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Functional consequences of RNA interference targeting *COMMD1* in a canine hepatic cell line in relation to copper toxicosis

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Summary

A deletion in the *copper metabolism* (*Murr1*) *domain containing 1* (*COMMD1*) gene is associated with hepatic copper toxicosis in dogs, yet evidence of copper retention in *COMMD1*-depleted hepatic cells has not been shown. In a dog hepatic cell line, we analysed the copper metabolic functions after an 80% (mRNA and protein) *COMMD1* reduction with *COMMD1*-targeting siRNAs. Exposure to ^{64}Cu resulted in a significant increase in copper retention in *COMMD1*-depleted cells. *COMMD1*-depleted cells were almost three times more sensitive to high extracellular copper concentrations. Copper-mediated regulation of *metallothionein* gene expression was enhanced in *COMMD1*-depleted cells. Based on the increased copper accumulation and enhanced cellular copper responses upon *COMMD1* reduction, we conclude that *COMMD1* has a major regulatory function for intracellular copper levels in hepatic cells.

Keywords copper, functional genomics, liver, metallothionein, siRNA.

The COMMD family is characterized by the presence of a conserved and unique motif called COMM (copper metabolism MURR1 containing) domain (Burstein *et al.* 2005). COMMD1, previously known as MURR1, functions as an interface for protein–protein interactions (de Bie *et al.* 2005, 2006). Several functions of COMMD1 have been reported such as the regulation of sodium transport and copper metabolism (Biasio *et al.* 2004; Struehler *et al.* 2004). *COMMD1* is mutated in Bedlington terriers and associated with massive accumulation of hepatic copper subsequently leading to increased oxidative stress, hepatitis and finally cirrhosis (van de Sluis *et al.* 2002). The deletion of *COMMD1* exon 2 in Bedlington terriers leads to the complete absence of the protein (Klomp *et al.* 2003). The phenotype of the affected Bedlington terriers does not indicate a role for COMMD1 in sodium transport.

In the past years, two studies on the genetic background of copper accumulation in Bedlington terriers have been reported (Rothuizen *et al.* 1999; Forman *et al.* 2005). Yet functional genomic studies on *COMMD1* in copper metabolism, preferentially in assays resembling the clinical situation, are limited. In a human embryonic kidney epithelial cell line (HEK293), mRNA interference experiments showed a moderately increased intracellular retention of copper during silencing of the gene (Burstein *et al.* 2004). *COMMD1*, which has high expression in liver, is also expressed in heart, kidney, muscle and placenta (Klomp *et al.* 2003). However, in the Bedlington terriers copper accumulation is restricted to the liver, and no signs of copper accumulation are observed in heart, kidney, muscle and placenta. Therefore, to further elucidate a direct or indirect role of *COMMD1* in hepatic copper metabolism, studies in liver cells derived from dogs are required.

In order to clarify the role of COMMD1 in copper metabolism of dog hepatic cells, we used an RNA interference (RNAi) strategy to target the *COMMD1* gene product. COMMD1-specific siRNA's were transfected into a hepatic cell line (bile-duct epithelial cells, BDE), which displays all characteristics of hepatocytes, such as production of serum albumin and ceruloplasmin (Oda *et al.* 1996). We investigated the differences in ^{64}Cu retention and copper-mediated cell death in BDE cells after *COMMD1* gene silencing with siRNAs. Differential expression of metallothionein, highly regulated by intracellular copper, was measured to evaluate functional effects of the loss of *COMMD1*. Taken together, this is the first functional genomics study on *COMMD1* that combines copper retention with biological consequences of *COMMD1* depletion.

We transfected BDE cells with siRNA specific for the canine *COMMD1* gene product (see Supplementary

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Material). The transfection efficiency was optimized as described previously (Spee *et al.* 2006). When treated with the optimal amount of siRNA (50 nM) for 72 h, *COMMD1* expression was markedly reduced. Cells treated with nonsense siRNA exhibited a similar expression level as the untreated control cells. Western blotting yielded a 25-kDa band immunoreactive to COMMD1 in the untreated control and nonsense siRNA-transfected samples. At 48 h after transfection, a marked decrease was observed in signal intensity for COMMD1 compared with controls. Densitometric analysis indicated an 80% reduction of COMMD1 protein levels in the siRNA treated samples. Gene-expression measurements and protein analysis did not indicate any effect on COMMD1 levels treated with different concentrations of extracellular copper over a 2-day period (Figure S1).

The sensitivity of COMDD1-depleted cells towards extracellular copper was determined by the frequently used MTT cell viability assay. Mock transfected cells and nonsense siRNAs were used as a control. Results showed that the ED_{50} of the COMMD1-depleted cells was reduced over twofold from approximately 64 to around 30 μg/ml (Figure S2).

BDE cells were incubated in the presence of different extracellular copper concentrations (0, 25, 50 and 100 μM) for 2 days. A MTT assay was run in parallel to correct for cellular death. Results (Fig. 1) showed the average amount of gamma-counts corrected for the percentage viable cells. After 2 days in the presence of 25 μM extracellular copper, copper retention was not statistically different between COMMD1-depleted and mock- and nonsense-treated control cells. With increasing extracellular copper concentrations, a significant difference between COMMD1-depleted and control cells occurred. Copper retention was increased 1.5-fold in the 100 μM copper treated *COMMD1* siRNA-treated group.

Metallothionein (MT1A) is known to be regulated by intracellular copper levels (Coyle *et al.* 2002). In order to show copper effects on *MT1A* gene expression, cells were exposed to the same copper concentration range as in the survival and copper accumulation experiment. As depicted in Fig. 2, *MT1A* mRNA expression was upregulated twofold after 50-μM copper treatment, and sevenfold after 100-μM copper treatment, in COMMD1-expressing cells. Interestingly COMMD1-depleted cells showed a 15-fold increase in *MT1A* expression after 100-μM copper treatment. A significant change in the 100-μM treated group between non-depleted and COMMD1-depleted cells was found.

COMMD1 was discovered after positional cloning and is responsible for an autosomal recessive form of hepatic copper toxicosis that affects Bedlington terriers (van de Sluis *et al.* 2002). Although an interaction with ATP7B has been described (Tao *et al.* 2003), the mechanism by which COMMD1 affects copper metabolism remains elusive. Here we report the characterization of COMMD1-depleted hepatic epithelial cells in order to perform functional genomics studies on the COMMD1 protein.

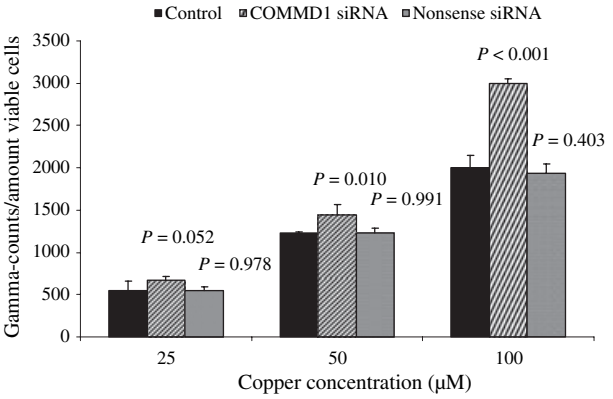

Figure 1 Copper isotope measurements in COMMD1-depleted cells. Copper isotope (^{64}Cu) measurements in mock transfected (control), COMMD1 siRNA-treated and nonsense-treated BDE cells after a two-day treatment of a 25 μM, 50 μM and 100 μM ^{64}Cu. Gamma-counts were corrected for cellular death by means of an MTT assay that ran in parallel. Statistical significance of differences in gamma-counts of the COMMD1-depleted cell lines compared to control and mock-treated cells were determined by a one-way ANOVA using the Dunnett multiple comparisons test. A *P*-value < 0.05 was considered statistically significant.

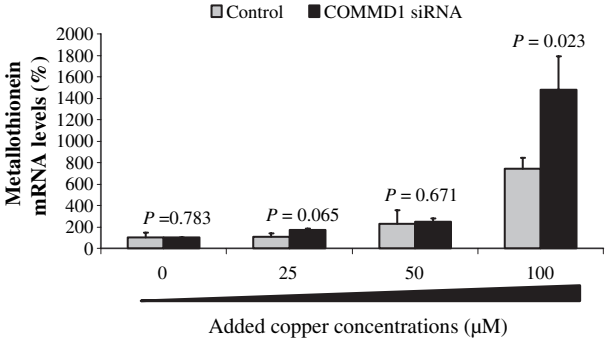

Figure 2 Gene expression of copper *metallothionein* (*MT1A*) 2 days after copper treatment. Data depicts mean + SD. Statistical significance of differences between treated and control samples were determined by using the Mann–Whitney *U*-test. A *P*-value < 0.05 was considered statistically significant.

At present, no human copper storage disease is associated with *COMMD1* mutations and no *COMMD1*$^{-/-}$ mice are available. In view of the hepatic features of the BDE cell line and the species used in this study, these *in vitro* data can be extrapolated to the only known clinical cases associated with *COMMD1* mutations, viz. hepatic copper accumulation in Bedlington terriers (Fuentealba & Aburto 2003). To facilitate future research, we have an in-house dog breed (mixed Bedlington with Beagle) that is homozygous for the *COMMD1* exon 2 deletion. This allows us to study longitudinally and in great detail how copper toxicosis develops *in vivo*. These dogs are potential animal models for chronic liver diseases (Mack 2005; Neff & Rine 2006).

COMMD1 is localized within the cytoplasm within perinuclear compartments that do not represent mitochondria

or lysosomes (Klomp *et al.* 2003). This observation, together with the observed copper accumulation in COMMD1-depleted cells, indicates an excretory function for COMMD1.

It is expected that cells that accumulate the highest levels of copper are affected the most and could detach from the culture dish. Because we removed the detached cells and consequently measured copper accumulation in only attached cells, it is possible that total copper retention was even higher in BDE cells. As copper is transferred to blood in the intestine, the liver is the first organ involved in the detoxification of high amounts of copper, indicating an even higher degree of importance of the used cell line.

We have shown that siRNA directed against canine *COMMD1* strongly down-regulated both *COMMD1* mRNA and protein levels. This effect cannot be reverted by high concentrations of extra-cellular copper (Figure S1). A reduction of COMMD1 levels resulted in a higher sensitivity of these cells for extra-cellular copper concentrations (Figure S2). Copper accumulation is higher in COMMD1-depleted cells than in the mock and nonsense siRNA treated cells (Fig. 1). Finally, *MT1A*, which is strongly inducible by copper, is twice as much increased in COMMD1-depleted cells (Fig. 2). Taken together, we demonstrate the importance of COMMD1 in copper metabolism and functional consequences of reduced COMMD1 levels in a canine hepatic cell line.

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References

Biasio W., Chang T., McIntosh C.J. & McDonald F.J. (2004) Identification of Murr1 as a regulator of the human delta epithelial sodium channel. *Journal of Biological Chemistry* **279,** 5429–34.

de Bie P., van de Sluis B., Klomp L. & Wijmenga C. (2005) The many faces of the copper metabolism protein MURR1/COMMD1. *Journal of Heredity* **96,** 803–11.

de Bie P., van de Sluis B., Burstein E., Duran K.J., Berger R., Duckett C.S., Wijmenga C. & Klomp L.W. (2006) Characterization of COMMD protein-protein interactions in NF-kappaB signalling. *Biochemical Journal* **398,** 63–71.

Burstein E., Ganesh L., Dick R.D. *et al.* (2004) A novel role for XIAP in copper homeostasis through regulation of MURR1. *EMBO Journal* **23,** 244–54.

Burstein E., Hoberg J.E., Wilkinson A.S., Rumble J.M., Csomos R.A., Komarck C.M., Maine G.N., Wilkinson J.C., Mayo M.W. & Duckett C.S. (2005) COMMD proteins, a novel family of structural and functional homologs of MURR1. *Journal of Biological Chemistry* **280,** 22222–32.

Coyle P., Philcox J.C., Carey L.C. & Rofe A.M. (2002) Metallothionein: the multipurpose protein. *Cellular and Molecular Life Sciences* **59,** 627–47.

Forman O.P., Boursnell M.E., Dunmore B.J. *et al.* (2005) Characterization of the *COMMD1* (*MURR1*) mutation causing copper toxicosis in Bedlington terriers. *Animal Genetics* **36,** 497–501.

Fuentealba I.C. & Aburto E.M. (2003) Animal models of copper-associated liver disease. *Comparative Hepatology* **2,** 5.

Klomp A.E., van de Sluis B., Klomp L.W. & Wijmenga C. (2003) The ubiquitously expressed MURR1 protein is absent in canine copper toxicosis. *Journal of Hepatology* **39,** 703–9.

Mack G.S. (2005) Cancer researchers usher in dog days of medicine. *Nature Medicine* **11,** 1018.

Neff M.W. & Rine J. (2006) A fetching model organism. *Cell* **124,** 229–31.

Oda D., Savard C.E., Nguyen T.D., Eng L., Swenson E.R. & Lee S.P. (1996) Dog pancreatic duct epithelial cells: long-term culture and characterization. *American Journal of Pathology* **148,** 977–85.

Rothuizen J., Ubbink G.J., van Zon P., Teske E., van den Ingh T.S.G.A.M. & Yuzbasiyan-Gurkan V. (1999) Diagnostic value of a microsatellite DNA marker for copper toxicosis in West-European Bedlington terriers and incidence of the disease. *Animal Genetics* **30,** 190–4.

van de Sluis B., Rothuizen J., Pearson P.L., van Oost B.A. & Wijmenga C. (2002) Identification of a new copper metabolism gene by positional cloning in a purebred dog population. *Human Molecular Genetics* **11,** 165–73.

Spee B., Jonkers M.D., Arends B., Rutteman G.R., Rothuizen J. & Penning L.C. (2006) Specific down-regulation of XIAP with RNA interference enhances the sensitivity of canine tumor cell-lines to TRAIL and doxorubicin. *Molecular Cancer* **5,** 34.

Struehler B., Reichert J., Stremmel W. & Schaefer M. (2004) Analysis of the human homologue of the canine copper toxicosis gene *MURR1* in Wilson disease patients. *Journal Molecular Medicine* **82,** 629–34.

Tao T.Y., Liu F., Klomp L., Wijmenga C. & Gitlin J.D. (2003) The copper toxicosis gene product Murr1 directly interacts with the Wilson disease protein. *Journal of Biological Chemistry* **278,** 41593–96.

Supplementary Material

The following supplementary material for this article is available online at http://www.blackwell-synergy.com/doi/full/10.1111/j.1365-2052.2007.01580.x

Figure S1 *COMMD1* gene expression and protein levels after siRNA treatment.

Figure S2 Cell viability after two-day copper treatment with a serial dilution of copper.

Methods S1

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