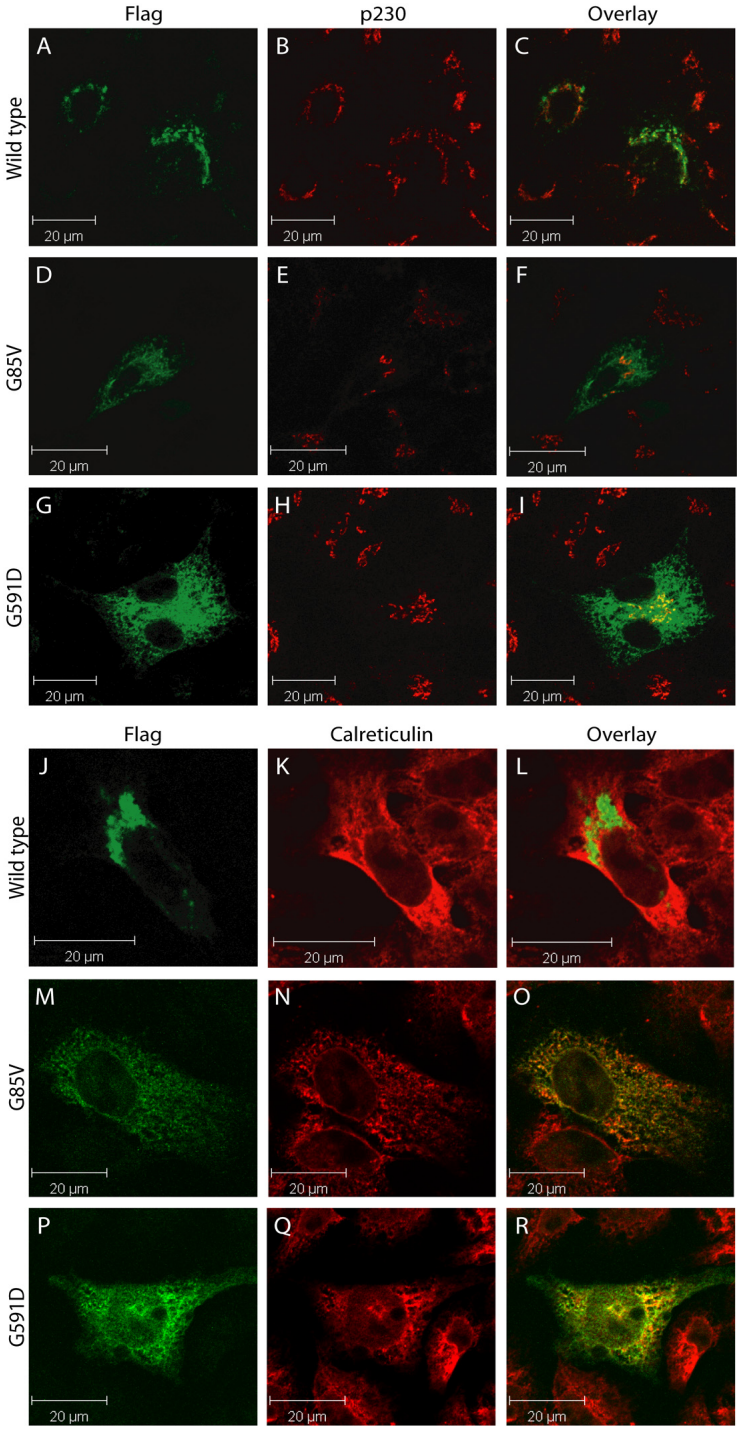


Figure 6. The G85V and G591D mutations lead to defective localization of ATP7B to the endoplasmic reticulum
HepG2 cells were transfected with ATP7B-Flag (A-C and J-L), ATP7B-G85V-Flag (D-F and M-O) or ATP7B-G591D-Flag (G-I and P-R) and analyzed using FITC conjugated antibodies against Flag (A, D, G, J, M, and P; green), and antibodies against p230 (B, E, and H; red) or Calreticulin (K, N, and Q; red), visualized by Alexa Fluor 568 conjugated donkey anti-mouse and Alexa Fluor 568 conjugated donkey anti-rabbit IgG respectively. Overlap in staining is depicted in yellow in the overlay images (C, F, I, L, O, and R).

degradation of G85V mutant ATP7B-Flag, suggesting that the increased proteolysis was at least partially mediated by the proteasome (Figure 7C).

Previous studies revealed that COMMD1 associates with several proteins to regulate the stability of such interacting proteins [184, 186, 187]. Therefore, we hypothesized that COMMD1 could also regulate the stability of ATP7B. To address this hypothesis, HEK293T cells were transfected with cDNA constructs encoding wild-type or G85V mutant ATP7B-Flag together with GST or COMMD1-GST. Pulse-chase analysis of ATP7B-Flag stability on these cells revealed that overexpression of COMMD1 results in increased proteolysis of wild-type ATP7B-Flag and, to a lesser extent also of G85V mutant ATP7B-Flag (Figure 7D).

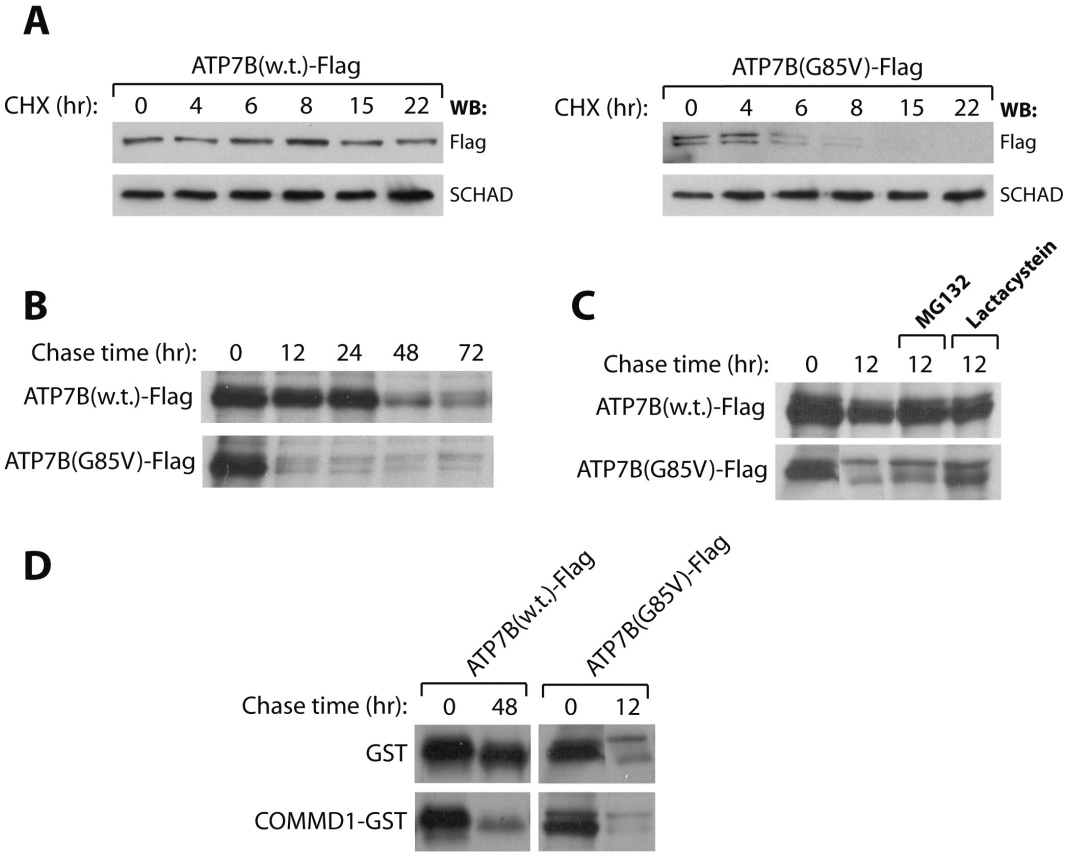


Figure 7. ATP7B-G85V is subject to increased degradation, partially facilitated by COMMD1.
A: HEK293T cells were transfected with cDNA constructs encoding for ATP7B-Flag or ATP7B-G85V-Flag and subsequently incubated with cycloheximide for the indicated time intervals. Cell lysates were generated and analyzed for expression of ATP7B-Flag and ATP7B-G85V-Flag. Equal loading was confirmed by immunoblotting for SCHAD. B: HEK293T cells were transfected with cDNA constructs encoding for ATP7B-Flag or ATP7B-G85V-Flag and biosynthetically labeled with ^{35}S-labeled methionine and cysteine for 90 minutes. After labeling, cells were chased with cold medium for the indicated time intervals. Cell lysates were subjected to immunoprecipitation using anti-Flag affinity gel before analysis by SDS-PAGE. Proteins were visualized with fluorography. C. HEK293T cells expressing ATP7B-Flag or ATP7B-G85V-Flag were subjected to biosynthetic labeling and immunoprecipitation as described in B. During 12 hr chase with cold media, cells were incubated with the proteasome inhibitors MG132 or Lactacystin as indicated. D. HEK293T cells expressing ATP7B-Flag or ATP7B-G85V-Flag together with GST or COMMD1-GST were subjected to biosynthetic labeling and immunoprecipitation as described in B.

DISCUSSION

The hepatic copper overload in Bedlington terriers with a homozygous deletion of the second exon of *COMMD1* implies a critical role of COMMD1 in the regulation of copper excretion [76]. In addition, knockdown of COMMD1 by RNA interference in several cell lines results in increased cellular copper levels [183, 333]. COMMD1 interacts with the copper transport protein ATP7B, which is mutated in WD, suggesting that these two proteins co-operate in the excretion of copper [182]. Consequentially, these observations suggest that COMMD1 could play a role in the development of WD. Consistent with this notion, heterozygosity for a silent missense mutation in *COMMD1* was possibly associated with an earlier onset of the disorder in patients with known *ATP7B* mutations [80]. Because COMMD1 appears to depend on protein-protein interactions for its functions (reviewed in [185]), we have characterized the effects of mutations in ATP7B associated with WD on its interaction with COMMD1. Several WD-causing mutations markedly increased the amount of ATP7B that interacted with COMMD1, suggesting that deregulation of this interaction is associated with the development of WD. In addition to previously reported effects of WD-causing mutations on ATP7B-mediated copper transport, cuproenzyme biosynthesis, ATP-binding and -hydrolysis, localization and copper-induced trafficking, post-translational modifications, and protein-protein interactions, this observation provides a novel molecular mechanism in the pathogenesis of WD.

Further characterization of the mutations that de-regulate the interaction of ATP7B with COMMD1 has led to valuable insights into the functionality of this interaction. The G85 and G591 residues in ATP7B are highly conserved at a position were a glycine is present in all six amino-terminal metal binding regions, indicating that these residues are of structural importance, which is underscored by our observation that mutation of other glycine residues also leads to increased binding to COMMD1. In addition, previous studies indicated that the G85V and G591D mutations markedly impair the interaction of ATP7B with ATOX1 [167]. Analysis of the localization of the G85V and G591D mutations demonstrated that both mutations result in defective localization of ATP7B to the ER in the cell. A similar defect has been demonstrated for several other WD-causing mutations (e.g. see references [123, 124]). ER mislocalization of proteins is often due to misfolding and associated with proteasomal degradation [157]. A well known example of this process in human disease development is the ER associated degradation of ΔF508 CFTR mutant in cystic fibrosis [158]. In addition, it has previously been shown that the common WD-causing mutation H1069Q results in increased proteolysis, associated with ER mislocalization of ATP7B [123]. Two complementary approaches indicated that the G85V mutant ATP7B protein has a dramatically shortened half-life. Incubation with the proteasome inhibitor lactacystin partially inhibited the degradation of the G85V mutant, indicating that this degradation is at least in part mediated by the proteasome.

The increased interaction of this mutant with COMMD1 led us to hypothesize that COMMD1 facilitates the degradation of ATP7B. This hypothesis is supported by several recent studies that have implicated COMMD1 as a negative regulator of the stability of a number of proteins in the HIF-1 and NF-κB pathways [184, 186, 187]. Within the NF-κB pathway, COMMD1

specifically promotes the ubiquitination and subsequent proteasomal degradation of RelA, whereas it protects IκBα from the same fate [184, 186]. COMMD1 has also been shown to inhibit the activity of the epithelial sodium channel (ENAC), through association with the αENaC and δENaC subunits [188]. The mechanism of this inhibition possibly involves ubiquitination and degradation, since it is well known that the ubiquitin-proteasome pathway plays an important role in the regulation of ENaC [334]. Furthermore, COMMD1 interacts with a number of subunits of E3 ubiquitin-ligase complexes, including Cullin1, Cullin2, Cullin3, Cullin5, and the X-linked inhibitor of apoptosis (XIAP), suggesting a direct role for COMMD1 in ubiquitin modifications [183, 184, 186, 187]. Taken together, these studies strongly implicate COMMD1 as a general negative regulator of protein stability in a number of cellular processes. Consistent with this proposed function, we have observed that overexpression of COMMD1 resulted in an increase in proteolytic turnover of newly synthesized ATP7B. The increased interaction with ATP7B as a result of several WD-causing mutations suggests that COMMD1 is involved in the quality control of ATP7B. The importance of quality control mechanisms is underscored by estimates that, in general, 30% of all newly synthesized proteins are targeted for degradation as a result of misfolding [335]. In this context, failure to maintain proper quality control of ATP7B in Bedlington terriers as a result of COMMD1 deficiency might be the mechanism that underlies the development of copper toxicosis. High copper intake through modern commercial dog food over the years has resulted in hepatic copper concentrations in healthy dogs that are over 10-fold higher than in humans [331]. This might predispose dogs to develop copper toxicosis, as evidenced by the occurrence of copper associated liver disease in a variety of dog breeds other than Bedlington terriers [64, 332]. We hypothesize that absence of COMMD1 in affected Bedlington terriers disrupts ATP7B quality control, resulting in a gradual build-up of sub-optimally functioning ATP7B, which perturbs the ability of the hepatocyte to cope with such high copper concentrations. This hypothesis is supported by the observation that, although serum ceruloplasmin levels appear normal, the incorporation of copper in ceruloplasmin is greatly reduced in a manner that inversely correlates with hepatic copper levels consistent with a gradual impairment of the function of ATP7B [262].

In conclusion, we have presented biochemical evidence that COMMD1, a protein identified through genetic studies in dogs with copper toxicosis, shows a markedly increased interaction with ATP7B affected by distinct mutations. These data suggest that this increased interaction contributes to the molecular basis of WD in patients harboring such mutations. This observation might partially explain the clinical heterogeneity observed in WD. In addition, our study indicates that COMMD1 regulates ATP7B stability, rather than copper-induced trafficking or interactions with ATOX1. Recently, nine human homologues of COMMD1 have been identified that share a common functional domain known as the COMM domain. These proteins have a similar inhibitory function in the NF-κB pathway as COMMD1 [85, 189]. These structural and functional similarities among COMMD proteins suggest that they might have a complementary function in copper homeostasis. This is supported by our observation that three COMMD proteins, other than COMMD1, also interact with ATP7B (P. de Bie et al., unpublished data). Further studies on the

molecular functions of COMMD1 and its homologues will provide valuable and novel insights into the regulation of ATP7B, and consequently copper homeostasis.

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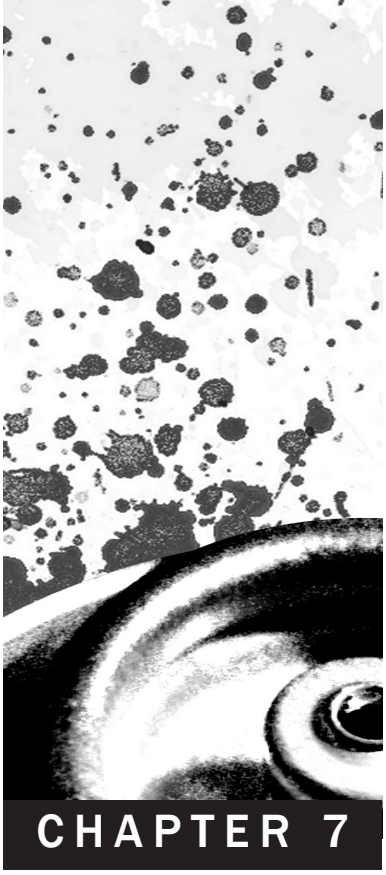


CHAPTER 7

Summarizing discussion

Protein-protein interactions form an essential mechanism through which many proteins exert their functions. Mapping of the protein-protein interactome provides a valuable framework to elucidate the functional organization of the human proteome, and consequently to understand the molecular pathology of human disease [162]. In this thesis, we have used this approach to study and characterize the molecular function of COMMD1, the gene product of the copper toxicosis gene in Bedlington terriers [76]. Our studies have revealed novel and significant insights into not only the functions of COMMD1 in copper metabolism, but also into the pathogenic mechanisms of copper overload disorders in man and dog. Scientists outside the field of copper metabolism have also contributed to our understanding of the functions of COMMD1, and have implicated COMMD1 in a variety of other cellular processes, including sodium transport, NF-κB signaling, and ubiquitin modifications [85, 183, 184, 186, 188, 189, 336] (figure 1, and reviewed in chapter 2 in this thesis). In addition, characterization of a COMMD1 knock-out mouse, carrying the same mutation that results in copper toxicosis in Bedlington terriers, has implicated COMMD1 as an inhibitor of hypoxia inducible factor 1 (HIF1) signaling [187].

COMMD PROTEINS; A FAMILY OF FUNCTIONAL HOMOLOGUES OF COMMD1

An open-minded screening approach, using yeast two-hybrid technology, allowed us to identify a number of putative interacting partners of COMMD1, including COMMD6. In a complementary study by Burstein et al., employing tandem affinity purification, COMMD3, COMMD4 and also COMMD6 were detected in complex with COMMD1 [85]. As a result of these protein-protein

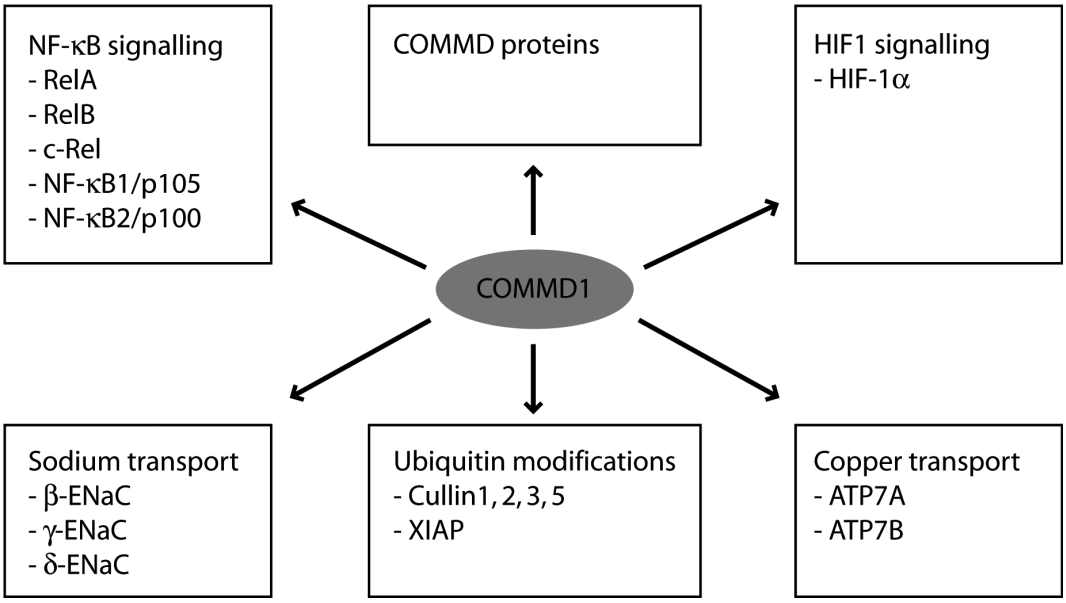


Figure 1: Schematic overview of the COMMD1 protein-protein interactome.
The identification of a large variety of interacting partners of COMMD1 has implicated COMMD1 in a variety of cellular processes including transport of copper and sodium, the HIF1 and NF-κB signalling pathways, and ubiquitin modifications.

interaction studies, COMMD proteins were described as a novel protein family sharing the conserved COMM domain [85] (figure 2). COMMD proteins appear to be not only structurally, but also functionally related; a number of COMMD proteins interact with ATP7A, ATP7B and several components of the NF-κB pathway ([85, 189] and chapters 3-5 in this thesis). Also an inhibitory function in NF-κB signaling appears to be conserved amongst most COMMD proteins [85, 189]. Further characterization of interactions among COMMD proteins indicated that both COMMD1 as well as COMMD6 can be detected in complex with any other COMMD protein ([85] and P. de Bie et al., unpublished observation). The composition of these complexes containing multiple COMMD proteins remains elusive, but given the differences in binding affinities for ATP7A, ATP7B and individual components of the NF-κB pathway ([85], and chapters 4 and 5 in thesis), it appears unlikely that COMMD proteins occur in the cell as one large complex containing all ten family members. It seems more likely that COMMD proteins form a variety of different complexes, the compositions of which will potentially determine the binding affinities for their interacting partners.

Characterization of the composition of COMMD-containing complexes under physiological conditions is important to further understand the functionality of the COMMD protein family as a whole. The sequence conservation among COMMD proteins suggests that these proteins contain distinct functional domains (figure 2). This is supported by the lobular

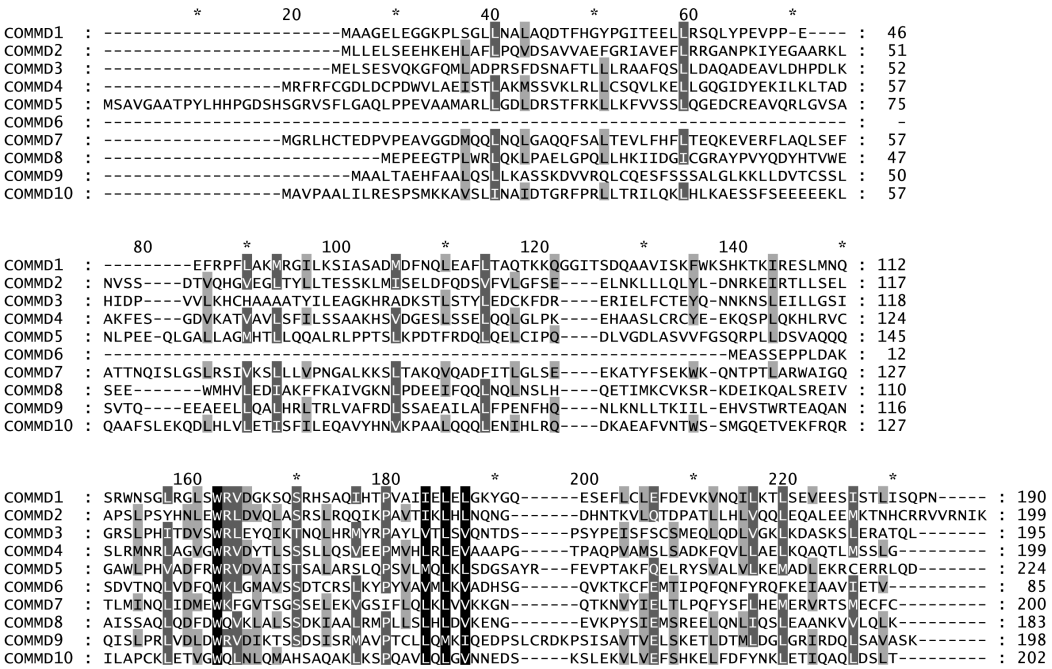


Figure 2. Alignment of the amino acid sequences of all ten human COMMD proteins.
Background shading indicates the degree of conservation of amino acid residues with similar properties (Black, similar residue conserved in all COMMD proteins; dark grey, similar residue conserved in at least eight out of ten COMMD proteins; light grey, similar residue conserved in at least six out of ten COMMD proteins).

structure of the amino terminal half of COMMD1 [337]. The conserved carboxy-terminal region of COMMD1, containing the COMM domain, mediates its interactions with COMMD3 and COMMD6, and for homo-dimerization of COMMD1 [85, 189]. In addition, the conserved tryptophan and proline residues in the COMM domain are essential for the inhibitory role of COMMD6 on the NF-kB pathway (chapter 3). The amino-terminal parts of COMMD proteins show a higher degree of variation, both in length and sequence conservation. This region might therefore play an important role in functions that are unique to each individual COMMD protein. For example, the amino-terminal region might determine the specificity of the interactions of COMMD proteins with other proteins. This hypothesis is supported by the observations that full-length COMMD1 is required for its interaction with RelA or ATP7B ([85] and P. de Bie et al., unpublished observation). Furthermore, the structure of the amino-terminal half of COMMD1 revealed several conserved patches that potentially mediate protein-protein interactions [337].

Another issue is the possibility of functional redundancy among COMMD family members. This possibility appears unlikely, as evidenced by the embryonic lethal phenotype of the *COMMD1* knockout mouse, indicating that COMMD1 has an essential and unique role in early embryogenesis. In addition, whereas all COMMD proteins inhibit NF-κB reporter activity, interaction data indicate functional differences; while COMMD1 interacts with all five NF-κB subunits (RelA, RelB, c-Rel, NF-κB1/p105, NF-κB2/p100) and the NF-κB inhibitor IκBα, COMMD3 interacts only with RelB and NF-κB1/p105, and COMMD6 only interacts with RelA and NF-κB/p105 [85, 189]. These differences in affinities for different NF-κB subunits suggest that the different COMMD proteins have distinctive functions within the NF-κB pathway. For example, COMMD proteins could mediate specificity in the transcriptional response upon NF-κB activation, a possibility that is currently being investigated in our laboratory using microarray technology.

COMMD PROTEINS AND COPPER METABOLISM

In the context of copper metabolism, distinct subsets of COMMD proteins interact with ATP7A and/or ATP7B. Not only do these data implicate COMMD proteins in the regulation of copper homeostasis, but they also indicate that COMMD proteins potentially act both on hepatic copper excretion as well as on copper uptake across the mucosal barrier. In addition, COMMD proteins might have a role in the biosynthesis of cuproenzymes, although this possibility could be disputed as affected Bedlington terrier have normal, if not elevated, serum ceruloplasmin levels. The copper overload phenotype in COMMD1-deficient Bedlington terriers indicates that COMMD proteins are not functionally redundant in the context of copper metabolism. Further studies should be undertaken to determine if COMMD proteins also interact with other copper transport proteins. Indeed, pilot experiments using tandem affinity purification identified the copper chaperone COX17 as a putative interacting partner of COMMD6 (P. de Bie et al., unpublished observation). Although this interaction needs to be confirmed and characterized further, this observation underscores the need for a thorough characterization of the roles of different COMMD proteins in copper homeostasis, and illustrates that many functional aspects of copper metabolism remain

to be uncovered. Taken together, these data implicate COMMD proteins as the first large family of proteins in the regulation of copper homeostasis. However, this statement could be criticized, as it is based on protein-protein interaction data. Therefore, functional studies undertaken to provide clarity and credibility for a potential role of COMMD proteins in copper homeostasis are of high priority. This thesis describes several studies aimed to elucidate the function of COMMD1 in this process (chapters 5 and 6), as further discussed below.

COMMD PROTEINS AND HUMAN COPPER OVERLOAD DISEASES

One of the main objectives of the work described in this thesis was to unravel the pathophysiological mechanisms of human copper overload disease, particularly to gain insight into copper overload of unknown etiology. The copper overload phenotype in COMMD1-deficient Bedlington terriers clearly illustrates the existence of a potential role for COMMD proteins in the pathogenesis of copper homeostasis disorders. In chapter 4 of this thesis, we have investigated the three *COMMD* genes whose gene products interact with ATP7B, as candidate genes for the human copper overload disorders Indian childhood cirrhosis (ICC), endemic Tyrolean infantile cirrhosis (ETIC), and idiopathic copper toxicosis (ICT). Several single nucleotide polymorphisms (SNPs) were detected in *COMMD2* and *COMMD10*. One non-synonymous coding SNP in COMMD2, encoding for either a leucine or an isoleucine at amino acid position 113, is of particular interest, as its genotype frequencies appeared remarkably different from that reported for a European control population. There is a growing awareness that common polymorphisms have an extensive modulatory role in the development of complex diseases, for example in alcoholic-induced liver disease [319]. Although excessive alcohol intake is the teratogenic cause of alcohol-induced liver disease, common polymorphisms in the *Tumor necrosis factor* (*TNF*), *Interleukin-1*β (*Il-1*β), *Interleukin-10* (*Il-10*), *CD14 endotoxin receptor* (*CD14*), *Manganese superoxide dismutase* (*MnSOD*), and *Glutathion peroxidase-1* (*GPx1*) genes are associated with the severity of this disease [319-326]. In analogy with alcohol-induced liver disease, besides a genetic predisposition, excessive copper intake is a required inducing factor for the development of ICC, ETIC, and ICT [5, 6, 8, 63, 317]. It is therefore likely that this genetic predisposition consists of either mild mutations or common polymorphisms, rather than more severe mutations that would disrupt gene function completely. Thus, the aforementioned SNP in *COMMD2* might indeed be associated with the development of ICC, ETIC or ICT. Unfortunately, it is difficult to test this hypothesis, as there are no sufficiently large cohorts of patients and controls with a defined copper intake. Biochemical studies are therefore important to assess if this SNP is associated with a perturbed function in copper homeostatic pathways, for example through altered binding with ATP7B. Care should be taken in the interpretation of such experiments, as the effects of the genotype for this SNP on COMMD2 function might be very marginal and thus very difficult to detect, or perhaps only in combination with high copper concentrations. Yet, our observation that several Wilson disease-causing mutations result in increased interaction of ATP7B with COMMD1 suggests that altered COMMD-ATP7B interactions could indeed underlie the development of copper overload (chapter

6). In addition to previously reported effects of WD-causing mutations on ATP7B-mediated copper transport, cuproenzyme biosynthesis, ATP-binding and -hydrolysis, localization and copper-induced trafficking, post-translational modifications, and protein-protein interactions (reviewed in chapter 1), this observation also provides a novel molecular mechanism in the pathogenesis of WD.

COMMD1, A REGULATOR OF COPPER TRANSPORT?

Since the identification of the genetic defect causing copper toxicosis in Bedlington terriers, COMMD1 has been postulated to be a direct regulator of copper transport [76]. This hypothesis was supported by observations that knockdown of COMMD1 by RNA interference resulted in increased copper levels in several cell lines [183, 271, 333]. The observed interactions of COMMD1 with ATP7A and ATP7B suggest that COMMD1 acts on the level of cellular copper excretion, which is consistent with the impaired excretion of copper through the bile in COMMD1-deficient Bedlington terriers [331]. However, using a luciferase-based method to detect intracellular copper levels, no effects of COMMD1 expression on the copper export activity of either ATP7A or ATP7B could be observed (Chapter 5 and P. van de Berghe, unpublished observation). Knockdown of COMMD1 also did not affect copper-induced relocalization of ATP7A or ATP7B. To solve the molecular function of COMMD1 in copper homeostasis, our observations on the effects of Wilson disease-causing mutations on the interaction of ATP7B with COMMD1 have been very helpful. Concomitant with an increased binding to COMMD1, these mutations in ATP7B resulted in mislocalization into the endoplasmic reticulum (ER). ER mislocalization of transmembrane proteins and proteins in the secretory pathway is generally a consequence of defects in proper folding or assembly of these proteins and usually precedes their degradation via the ubiquitin-proteasome system. This process, known as ER-associated protein degradation (ERAD), serves as a general quality control of newly synthesized proteins [157]. A well-known substrate for ERAD is the ΔF508 CFTR mutant that causes cystic fibrosis, underscoring the importance of this process in human disease development [158]. In addition, ERAD potentially plays an important role in the development of Wilson disease, as evidenced by our results showing that the ATP7B-G85V mutant is prone to rapid degradation. All of our observations led us to hypothesize that COMMD1 is involved in regulating the degradation of ATP7B. This hypothesis is supported by observations on the function of COMMD1 in other cellular processes: several recent studies have implicated that COMMD1 exerts its inhibitory function on HIF-1 and NF-κB signaling by acting as a regulator of the stability of a number of proteins in these pathways [184, 186, 187]. In chapter 6 of this thesis, we have provided evidence that COMMD1 promotes the degradation of newly synthesized ATP7B. The exact mechanisms of COMMD1-facilitated degradation of ATP7B remain to be determined, but they may involve ubiquitination and proteasomal degradation as proteolysis of ATP7B could be partially inhibited by the proteasome inhibitors MG132 and lactacystein. Our observations fit into a model in which COMMD1 exerts its function in hepatic copper homeostasis by regulating the degradation of ATP7B. Possibly, COMMD1 mediates the

link between quality control and the ubiquitin-proteasome system in the process of ERAD. In this light, failure to ensure proper quality control of newly synthesized ATP7B due to COMMD1 deficiency might underlie the development of copper toxicosis in Bedlington terriers; this could result in a gradual build-up of sub-optimally functioning ATP7B perturbing the ability of the hepatocyte to cope with the high copper concentrations that dogs are exposed to through their copper-rich diets. Further research characterizing the localization, expression and degradation of ATP7B in liver biopsies of affected Bedlington terriers are currently being performed to test this hypothesis. It would also be interesting to explore if COMMD1, or other COMMD proteins, could have a role in the degradation of other ERAD substrates, such as ΔF508 CFTR.

TOWARDS A GENERAL MODEL FOR THE MOLECULAR FUNCTION OF COMMD1

One of the most striking and puzzling observations regarding COMMD1 concerns the phenotype present in COMMD1-deficient mice carrying the same mutation that causes copper overload in Bedlington terriers. To our surprise, this mutation resulted in embryonic lethality at E9.5. This phenotype appeared unrelated to defects in copper homeostasis, but rather a deregulation of HIF1 signaling [187]. The analysis of *COMMD1*$^{-/-}$ mice supported the emerging view that COMMD1 exerts a pleiotropic function that extends beyond the regulation of hepatic copper metabolism, which appears consistent with its ubiquitous expression pattern. With this growing realization, it is striking that COMMD1-deficient Bedlington terriers display a phenotype that is restricted to hepatic copper metabolism. The inconsistency in the phenotypes of COMMD1-deficient Bedlington terriers and *COMMD1*$^{-/-}$ mice has currently not been solved. We could speculate, however, that this could be explained by different levels of redundancy among COMMD proteins in Bedlington terriers and mice, or by differences in embryogenesis in these species. The fact that affected Bedlington terriers gradually accumulate copper could be associated with a high copper intake through their diet. Further investigations are required to unravel the functional differences of COMMD1 in mice and Bedlington terriers, in which a conditional knock out mouse should prove very helpful.

To unravel the molecular function of COMMD1, its role in all cellular processes in which it has been implicated should be taken in consideration. The work described in this thesis and by others, suggests that within copper homeostasis pathways and the HIF1 and NF-κB signaling pathways, COMMD1 exerts its function through the regulation of protein degradation (Figure 3; [184, 186, 187], and chapter 6 in this thesis). Within the NF-κB pathway, COMMD1 specifically promotes the ubiquitination and subsequent proteasomal degradation of RelA, whereas it protects the NF-κB inhibitor IκBα from the very same fate [184, 186]. COMMD1 has also been shown to inhibit the activity of the epithelial sodium channel (ENAC), through association with the αENaC and δENaC subunits [188]. The mechanism of this inhibition is currently unknown, but may involve ubiquitination and degradation, since it is well known that the ubiquitin-proteasome pathway plays an extensive role in the regulation of ENaC [334]. Finally, COMMD1 interacts with a number of subunits of E3 ubiquitin-ligase complexes, including Cullin1, Cullin2, Cullin3, Cullin5,

and the X-linked inhibitor of apoptosis (XIAP), suggesting a direct role for COMMD1 in ubiquitin modifications [183, 184, 186, 336].

Interestingly, XIAP has recently been implicated as a regulator of copper homeostasis by two distinct observations. The first observation identified COMMD1 as a substrate for XIAP-mediated ubiquitination, resulting in the proteasomal degradation of COMMD1. Through regulation of COMMD levels, XIAP might play a role in the regulation of copper homeostasis. The second observation implicated an even more direct role for XIAP in copper metabolism. It was demonstrated that XIAP specifically binds copper, and that copper binding results in an extensive conformational alteration as evidenced by differences in SDS-PAAGE mobility of XIAP upon copper binding [338]. Further observations that expression of XIAP is de-regulated in Wilson disease and copper toxicosis in Bedlington terriers indicate that XIAP might be involved in the pathogenesis of hepatic copper overload [338]. The possibility that XIAP, as an E3 ubiquitin ligase, has a role in COMMD1-facilitated degradation of ATP7B merits further investigation.

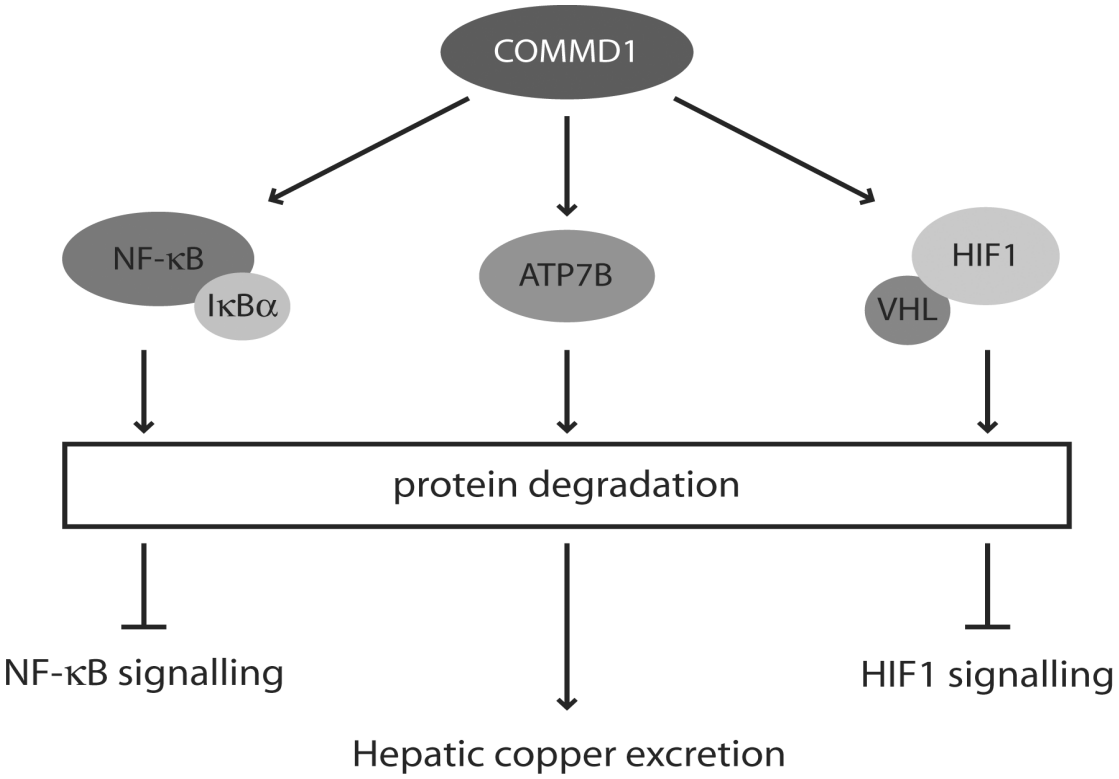


Figure 3. Model for the general function of COMMD1.
In copper homeostasis pathways and the HIF1 and NF-κB signaling pathways, COMMD1 exerts its function through the regulation of protein degradation, indicating that COMMD1 has a general molecular function as a modulator of protein stability.

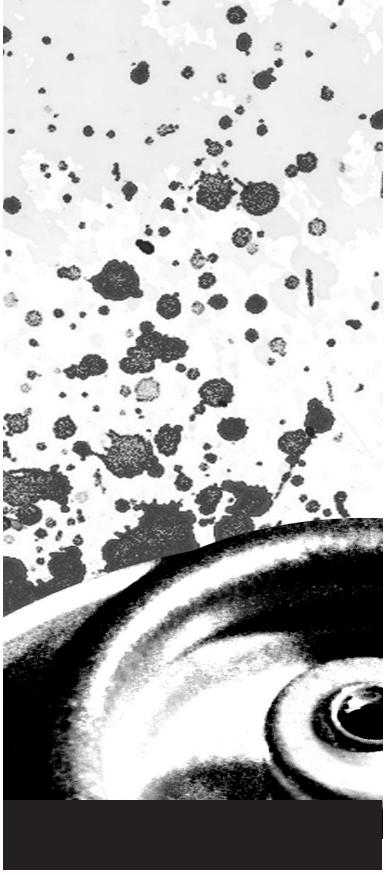
Cullin-containing ubiquitin ligase complexes have a regulatory role in a variety of cellular processes including the cell cycle, signal transduction (including the HIF-1 and NF-κB pathways), and protein quality control [339]. Specific receptor protein families rich in protein-protein interaction domains facilitate the substrate specificity of these Cullin-containing complexes [339, 340]. These receptor proteins include F-box proteins in Cullin1 and Cullin7 complexes, SOCS-box proteins in Cullin2 and Cullin 5 complexes, and BTB-domain proteins in Cullin3 complexes [339]. In analogy to these receptor proteins, COMMD proteins appear to undergo many protein-protein interactions. A possible role for COMMD proteins in ubiquitin modification pathways might therefore involve substrate specificity determination. In fact, COMMD1 was shown to facilitate the interaction between SOCS1 and RelA, thereby promoting the ubiquitination of RelA [186]. In order to further characterize this possibility, it is again important to determine the composition of endogenous COMMD-containing complexes relating to different Cullin proteins. Recently, a tandem affinity purification method was developed to identify substrates of specific Cullin-containing E3-ubiquitin ligase complexes. This method allowed identification of substrates to specific F-box proteins [341-343]. Such an approach could be valuable in further dissecting the role of COMMD proteins in ubiquitin modification pathways, and particularly in identifying new substrates that are targeted by COMMD facilitated ubiquitination. As this aspect potentially forms the basic molecular mechanism through which COMMD proteins exert their functions in various cellular processes, it is of importance to further characterize it in future studies. Taken together, the identification of the COMMD protein family potentially provides a new avenue to study ubiquitin modifications and regulated protein degradation, which will provide valuable new insights into this important regulatory mechanism that underlies various cellular processes.

CONCLUDING REMARKS

The studies in this thesis aimed to dissect the molecular pathogenesis of human copper homeostasis disorders. The detected interactions of COMMD proteins with ATP7A and/or ATP7B implicate this novel protein family in pathways of copper metabolism. In addition, these data suggest that COMMD proteins could play a role in the pathogenesis of copper homeostasis disorders, which is supported by the detected genotype frequencies of SNPs in *COMMD2* and *COMMD10* in patients with ICC, ETIC and ICT. The next challenge is to provide proof of the involvement of COMMD proteins in human diseases of copper homeostasis. Biochemical studies characterizing the effects of common polymorphisms in *COMMD* genes on the functions of their gene products, and studies combining genetic and environmental factors, are essential to reach this goal.

Our functional studies suggest that deregulation of the degradation of ATP7B underlies the copper overload phenotype in Bedlington terriers. In addition, through this function, COMMD1 might be involved in the pathogenesis of Wilson disease. Combining the results of our studies and those of other scientists, it appears that in most cellular processes it is involved in, COMMD1 exerts its function through the regulation of protein degradation, possibly through the ubiquitin-

proteasome system. Further studies should aim to dissect the molecular function of COMMD1, and its family members, in the ubiquitin-proteasome system. One important issue to address in this matter is the identification of substrates of COMMD1 mediated protein degradation. Since a regulated protein degradation is essential for the adaptability of the cell, these studies could lead to new insights into the molecular mechanisms of human disease.



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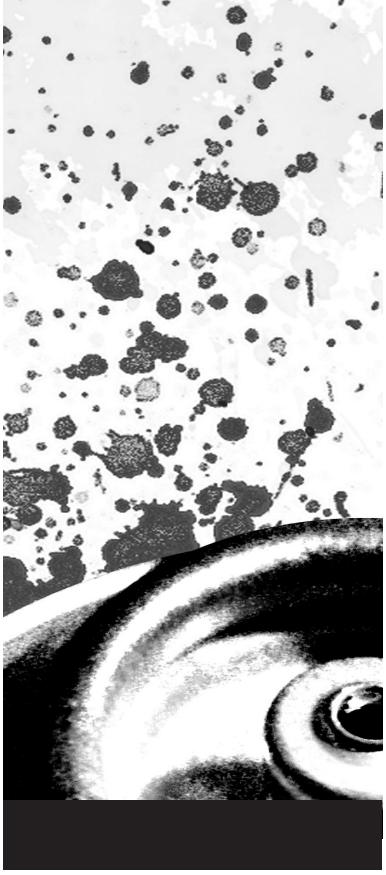
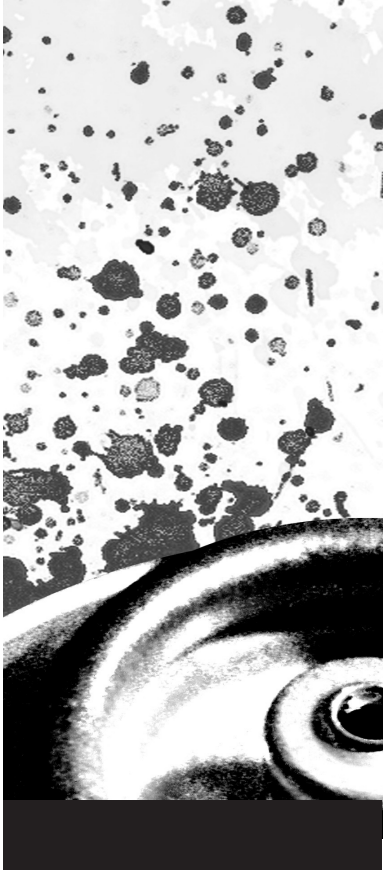


Abbreviations

A-domain: actuator domain
AIPP1: ATPase interacting PDZ protein 1
CCS: copper chaperone for superoxide dismutase 1
COMM domain: copper metabolism gene MURR1 domain
CT: copper toxicosis in Bedlington terriers
CTR1: copper transporter 1
ER: endoplasmic reticulum
ETIC: endemic Tyrolian infantile cirrhosis
GST: glutathione-S-transferase
HA: hemagglutinin
HEK293T: human embryonic kidney 293T cells
HIF1: hypoxia inducible factor 1
HIV-1: Human immunodeficiency virus type 1
ICC: Indian childhood cirrhosis
ICT: idiopathic copper toxicosis
LEC rat: Long Evans Cinnamon rat
MBS: metal binding site
MD: Menkes disease
MRE: metal responsive element
MTF-1: metal responsive transcription factor-1
N-domain: nucleotide binding domain
NF-κB: nuclear factor κB
OHS: occipital horn syndrome
P-domain: phosphorylation domain
PLZF: promyelocytic leukemia zinc finger protein
RLU: relative light units
RT-PCR: reverse transcriptase polymerase chain reaction
SOD1: superoxide dismutase 1
shRNA: short hairpin RNA
SNP: single nucleotide polymorphism
TGN: *trans* Golgi network
TNF: tumour necrosis factor
YFP: yellow fluorescent protein
WD: Wilson disease



Nederlandse samenvatting

Eén van de meest belangrijke biochemische eigenschappen van koper is dat dit metaal in ionische vorm in twee verschillende oxidatieve staten kan voorkomen. Door deze eigenschap kan koper dienen als elektrondonor of elektronacceptor en is zeer geschikt als co-factor bij chemische redoxreacties. Als een dergelijke co-factor is koper betrokken bij een groot aantal zeer belangrijke cellulaire processen. Onder deze processen bevinden zich onder andere de energie voorziening van de cel door middel van de mitochondriale ademhalingsketen, bescherming tegen vrije zuurstofradicalen, neurotransmitter synthese, en de productie van bindweefsel en pigment. Vanwege de belangrijke rol van koper is een voldoende beschikbaarheid van dit element essentieel voor het leven. Echter, dezelfde eigenschappen die koper zo essentieel maken, zorgen er ook voor dat koper potentieel zeer gevaarlijk kan zijn voor de cel, doordat het de vorming van uiterst schadelijke vrije zuurstofradicalen katalyseert. Het is dan ook van extreem belang dat een overvloed aan koper wordt voorkomen. Tijdens de evolutie zijn geraffineerde mechanismen ontwikkeld om ervoor te zorgen dat op cellulair niveau, maar ook binnen het gehele organisme, de hoeveelheid koper in een strikte homeostatische balans wordt gehouden. Stoornissen in deze balans, veroorzaakt door genetische defecten in de genen die de koperhomeostase waarborgen, resulteren in ernstige ziektebeelden bij zowel mens als dier. Het doel van de studies beschreven in dit proefschrift, is het karakteriseren van de moleculaire defecten die ten grondslag liggen aan stoornissen in de koperhomeostase bij de mens.

Een van de grote doorbraken in het onderzoek aan kopermetabolisme was het kloneren van de eerste twee genen die geassocieerd zijn met erfelijke stoornissen van de koperhomeostase in de mens. Deze genen, *ATP7A* en *ATP7B*, coderen voor twee homologe kopertransporterende P-type ATPases. Ondanks de grote homologie in zowel de structuur als de functie van ATP7A en ATP7B, leiden mutaties in de genen die voor deze eiwitten coderen tot tegengestelde ziektebeelden. Mutaties in *ATP7A* veroorzaken de ziekte van Menkes, die gekenmerkt wordt door een systemische koperdeficiëntie. Mutaties in *ATP7B*, daarentegen, veroorzaken de ziekte van Wilson, die juist gekenmerkt wordt door een stapeling van koper in de lever en in het brein. Een zeer groot aantal mutaties in ATP7A en ATP7B zijn geassocieerd met respectievelijk de ziektes van Menkes en Wilson. Deze mutaties kunnen de functies van ATP7A en ATP7B op verscheidene manieren verstoren en kunnen aangrijpen op het ATP7A en ATP7B afhankelijke koper transport en incorporatie van koper in koperafhankelijke enzymen. Tevens kunnen deze mutaties leiden tot verstoringen in de cellulaire lokalisatie, post-translationele modificaties en eiwit-eiwit interacties van ATP7A en ATP7B. In **hoofdstuk 1** hebben we deze effecten uiteengezet alsmede hoe verschillende mutaties in *ATP7A* en *ATP7B* relateren met de variabiliteit in de ziektebeelden van respectievelijk de ziektes van Menkes en Wilson.

Recentelijk is binnen onze afdeling *COMMD1* geïdentificeerd als een nieuw gen met een rol in de koperhomeostase op basis van de observatie dat een deletie binnen *COMMD1* in Bedlington terriërs een koperstapelingsziekte veroorzaakt die sterke gelijkenissen vertoont met de ziekte van Wilson. Het experimentele deel van dit proefschrift heeft als doel de inzichten in de mechanismen van koperhomeostase te vergroten, middels het karakteriseren van de functies

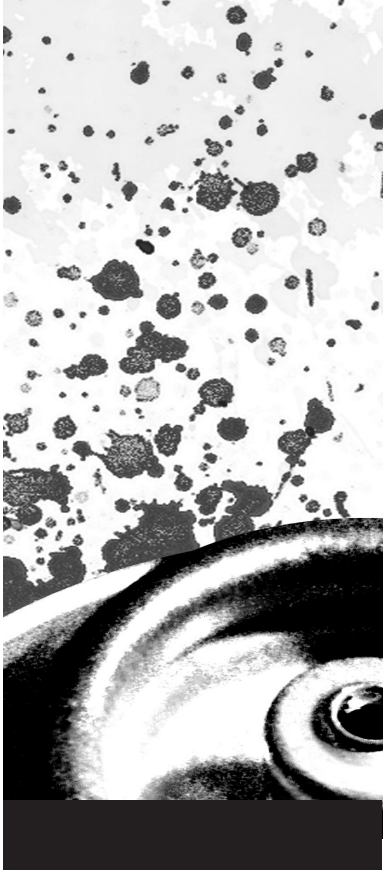
van COMMD1. Het behalen van dit doel zal helpen om de pathogenese van koperstapeling in Bedlington terriërs te begrijpen en ook de oorzaken en pathogenese van koperstapeling bij de mens te verhelderen. Om dit doel te verwezenlijken hebben we getracht eiwitten die interacties met COMMD1 aangaan te identificeren en te beschrijven. Deze aanpak is tevens succesvol toegepast door onderzoekers uit andere wetenschappelijke velden, wat heeft geleid tot de identificatie van een groot aantal interacterende partners voor COMMD1 in de periode dat dit promotieonderzoek heeft plaatsgevonden. De implicaties van deze studies hebben we uiteengezet in **hoofdstuk 2** en zij duiden aan dat COMMD1 naast zijn rol in koperhomeostase, ook functies heeft in de regulatie van natrium transport, van de activiteit van de transcriptiefactoren HIF1 en NF-κB, en van ubiquitine modificaties.

Om nieuwe interacterende partners voor COMMD1 te identificeren met een mogelijke rol in de koperhomeostase hebben we yeast two-hybrid technologie toegepast. Met deze techniek hebben we het toenmalig onbekende eiwit COMMD6 geïdentificeerd, dat een zeer sterke en specifieke interactie aangaat met COMMD1. Gebruikmakend van een door ons gegenereerd specifiek antiserum tegen COMMD6, waren we in staat om deze interactie op endogeen niveau aan te tonen. Deze resultaten staan beschreven in **hoofdstuk 3** en hebben samen met resultaten van Burstein *et al*. bijgedragen aan het karakteriseren van de COMMD eiwitfamilie. Deze eiwitfamilie bestaat naast COMMD1 uit negen andere COMMD eiwitten, waaronder COMMD6, die worden gekenmerkt door de aanwezigheid van het geconserveerde COMM domein. Onderzoeksresultaten van Burstein *et al*. hebben aangetoond dat alle COMMD eiwitten, net zoals COMMD1, een remmende werking hebben op de activiteit van de transcriptiefactor NF-κB. Deze functie van COMMD6 hebben we verder onderzocht, waarbij de bevindingen (beschreven in **hoofdstuk 3**) erop wijzen dat COMMD6 voor zijn remmende activiteit op NF-κB afhankelijk is van twee zeer geconserveerde residuen in het COMM domein.

Vervolgens hebben we onderzocht of COMMD eiwitten een rol spelen binnen de koperhomeostase op basis van mogelijke interacties met ATP7A en ATP7B. Onze bevindingen, zoals beschreven in **hoofdstukken 4 en 5**, duiden erop dat meerdere COMMD eiwitten een interactie aangaan met ATP7A en/of ATP7B. Deze resultaten suggereren ook dat deze COMMD eiwitten een rol zouden kunnen spelen in de ontwikkeling van stoornissen van koper homeostase bij de mens. Naast de ziekte van Wilson bestaan nog drie andere ziektebeelden gekenmerkt door koperstapeling in de lever, waarvan het onderliggende genetische defect nog niet is gekarakteriseerd. Om het inzicht in de genetische oorsprong van deze ziektes te vergroten, hebben we de *COMMD* genen wier genproducten een interactie aangaan met ATP7B onderzocht als kandidaatgenen voor deze niet-Wilson vormen van koperstapeling. In dit onderzoek (beschreven in **hoofdstuk 4**) hebben we een aantal polymorfismen in de DNA sequenties van *COMMD2* en *COMMD10* geïdentificeerd, die afwijken in de patiëntengroep ten opzichte van de normale populatie en dus mogelijk kunnen bijdragen aan de ontwikkeling van koperstapeling in deze patiënten. De functionele gevolgen van deze polymorfismen zijn onderwerp van vervolgonderzoek, waarmee de rol van COMMD2 en COMMD10 in de ontwikkeling van koperstapelingsziekten bij de mens verder kan worden getest.

De functionaliteit van de waargenomen interacties van COMMD1 met ATP7A en ATP7B hebben we onderzocht in de studies beschreven in de **hoofdstukken 5 en 6**. Onze experimentele data wijzen erop dat COMMD1 een stimulerende rol heeft in de proteolyse van ATP7B. Deze waarneming is consistent met de bevindingen dat COMMD1 ook de stabiliteit reguleert van meerdere componenten van de HIF1 en NF-κB signaaltransductie paden. De data beschreven in **hoofdstuk 6** onthullen tevens een ontregeling van de interactie tussen COMMD1 en ATP7B ten gevolge van een aantal specifieke mutaties in *ATP7B* die de ziekte van Wilson veroorzaken. Deze bevinding duidt erop dat COMMD1, in ten minste een deel van de patiënten, betrokken is bij de pathogenese van de ziekte van Wilson.

Samengevat heeft het onderzoek beschreven in dit proefschrift bijgedragen aan de identificatie en karakterisering van de COMMD eiwit familie. Onze data duiden op een belangrijke rol voor deze familie in de regulatie van de koperhomeostase door eiwit-eiwit interacties met ATP7A en/of ATP7B. Tevens tonen onze data aan dat COMMD eiwitten mogelijk betrokken zijn bij de ontwikkeling van koperstapelingsziekten bij de mens.

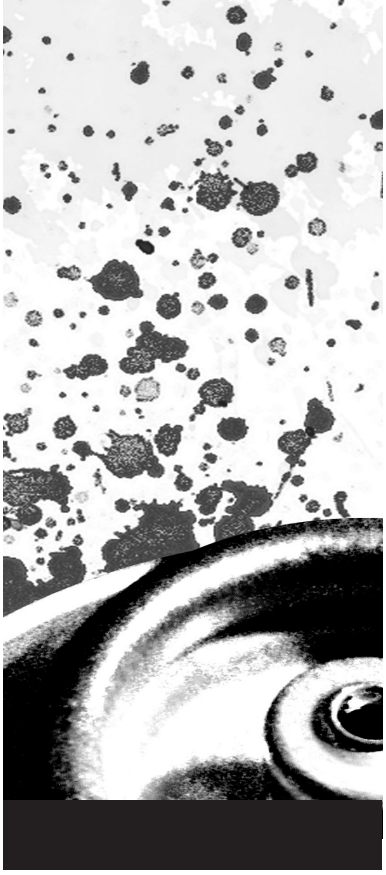


Curriculum Vitae

Prim de Bie is geboren op 27 december 1978 te Goirle. Na het behalen van zijn VWO diploma aan de Scholen Gemeenschap Huizermaat in Huizen is hij in 1997 Medische Biologie gaan studeren aan de Universiteit Utrecht. In 2001 heeft hij zijn negen maanden durende hoofdvak stage doorlopen bij het Eijkman-Winkler Instituut in het UMC Utrecht onder begeleiding van Dr. Niki A. Georgiou, Dr. B. Sweder van Asbeck en Prof. Dr. Jo J.M. Marx. Tijdens deze stage onderzocht hij de remming van HIV-1 replicatie door het gebruik van ijzerchelatoren. Vervolgens doorliep hij zijn zes maanden durende bijvak stage bij de Afdeling Medische Genetica in het UMC Utrecht onder begeleiding van Dr. Bart van de Sluis en Prof. Dr. Cisca Wijmenga. Tijdens deze stage heeft hij nieuwe eiwitten geïdentificeerd die een interactie aangaan met het koper toxicose eiwit COMMD1. Na het behalen van zijn doctoraal diploma in Augustus 2002, heeft hij dit onderzoek vervolgd als AIO bij de afdeling Medische Genetica en het Laboratorium voor Metabole en Endocriene Ziekten in het UMC Utrecht, onder begeleiding van Dr. Leo W.J. Klomp, Prof. Dr. Cisca Wijmenga en Prof. Dr. Ruud Berger. Als onderdeel van dit onderzoek heeft hij in 2004 drie maanden gewerkt in het laboratorium van Dr. Colin S. Duckett in Ann Arbor, MI, USA. De resultaten van dit onderzoek staan beschreven in dit proefschrift.
Prim de Bie zal zijn academische carrière voortzetten als postdoctoraal medewerker in het Technion Instituut te Haifa in Israël, onder begeleiding van Prof. Dr. Aaron Ciechanover. Hier zal hij de mechanismen van eiwit afbraak volgens het ubiquitine-proteasoom systeem bestuderen.

Prim de Bie was born on December 27th 1978 in Goirle, The Netherlands. He attended the S.G. Huizermaat secondary school, after which he started to study Medical Biology at the Utrecht University, The Netherlands in 1997. In 2001 he performed a nine-month practical training rotation ate the Eijkman-Winkler Institute at the University Medical Center Utrecht, under supervision of Dr. Niki A. Georgiou, Dr. B. Sweder van Asbeck and Prof. Jo J.M. Marx, where he studied the inhibition of HIV-1 replication by iron chelators. In 2002 he performed a six-month practical training rotation at the department of Medical Genetics at the University Medical Center Utrecht, under supervision of Dr. Bart van de Sluis and Prof. Cisca Wijmenga, where he identified novel interacting partners for the copper toxicosis protein COMMD1. After graduating as MSc in Medical Biology in august 2002, he continued his research on the copper toxicosis protein COMMD1 as a PhD student at the department of Medical Genetics and the Laboratory of Metabolic and Endocrine Diseases at the University Medical Center Utrecht, under supervision of Dr. Leo W.J. Klomp, Prof. Cisca Wijmenga and Prof. Ruud Berger. As part of his research he has also worked three months in the laboratory of Dr. Colin S. Duckett in Ann Arbor, MI, USA. The results of his research are described in this thesis.

Prim de Bie will continue his academic career as a postdoctoral fellow in the Technion Institute in Hafa, Israel, in the laboratory of Prof. Aaron Ciechanover. Here, he will study the mechanisms of the ubiquitin-proteasome system.



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De Bie P, Muller PAJ, Wijmenga C, Klomp LWJ. Molecular pathogenesis of Wilson and Menkes disease; correlation of mutations with molecular defects and disease phenotypes. *Journal of Medical Genetics, in press.*

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De Bie P, Van de Sluis B, Müller T, Burstein E, Berger R, Wijmenga C, Klomp LWJ. Protein-protein interactions with ATP7B implicate *COMMD* genes as candidate genes for human disorders of hepatic copper overload. *Submitted*

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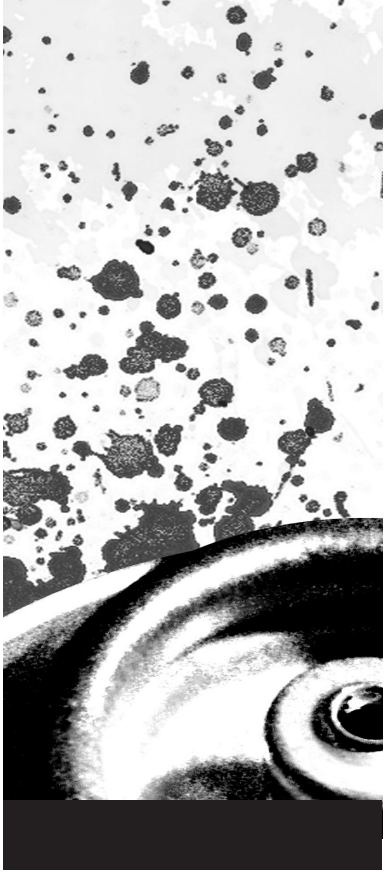

Dankwoord

Nu rest mij nog een laatste, en toch heel belangrijke, deel van mijn proefschrift. In de afgelopen jaren waarin ik mijn promotieonderzoek heb uitgevoerd heb ik veel geleerd en steun en hulp gekregen van al mijn collega's, vrienden en familie. Hierbij wil ik de gelegenheid nemen om iedereen daarvoor bedanken en een aantal mensen in het bijzonder.

Te beginnen bij de drijvende krachten achter dit onderzoek, mijn co-promotor Dr. Leo Klomp en mijn promotoren Prof. Dr. Cisca Wijmenga en Prof. Dr. Ruud Berger, voor de mogelijkheid die zij mij hebben gegeven om dit promotieonderzoek uit te voeren.

Beste Leo, ik wil je voornamelijk bedanken voor de zelfstandigheid die je me hebt gegeven in het uitvoeren en uitwerken van het onderzoek. Ik heb grote waardering voor je didactische kwaliteiten, waarmee je de ontwikkeling van de AIO vooropstelt. Hierdoor heb ik mijn tijd als AIO als enorm leerzaam ervaren. Ik vond het erg prettig met je te werken, niet in de minste plaats omdat je altijd een dankbaar doelwit blijkt te zijn voor onze lab-terreur (waarbij je het ook niet na laat om op gepaste wijze wraak te nemen). Beste Cisca, het hele avontuur is begonnen toen ik als student in jouw lab terecht kwam. Ik ben erg blij dat ik de mogelijkheid heb gekregen om het project waar ik toen mee ben begonnen als promotieonderzoek voort te kunnen zetten. Ik heb vooral veel bewondering voor jouw strategische- en managementskwaliteiten, waarmee je het lab enorm hebt doen groeien in de tijd dat ik er heb gewerkt. Ik heb daar veel van geleerd, voornamelijk om buiten mijn experimenten, ook mijn academische carrière strategisch te plannen. Het is jammer dat jouw rol in de kopergroep kleiner is geworden met jouw vertrek naar Groningen. Ik wens je heel veel succes met de start van je nieuwe lab. Beste Ruud, ondanks dat jouw betrokkenheid tot mijn onderzoek zich meer op de achtergrond afspeelde, wil ik je bedanken voor alle scherpe commentaren en suggesties bij de werkbesprekingen. Ik heb altijd met veel plezier binnen jouw afdeling gewerkt.

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afdeling. Door de tijd heen zijn er ook een hoop mensen gekomen en gegaan van het lab, wat ons lab altijd een enorm leuke en dynamische werkplek heeft gemaakt. Iedereen enorm bedankt voor alle leuke collegialiteit! Een aantal oud-collega's wil ook nog even noemen: Sjaacie (of is het tegenwoordig Sjansie, of gewoon Mevrouw Jansen?), Joep (dude) en Denis voor de "week of the practical joke". Vrijdagmiddag 15.30 is niet meer hetzelfde geweest na jouw vertrek.

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