The Menkes and Wilson disease genes counteract in copper toxicosis in Labrador retrievers: a new canine model for copper-metabolism disorders

Hille Fieten1,*, Yadvinder Gill2, Alan J. Martin2, Mafalda Concilli3, Karen Dirksen1, Frank G. van Steenbeek1, Bart Spee1, Ted S. G. A. M. van den Ingh4, Ellen C. C. P. Martens1, Paola Festa3, Giancarlo Chesi3, Bart van de Sluis5, Roderick H. J. H. Houwen6, Adrian L. Watson2, Yurii S. Aulchenko7,8, Victoria L. Hodgkinson9,10, Sha Zhu9,10, Michael J. Petris9,10,11, Roman S. Polishchuk3, Peter A. J. Leegwater1 and Jan Rothuizen1

ABSTRACT
The deleterious effects of a disrupted copper metabolism are illustrated by hereditary diseases caused by mutations in the genes coding for the copper transporters ATP7A and ATP7B. Menkes disease, involving ATP7A, is a fatal neurodegenerative disorder of copper deficiency. Mutations in ATP7B lead to Wilson disease, which is characterized by a predominantly hepatic copper accumulation. The low incidence and the phenotypic variability of human copper toxicosis hamper identification of causal genes or modifier genes involved in the disease pathogenesis. The Labrador retriever was recently characterized as a new canine model for copper toxicosis. Purebred dogs have reduced genetic variability, which facilitates identification of genes involved in complex heritable traits that might influence phenotype in both humans and dogs. We performed a genome-wide association study in 235 Labrador retrievers and identified two chromosome regions containing ATP7A and ATP7B that were associated with variation in hepatic copper levels. DNA sequence analysis identified missense mutations in each gene. The amino acid substitution ATP7B:p.Arg1453Gln was associated with copper accumulation, whereas the amino acid substitution ATP7A:p.Thr327Ile partly protected against copper accumulation. Confocal microscopy indicated that aberrant copper metabolism upon expression of the ATP7B variant occurred because of mis-localization of the protein in the endoplasmic reticulum. Dermal fibroblasts derived from ATP7A:p.Thr327Ile dogs showed copper accumulation and delayed excretion. We identified the Labrador retriever as the first natural, non-rodent model for ATP7B-associated copper toxicosis. Attenuation of copper accumulation by the ATP7A mutation sheds an interesting light on the interplay of copper transporters in body copper homeostasis and warrants a thorough investigation of ATP7A as a modifier gene in copper-metabolism disorders. The identification of two new functional variants in ATP7A and ATP7B contributes to the biological understanding of protein function, with relevance for future development of therapy.

KEY WORDS: ATP7A, ATP7B, Dog, Liver

INTRODUCTION
Copper is an essential trace element for a wide range of biochemical processes in the body. The P-type copper-transporting ATPases ATP7A (Vulpe et al., 1993) and ATP7B (Bull et al., 1993) are crucial for copper homeostasis. At the cellular level, these proteins have a biosynthetic role in the trans-Golgi network, where they facilitate incorporation of copper into proteins. Furthermore, they prevent toxic accumulation of cellular copper by redistribution to a vesicular compartment, resulting in excretion of copper through either the apical membrane (ATP7B) or the basolateral membrane (ATP7A). This redistribution involves metal-binding domains (MBDs) in cytoplasmic domains of the proteins and is regulated in part by their phosphorylation state (Voskoboinik et al., 2002).

Body copper homeostasis is maintained by balancing the rates of dietary copper absorption and biliary copper excretion. ATP7A is highly expressed in enterocytes, where it is involved in copper uptake across the basolateral membrane into the portal circulation for delivery to the liver, where copper is stored (Linder and Hazegh-Azam, 1996). Besides its function in intestinal epithelial cells, ATP7A was recently identified to play an important role in hepatic copper mobilization in response to copper demands of peripheral tissues (Kim et al., 2010). Excess hepatic copper is excreted into the bile and requires expression of functional ATP7B in hepatocytes (Gitlin, 2003).

The severe effects of copper-metabolism imbalance are illustrated by inherited disorders resulting from mutations in ATP7A and ATP7B. Mutations in ATP7A result in a fatal, X-linked copper-deficiency disorder in infants known as Menkes disease. The disease is characterized by cerebral and cerebellar degeneration, failure to thrive, coarse hair and connective tissue abnormalities (Kaler, 2011).

Wilson disease results from mutations in ATP7B (Gitlin, 2003) and is associated with copper accumulation in the liver and secondarily in the brain, resulting in hepatic cirrhosis and neuronal degeneration. The age of onset and the clinical manifestations vary greatly between individuals affected by Wilson disease. This lack of
text: 2002). However, convincing evidence of involvement of hepatic copper accumulation in infants include Indian childhood cirrhosis (Tanner, 1998) and endemic Tyrolean infantile cirrhosis (Müller et al., 1996). In these diseases, the causal genes are currently unknown, and dietary copper intake is believed to contribute significantly to disease progression.

In order to develop new treatment strategies for copper-metabolism disorders, several rodent models were investigated, including natural models, such as the mottled mouse (Grimes et al., 1997), the toxic milk mouse (Theophilos et al., 1996) and the Long–Evans cinnamon rat (Li et al., 1991), in addition to ATP7A (Wang et al., 2012) and ATP7B knockouts (Buiakova et al., 1999). Although rodent models are invaluable for studying diseases, the dog as a large-animal model represents a unique translational bridge between rodents and humans. The best-characterized canine model for copper toxicity is the Bedlington terrier, in which severe hepatic copper accumulation is caused by a deletion in the COMMD1 gene (Van de Sluis et al., 2002). However, convincing evidence of involvement of the COMMD1 gene in human copper-metabolism disorders is lacking (Coronado et al., 2005; Lovicu et al., 2006; Müller et al., 2003).

The Labrador retriever dog breed was recently characterized as a new mammalian model for copper toxicosis, distinct from COMMD1-associated autosomal recessive copper toxicosis. In predisposed Labrador retrievers, hepatic copper accumulation induces hepatic cirrhosis, usually in middle-aged dogs. Female Labrador retrievers are at increased risk for the disease (Fieten et al., 2012b).

The genes underlying the disease in Labrador retrievers were not known. We hypothesized that identification of these genes would contribute to the understanding of copper-metabolism biology in dogs and other mammals. Given that the copper genome is highly conserved across species, this study might reveal genes that are candidate modifiers in human copper-metabolism disorders. Furthermore, elucidating the genetic background of copper toxicosis in Labrador retrievers contributes to the characterization of this new model for copper-metabolism disorders, necessary to exploit its possibilities fully.

Whereas gene-mapping studies in human complex diseases require thousands of affected individuals and millions of single nucleotide polymorphism (SNP) markers, smaller numbers suffice for a genome-wide association study (GWAS) of inbred pedigree dogs owing to the reduced genetic and phenotypic heterogeneity. Therefore, the dog is a useful model for identifying genes in naturally occurring complex hereditary diseases that are phenotypically similar in dogs and humans (Boyko, 2011).

The aims of the present study were to identify gene variants involved in copper toxicosis in the Labrador retriever and to provide biological evidence for their involvement in the disease.

RESULTS

Dogs

The initial GWAS cohort consisted of 235 Labrador retrievers. The independent replication cohort consisted of 59 Labrador retrievers. The median copper score in the 294 Labrador retrievers included in the study was 1.5 (range 0-5). A summary of characteristics of the dogs, including age, sex and hepatic copper scores, is provided in Table S1.

Electron microscopic evaluation of copper toxicosis in Labrador retrievers

To complement the phenotype description of copper toxicosis in Labrador retrievers, liver biopsies of two Labrador retrievers affected with copper toxicosis and a control were assessed by electron microscopy. Ultrastructural changes in the biopsies of the affected dogs corresponded to copper-laden lysosomes, comparable to what is recognized by electron microscopy of the liver of human individuals with Wilson’s disease (Fig. 1).

Genome-wide association study of copper toxicosis in Labrador retrievers

After quality control of genotype data used for the GWAS, 35.7% of SNPs were removed because of a low minor allele frequency and 0.7% of SNPs had a call rate of less than 98%, leaving 109,496 SNPs and 235 dogs for analysis. Population stratification was determined by a multidimensional scaling plot (Fig. S1), and inflation of the test statistic was inspected by a QQ-plot (Fig. S2). The heritability of hepatic copper score was estimated to be 0.48. The highest association signal using hepatic copper level as a quantitative trait in a linear mixed model was identified on chromosome 22, with a P-value of 2.4 × 10^{-17} and a β of 0.65 (s.e. 0.14; Fig. 2A). Inspection of the region showed a linkage disequilibrium (LD) block at the first 10 Mb of the chromosome. The crucial interval was determined to be 22:152034-10753911, which contained 83 genes, including the candidate gene ATP7B, located at position 22:162769-225266 (Canfam 3.1; Fig. 2B).

A stratified analysis for males and females was performed (Fig. 2C,D). In the male subset, the crucial region was located at X:54322875-65713151 and contained 77 genes. The highest association signal ([P=1.1 × 10^{-14} and a β of ~0.57 (s.e. 0.15)]) was caused by six SNPs that formed an LD block of 8.2 Mb, including the candidate gene ATP7A at position X:60203319-60356690 (Fig. 2C).

Given the fact that ATP7A and ATP7B code for copper transporters, they were strong candidate genes for involvement in copper toxicosis; therefore, we focused on these genes for in-depth DNA sequence analysis. A list of observed DNA variants and their effect on the hepatic copper score is presented in Table 1.

The five nominally significant variations (P≤0.05) were genotyped in an independent cohort of 59 Labrador retrievers (Table 1; Table S1). The association with hepatic copper score was replicated for the non-synonymous nucleotide substitution of ATP7A at X-chromosomal position 60279238 (ENSCAFT000000049745 ATP7A:c.980C>T), the intronic variation in ATP7A (position X:60338569) and the non-synonymous nucleotide substitution of ATP7B at chromosome 22, position 225112 (ENSCAFT0000006859 ATP7B:c.4358G>A). When analysed in the complete data set (n=294), the P-values were similar to or lower than initially derived in the GWAS, 1.6 × 10^{-7} and 4.9 × 10^{-7} for ATP7A:c.980C>T and ATP7B:c.4358G>A, respectively (Table 1).

When inspecting the effect of the combined genotypes for ATP7A and ATP7B on hepatic histological copper levels (Table 2), we appreciated that for both sexes ATP7B:c.4358A was associated with increased hepatic copper levels in an additive way. This effect is most clear in female dogs, leading to a significant difference between copper levels of dogs homozygous for ATP7B:c.4358A and any of the other dogs. Concurrent presence of ATP7A:c.980T attenuated the copper accumulation effect in both sexes. This effect was most notable in males, leading to a significant difference between dogs with and without the mutant allele of ATP7A (Table 2).

The heritability for hepatic copper score in the model including the co-variates sex, ATP7A:c.980C>T and ATP7B:c.4358G>A was calculated to be 0.42. Thus, the explained genetic variability by the two non-synonymous mutations is [(0.48−0.42)/0.48] 12.5%, of
which 4.3% could be attributed to \( ATP7A: c.980C>T \) and 8.2% could be attributed to \( ATP7B: c.4358G>A \).

\( ATP7A: c.980C>T \) resulted in an amino acid substitution from a threonine to an isoleucine in the third MBD of \( ATP7A \) at position 327 (Ensembl: ENSCAFT00000049745, \( ATP7A: p.Thr327Ile \)). \( ATP7B: c.4358G>A \) caused an amino acid substitution from an arginine to a glutamine in the C-terminus of the \( ATP7B \) protein at position 1453 (Ensembl: ENSCAFT00000006859, \( ATP7B: p.Arg1453Gln \)). Alignment of the amino acid sequence adjacent to canine \( ATP7A: p.Thr327Ile \) (\( ATP7A^{Thr327Ile} \)) and \( ATP7B: p.Arg1453Gln \) (\( ATP7B^{Arg1453Gln} \)) indicated strong conservation of the domains in mammals (Fig. 3A, B).

**Functional evaluation of \( ATP7B^{R1415Q} \)**

\( ATP7B^{R1415Q} \) is partly mis-localized to the endoplasmic reticulum

To analyse how the canine \( ATP7B: p.Arg1453Gln \) might affect copper homeostasis at the cellular level, we expressed the mutant protein exogenously in a cell line. To investigate the consequences of canine \( ATP7B: p.Arg1453Gln \) substitution on the subcellular localization of \( ATP7B \), the conserved arginine (Arg) at position 1415 (Ensembl: ENST00000242839) of human GFP-tagged \( ATP7B \) was replaced by a glutamine (Gln). Both human \( ATP7B: p.Arg1415-GFP \) (\( ATP7B^{WT} \)) and \( ATP7B: p.Gln1415-GFP \) (\( ATP7B^{R1415Gln} \)) were expressed in HeLa cells and and treated with either bathocuproine disulphonate (BCS) or 200 \( \mu \)M CuSO\(_4\). Confocal microscopy revealed that in low-Cu\(^{2+} \) conditions, \( ATP7B^{WT} \)-GFP exhibited significant overlap with the \( \text{trans} \)-Golgi marker TGN46 as reported before (Barnes et al., 2009), whereas stimulation with CuSO\(_4\) induced \( ATP7B^{WT} \)-GFP trafficking to the cell surface and numerous cytosolic vesicular structures (Fig. 4A; Fig. S3). In contrast, a significant amount of \( ATP7B^{R1415Gln} \)-GFP was detected in the endoplasmic reticulum (ER) network-like membranes in both low- and high-Cu\(^{2+} \) conditions (Fig. 4A; Fig. S3), although substantial amounts of the protein were delivered to the Golgi and post-Golgi compartments. Morphometric analysis confirmed an increase in the percentage of cells that exhibited GFP signal in the ER upon expression of \( ATP7B^{R1415Gln} \), whereas the number of cells with \( ATP7B^{WT} \)-GFP trafficking to the cell surface and numerous cytosolic vesicles was very low (Fig. 4C). Partial mis-localization of \( ATP7B^{R1415Gln} \) to the ER compartment was also observed in an HepG2 cell line (Fig. 4B, C). This indicated that the substitution of arginine to glutamine impairs (at least partly) appropriate compartmentalization of \( ATP7B \).

\( ATP7B^{R1415Gln} \) fails to reach the canalicular domain of polarized HepG2 cells

To verify whether \( ATP7B^{R1415Gln} \) can be targeted to the apical (canalicular) domain of hepatic cells, which is necessary to remove...
excess $\text{Cu}^{2+}$ from hepatocytes into bile, we grew HepG2 cells in the conditions that allow their maximal polarization (Slimane et al., 2003). Then, the cells were transfected with either ATP7B$^{\text{WT}}$-GFP or ATP7B$^{R1415Q}$-GFP and subsequently incubated with 200 $\mu$M CuSO$_4$. Stimulation with copper resulted in delivery of ATP7B$^{\text{WT}}$-GFP to the apical surface domains, which was labeled with the canalicular marker multidrug resistance protein 2 (MRP2) (Fig. 4B; Fig. S4). On the contrary, ATP7B$^{R1415Q}$-GFP failed to arrive at the canalicular surface of the cells (Fig. 4B; Fig. S4) and remained mainly in the intracellular cytoplasmic structures, as it has been already demonstrated for another ER-retained mutant, ATP7B$^{H1069Q}$ (Polishchuk et al., 2014). These results indicated that the mutant cannot be delivered efficiently to regular copper-excretion sites in polarized hepatocytes.

ATP7B$^{R1415Q}$ exhibits copper-transporting activity

To assess the ability of ATP7B$^{R1415Q}$ to transport copper, we transfected HeLa cells with the metal-responsive element (MRE)-luciferase reporter, a copper sensor based on the metallothionein-I promoter that responds to bioavailable cytosolic copper and that was previously used to characterize cellular copper import (van den Berghe et al., 2007). Co-expression of MRE-luciferase reporter with empty GFP vector resulted in strong induction of luciferase activity (Fig. 4D) upon exposure of the cells to CuSO$_4$. The induction of this copper-dependent reporter was significantly reduced when ATP7B$^{\text{WT}}$ was co-expressed in the cells, whereas transfection of the partly active ATP7B$^{H1069Q}$ mutant resulted in only modest inhibition of MRE-luciferase activation (Fig. 4D). Cells expressing ATP7B$^{R1415Q}$ exhibited substantial reduction of...
### Table 1. Mutations and effect estimates with regard to hepatic copper scores

| Gene     | Variant | Location | Chr. | Position (CF 3.1) | Allele ref. | Allele non-ref. | AA ref. | AA non-ref. | Effect estimate | s.e. | P-value |
|----------|---------|----------|------|------------------|-------------|----------------|---------|-------------|----------------|------|---------|
| **ATP7A** | SNP     | Exon 4   | X    | 60279238         | C           | T              | I       | I           | −0.44          | 0.18 | 0.02    |
| **ATP7A** | SNP     | Intron 5 | X    | 60289392         | C           | A              |         |             | 0.059          | 0.17 | 0.037   |
| **ATP7A** | SNP     | Intron 14| X    | 60326659         | G           | T              |         |             | 0.069          | 0.15 | 0.065   |
| **ATP7A** | SNP     | Exon 15  | X    | 60327032         | C           | T              |         |             | 0.056          | 0.17 | 0.074   |
| **ATP7A** | SNP     | Intron 18| X    | 60338569         | C           | T              |         |             | 0.052          | 0.18 | 0.005   |
| **ATP7A** | SNP     | Intron 19| X    | 60348365         | C           | T              |         |             | 0.003          | 0.17 | 0.098   |
| **ATP7A** | SNP     | Intron 20| X    | 60348994         | A           | G              |         |             | 0.063          | 0.18 | 0.00079 |
| **ATP7A** | SNP     | Intron 21| X    | 60351656         | G           | A              |         |             | 0.01           | 0.17 | 0.95    |
| **ATP7B** | SNP     | Intron 1 | 22   | 191461           | C           | T              |         |             | −0.06          | 0.2  | 0.76    |
| **ATP7B** | indel   | Intron 1 | 22   | 191502           | _            | Ins AGCTCAG    |         |             | −0.022         | 0.2  | 0.91    |
| **ATP7B** | SNP     | Exon 2   | 22   | 192722           | G           | A              |         |             | −0.52          | 0.22 | 0.019   |
| **ATP7B** | indel   | Exon 2   | 22   | 192865           | gccccgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgcgccccc | | | | | 0.09 | 0.61 | 0.31 | 0.05 | 0.09 | 0.09 | 0.61 | 0.31 | 0.05 |
| **ATP7B** | SNP     | Exon 2   | 22   | 193128           | C           | T              |         |             | −0.67          | 0.91 | 0.46    |
| **ATP7B** | SNP     | Exon 3   | 22   | 199368           | G           | A              |         |             | 0.034          | 0.19 | 0.86    |
| **ATP7B** | SNP     | Exon 3   | 22   | 199395           | C           | A              |         |             | 0.034          | 0.19 | 0.86    |
| **ATP7B** | SNP     | Exon 4   | 22   | 201943           | C           | T              |         |             | 0.052          | 0.19 | 0.79    |
| **ATP7B** | SNP     | Intron 4 | 22   | 203200           | A           | G              |         |             | 0.056          | 0.19 | 0.77    |
| **ATP7B** | SNP     | Intron 6 | 22   | 204445           | G           | A              |         |             | 0.065          | 0.19 | 0.74    |
| **ATP7B** | SNP     | Intron 8 | 22   | 207706           | C           | T              |         |             | −0.021         | 0.19 | 0.91    |
| **ATP7B** | indel   | Intron 11| 22   | 213592           | delT        |         |         |             | 0.067          | 0.91 | 0.46    |
| **ATP7B** | SNP     | Intron 11| 22   | 213626           | G           | A              |         |             | 0.096          | 0.19 | 0.62    |
| **ATP7B** | SNP     | Intron 14| 22   | 218433           | C           | T              |         |             | 0.052          | 0.19 | 0.79    |
| **ATP7B** | SNP     | Intron 16| 22   | 220086           | C           | T              |         |             | 0.014          | 0.19 | 0.94    |
| **ATP7B** | SNP     | Intron 16| 22   | 220214           | G           | A              |         |             | −0.062         | 0.9  | 0.49    |
| **ATP7B** | indel   | Intron 19| 22   | 223949           | ins C       |         |         |             | −0.047         | 0.18 | 0.8     |
| **ATP7B** | indel   | Intron 20| 22   | 224511           | ins C       |         |         |             | 0.0091         | 0.19 | 0.96    |
| **ATP7B** | SNP     | Exon 21  | 22   | 225112           | G           | R              |         |             | 0.29           | 0.41 | 0.039   |

**Summary of identified mutations in ATP7A and ATP7B in 96 Labrador retrievers that were randomly selected from the cohort of 235 Labrador retrievers used for the GWAS.** Effect estimates for the non-reference allele on copper score, corrected for sex effect, standard errors and P-values are reported. Significant mutations (P < 0.05) were replicated in an independent replication cohort consisting of 59 Labrador retrievers. Significantly replicated mutations were genotyped in the entire cohort of 294 Labrador retrievers, and allele frequencies, effect estimates and P-values are reported in the last column. AA, amino acid; All freq., allele frequency; Chr., chromosome; CF 3.1, CanFam 3.1 genome build; non-ref., non-reference; ref., reference; s.e., standard error; SNP, single nucleotide polymorphism; indel, small insertion or deletion.

**Disease Models & Mechanisms**
reporter activity (Fig. 4D), while expressing a similar amount of protein to ATP7BWT-transfected cells (Fig. S5). This observation indicates that ATP7BR1415Q is still capable of transporting copper out of the cytosol and that observed differences are not the result of differences in protein expression.

Taken together, our findings indicate that accumulation of copper upon ATP7BR1415Q expression occurs mainly as a result of failure of the mutant to reach the appropriate copper-excretion compartments rather than as a result of a reduction in its copper-translocating activity.

Table 2. Hepatic histological copper score in relation to ATP7A and ATP7B genotype in male and female Labrador retrievers

| ATP7B c.4358G>A genotypes | Males | ATP7A c.980C>T genotypes | Females |
|-----------------------------|-------|---------------------------|---------|
|                            | CY    | TY | CC | CT | TT |
| AA | n=10 | n=0 | 2.25 (0-4) a | 3.5 (0.5-5) y | n=7 | n=9 | n=0 |
| AG | n=41 | n=11 | 0.5 (0-2) b,c | 2 (0-3.5) z | n=53 | n=24 | n=4 |
| GG | n=28 | n=11 | 1.25 (0-3.5) a,b | 1.5 (0-3.5) z | n=55 | n=34 | n=7 |

Combinations of genotypes of individual dogs for ATP7A c.980C>T and ATP7B c.4358G>A are indicated. Data are presented for male dogs and female dogs separately. The number of dogs (n) and median and range (in parentheses) for hepatic copper score per genotype category are summarized. Significant differences between genotype categories are indicated by letters in males (a,b,c) and females (y,z). Genotypes for which fewer than five animals were present were omitted from the statistical analysis.

Fig. 3. Protein domains of ATP7A and ATP7B involved in copper toxicosis in Labrador retrievers. Overview of the ATP7A (A) and ATP7B (B) proteins with the N-terminus, metal-binding domains (MBDs), actuator domain (A), nucleotide-binding domain (N), phosphorylation domain (P) and the C-terminus. The green asterisk indicates the position of the mutations. (A) Alignment of the region containing ATP7AT327I (in green) shows a strong conservation of this amino acid position in the human, rat, mouse, cow, cat and horse. The copper-binding site XMXCXXC (boxed), predicted α-helix (red) and β-sheets (blue) are indicated. (B) Alignment of the region containing ATP7BR1453Q (in green) shows a strong conservation of this amino acid position in the human, rat, mouse, cow, cat and horse. The predicted α-helix (red) and β-sheets (blue) are indicated.
Canine dermal fibroblasts expressing ATP7AT327I accumulate more copper than wild-type fibroblasts
To ascertain whether ATP7A:9.Thr327Ile (ATP7AT327I) influenced cellular copper accumulation, a copper uptake and retention assay (Moller et al., 2011) was performed using copper 64 isotope ($^{64}$Cu) in dermal fibroblasts. Fibroblasts with ATP7AWT were derived from five female and three male dogs, and fibroblasts with the variant ATP7AT327I were...
derived from four female and three male dogs, and used for the $^{64}$Cu experiments. For the $^{65}$Zinc experiments, fibroblasts from three ATP7A$^{WT}$ dogs (two females, one male) and three ATP7AT$^{327I}$ dogs (one female, two males) were used. All dogs used for the fibroblast studies that were homo- or hemizygous for $ATP7A$: c.980C were homo- or hemizygous for the reference allele for the SNP in intron 18 (position 60338569), and all dogs that were homo- or hemizygous for $ATP7A$: c.980T were homo- or hemizygous for the non-reference allele.

The model for $^{64}$Cu accumulation in fibroblasts that included the fixed factors time and mutation had the lowest Akaike’s information criterion (AIC). Overall, a significant increase of copper at time points 22 and 30 h was present. The fibroblasts derived from the ATP7AT$^{327I}$ dogs accumulated significantly more copper than fibroblasts derived from ATP7A$^{WT}$ dogs (Fig. 5A).

To establish whether the differences in $^{64}$Cu levels between fibroblasts were the result of abrogated efflux, a chase method was used, after preloading of cells with copper for 22 h. The average export rate of fibroblasts with the ATP7A$^{327I}$ variant was significantly lower compared with the average export rate in fibroblasts derived from ATP7A$^{WT}$ (Fig. 5B). Western blots of dermal fibroblasts derived from ATP7A$^{WT}$ and ATP7AT$^{327I}$ dogs did not show differences in ATP7A protein expression (Fig. S6).

As a control, fibroblasts from a human fibroblast cell line (ATP7A$^{WT}$) and a human Menkes disease-affected individual (Menkes; both $n=1$) were included in the experiments. Fibroblasts derived from the human with Menkes disease accumulated considerably more $^{64}$Cu than the ATP7A$^{WT}$ fibroblasts (Fig. 5C) and showed abrogated efflux (Fig. 5D).

Handling of an alternative trace metal in canine fibroblasts was not influenced by the $ATP7A$ genotype. Incubation of fibroblasts with $^{65}$Zn resulted in similar patterns of accumulation of isotope over time (Fig. S7A), and the release kinetics of newly accumulated $^{65}$Zn were comparable between the fibroblasts derived from ATP7A$^{WT}$ dogs and dogs with ATP7AT$^{327I}$ (Fig. S7B). In summary, this experiment showed that fibroblasts derived from dogs with the ATP7AT$^{327I}$ accumulate more copper than fibroblasts derived from dogs with ATP7A$^{WT}$ and that copper accumulation might be the result of decreased copper excretion by ATP7AT$^{327I}$ fibroblasts.

**Copper-induced trafficking is not changed in cells expressing ATP7AT$^{327I}$**

To investigate whether the observed decrease in copper excretion was a result of aberrant trafficking, immunofluorescence studies were performed in canine dermal fibroblasts derived from ATP7A$^{WT}$ and ATP7AT$^{327I}$ dogs. Both variants reside in the...
Disease Models & Mechanisms (2016) 9, 25-38 doi:10.1242/dmm.020263

Golgi in low-copper circumstances. After 2 h incubation with copper, both ATP7AWT and ATP7AT327I exhibited substantial redistribution from the Golgi to the small post-Golgi vesicles and plasma membrane (Fig. S8).

To understand whether basolateral targeting of ATP7AT327I was compromised, apical-basal distribution of both ATP7AWT and ATP7AT327I was investigated in filter-grown polarized Madin–Darby canine kidney (MDCK) cells. The MDCK cells were transfected with GFP-tagged versions of either ATP7AWT or ATP7AT327I and exposed to CuSO4 for 4 h. Confocal microscopy revealed efficient delivery of ATP7AWT to the lateral cell surface domain of MDCK located below the occludin-labelled belt of tight junctions. As did the wild-type protein, ATP7AT327I exhibited efficient targeting to the lateral walls of MDCK cells (Fig. S9), indicating that its polarized delivery in epithelial cells remained unaffected.

**DISCUSSION**

We performed a GWAS in 235 Labrador retrievers to identify causal genes for copper toxicity. We identified two chromosomal loci that were positively and negatively associated with hepatic copper levels. The two loci respectively harboured the genes coding for the copper transporters ATP7B and ATP7A. Subsequent Sanger sequencing in 96 Labrador retrievers and validation of mutations in an independent cohort of 59 Labrador retrievers identified a non-synonymous SNP in ATP7B associated with an increase in hepatic copper levels, and a non-synonymous and intronic SNP in ATP7A associated with a decrease in hepatic copper levels. Functional follow-up studies for the non-synonymous mutations provided biological evidence for their involvement in a disturbed copper metabolism.

Copper toxicity in Labrador retrievers and Wilson disease are similar with regard to the amount of hepatic copper accumulation, presence of iron accumulation and progression to liver cirrhosis. Electron microscopy of liver sections of affected Labrador retrievers revealed ultrastructural changes that are similar to those observed in humans with Wilson disease. We show, for the first time, that Labrador retrievers and humans with Wilson disease share ATP7B as the causal gene. However, histopathological differences between copper toxicity in Labrador retrievers and Wilson disease are present and include predominant centrolobular copper accumulation in Labrador retrievers versus a perportal distribution in humans with Wilson disease. Furthermore, fatty degeneration and Mallory bodies are not recognized in liver histopathology from Labrador retrievers. In both diseases, the age of onset is usually in middle or older age, but neurological symptoms and Kaiser–Fleisher rings have not been recognized in Labrador retrievers.

Dietary intake of copper seems important in disease progression in Labrador retrievers (Fieten et al., 2014, 2012a) and, in this regard, Labrador retriever copper toxicity is comparable to endemic Tyrolean infantile cirrhosis and Indian childhood cirrhosis. The observed differences among copper toxicity diseases in humans and Labrador retrievers are intriguing, and further studies are needed to identify whether they can be attributed to general species differences in copper metabolism or to other, yet to be identified gene mutations in either species.

ATP7B is highly expressed in hepatocytes, where it is located in the trans-Golgi network in low-copper conditions. With increasing intracellular copper levels, ATP7B traffics to endo-lysosomal vesicular structures and to the canalicular surface of hepatocytes (Guo et al., 2005; Polishchuk et al., 2014; Schaefer et al., 1999) to facilitate copper excretion into the bile. The C-terminus has an important role in regulating copper-responsive trafficking. Mutations of residues in this region are suggested to contribute to aberrant Golgi retention of ATP7B in high-copper circumstances (Braiterman et al., 2011). Our fluorescence studies did not show a significant difference in GFP signals in both HeLa and polarized HepG2 cells between the wild type and ATP7BR1415Q after copper treatment. However, ATP7BR1415Q showed increased retention in the ER and failure to reach the canalicular surface in polarized hepatic cells in high-copper conditions, which might occur because of mis-folding of the protein. Mis-folding and aberrant retention in the ER have been previously reported in Wilson disease-associated ATP7B mutations (Gupta et al., 2011; van den Bergh et al., 2009). The luciferase assay demonstrated that the copper-transport capacity of ATP7BR1415Q showed only a modest impairment, indicating that the functional effect of ATP7BR1415Q in hepatic copper accumulation in Labrador retrievers results from mis-localization of the protein, rather than from severely decreased copper-transport capacity.

In Labrador retrievers, there is a strong female predisposition for disease (Fieten et al., 2012b). X-chromosomal mapping in a combined data set can be hampered by hemizygosity in males, and thereby, possibly cause disturbance of association signals. Therefore, we analysed males and females separately. In the region harbouring ATP7A, we identified a suggestive signal of association with lower hepatic copper levels in male dogs. We hypothesized that mutations in ATP7A might modify copper accumulation, and this gene was therefore analysed by DNA sequence analysis. The identified missense mutation ATP7A:c.980C>T results in the amino acid substitution ATP7A:p.Thr327Ile in MB3 of ATP7A and was negatively associated with hepatic copper levels. MB3 is located in the cytoplasm and has the ability to receive copper from the Atox1 chaperone (Strausak et al., 2003). MB3 of ATP7A is only metallated in elevated copper conditions (Banci et al., 2010) and is thought to be an important domain for regulating ATP7A activity via cell-signalling pathways (Veldhuis et al., 2011). It has been shown that ATP7A:p.T327 is an important phosphorylation site of ATP7A (Veldhuis et al., 2009).

To investigate whether ATP7AT327I influenced cellular copper accumulation, we studied copper uptake and retention in dermal fibroblasts derived from Labrador retrievers hemi- or homozygous for either ATP7AWT or ATP7AT327I. Mutant fibroblasts accumulated significantly more copper and showed a decreased efflux rate without a change in protein expression levels, indicating a functional impairment of ATP7A. We also investigated whether this could be a result of aberrant trafficking or sorting of the mutant upon copper stimulation. Hereto, we used wild-type and ATP7AT327I dermal fibroblasts and polarized MDCKs transfected with either ATP7AWT or ATP7AT327I. In both experiments, correct trafficking to the plasma membrane and delivery to the basolateral membrane (in polarized MDCKs) upon copper stimulation was observed. These results are in line with previous observations that the phosphorylation site ATP7A:p.T327 is not involved in aberrant trafficking of ATP7A (Veldhuis et al., 2009), but is rather predicted to stabilize a conformational change in ATP7A, exposing the CxxC copper-binding region of MB3, which might affect catalytic activity of ATP7A (Veldhuis et al., 2011). This might indicate that catalytic activity, rather than aberrant trafficking of ATP7A, is responsible for the observed decrease in hepatic copper levels in Labrador retrievers.

In humans, disease-causing mutations in the third metal-binding domain of ATP7A were not reported (Kaler, 2011). For ATP7B, 15 (three silent, 12 Wilson disease-causing) variants in MB3 are
described. Two mutations, c.915T>A (Loudianos et al., 1998) and
c.918-931del (Davies et al., 2008), lead to a premature stop and a
concomitant absence of ATP7B:p.Ser306 (corresponding to
ATP7A:p.Thr327). Both of these mutations cause Wilson disease.

Another single base-pair substitution, C>T, located in intron 18
(position 60338569, CF 3.1), was also inversely related to hepatic copper
score. In the dogs from which the fibroblasts were derived, the
SNP in intron 18 and ATP7A:c.980C>T were in complete LD, and
therefore we cannot rule out the possibility that the intronic variation
exerts an effect on ATP7A gene function; however, we consider it less
likely. The alleles are both pyrimidines within a stretch of
pyrimidines, are not situated close to an intron-exon junction, and
the mutation is not expected to activate a cryptic splice site. In the
total data set, the intronic SNP was in high LD (r² 0.92, D’ 0.97) with
ATP7A:c.980C>T, making it likely that the observed association is a
result of the fact that this SNP is in LD with ATP7A:c.980C>T.

ATP7B:c.4358G>A showed an additive effect on hepatic copper
levels, which was most significant in female dogs (Fig. 2). Copper-
accumulating effects of ATP7B:c.4358G>A were modified by the
presence of ATP7A:c.980C>T and this effect was most clear in
male dogs (Table 2). In accordance with observations in humans
with Menkes disease, where aberrant ATP7A function results in an
impaired intestinal copper uptake, we suggest that in Labrador
 retrievers ATP7A:327I might result in decreased intestinal copper
uptake, thereby attenuating hepatic copper accumulation because of
ATP7B1453Q. Other possible mechanisms influencing copper
homeostasis in Labrador retrievers and, more specifically, the
interplay between ATP7A and ATP7B might include regulation by
hormones, inflammatory cytokines, growth factors or a yet
unidentified small molecule acting as a regulator of systemic
copper metabolism (Kim et al., 2010).

In humans with Wilson disease, a wide variation in clinical
symptoms and hepatic copper accumulation is noticed, which is
currently unexplained (de Bie et al., 2007; Lee et al., 2011; Riordan
and Williams, 2001; Shah et al., 1997). When we translate our
observations in the Labrador retrievers to the human disease, we
might think of ATP7A as a possible modifier gene in Wilson disease.
Variations in ATP7A with a small effect on protein function might
reduce the rate of copper accumulation in humans with Wilson
disease, thereby modifying the disease phenotype, for example by
delaying the age at onset of clinical symptoms. In fact, part of the
apparent discrepancy between the genetic incidence of Wilson
disease and the number of affected individuals diagnosed
coffey et al., 2013) might be a result of a slower rate of copper
accumulation that does not reach toxic levels in the life span of
some individuals. In a recent pilot study, two SNPs in ATP7A were
genotyped in humans with Wilson disease. No association was
identified between the ATP7A polymorphisms and the presence of
Wilson disease or clinical phenotypic parameters (Przybyłkowski
et al., 2014). However, these results clearly do not exclude ATP7A
as a candidate modifier, because it was a very limited investigation
of ATP7A and a small data set.

In conclusion, the present study illustrates the potential of canine
inbred populations for the unravelling of complex hereditary
diseases. We identified the involvement of the copper transporter
ATP7B in copper toxicosis in the Labrador retriever, identifying this
breed as the first naturally occurring non-rodent mammalian model
for Wilson disease. The concurrent identification of a functional
mutation in ATP7A suggests a role for the Menkes gene as a disease
modifier in copper-metabolism disorders. Owing to its long life
span and large body size, the Labrador retriever will be an
invaluable large-animal model for the development of new
therapies, including gene therapy, beneficial to both human and
canine affected individuals.

MATERIALS AND METHODS

Dogs

Samples for the initial GWAS and DNA sequencing study were collected
between 2003 and 2009, and samples for the replication cohort were
collected between 2009 and 2013 at the Faculty of Veterinary Medicine,
Utrecht University, with informed consent of the owners. The procedures
were approved by the Utrecht University Ethical Committee as required
under Dutch legislation. In addition, procedures were reviewed and
approved by the WALTHAM Centre for Pet Nutrition. Each dog
underwent a physical examination. An EDTA blood sample of 4 ml was
used for DNA extraction from peripheral white blood cells by a salt
extraction procedure (Miller et al., 1988). For diagnostic purposes, liver
biopsies were obtained by the Menghini technique, ultrasound guided with a
Tru-cut device and 14 gauge needle, during laparoscopy or laparotomy
(Rothuizen et al., 2006). Local anaesthetics (infiltration of the skin and
subcutaneous tissues with lidocaine) were applied for Tru-cut liver biopsies.
When necessary, dogs were sedated with propofol (doseage to effect)
during the liver biopsy procedure. For liver biopsies obtained by laparoscopy
or laparotomy, dogs underwent general anaesthesia. The anaesthesia protocols
used were individually designed based on the anaesthetic risk classification of
the American Association of Anesthesiologists. Tissue specimens were
embedded in paraffin, and 4 μm thick sections were mounted on glass slides.
Sections were stained with haematoxylina and cosin, rubeanic acid (Uzman,
1956) for copper and reticulin (Gordon and Sweets, 1936). All samples were
evaluated and graded by one board-certified pathologist (T.S.G.A.M.v.d.L.)
according to World Small Animal Veterinary Association standards (Van
Den Ingh et al., 2006). Hepatic copper accumulation was histologically
graded on a scale from zero to five as described previously (Fieten and
Rothuizen, 2014).

Electron microscopy

For routine electron microscopic analysis, liver specimens were obtained
from two Labrador retrievers affected with copper toxicosis and one healthy
dog of mixed breed. Biopsies were fixed in 1% glutaraldehyde in 0.2 M
HEPES buffer. Small blocks of liver tissue were post-fixed in uranyl acetate
and in OsO4. After dehydration through a graded series of ethanol, the
tissue samples were cleared in propylene oxide, embedded in the Epoxy resin
(Epon 812) and polymerized at 60°C for 72 h. From each sample, thin
sections were cut with a Leica EM UC7 ultra-microtome. Thin sections were
investigated further using a FEI Tecnai-12 (FEI; Eindhoven, The
Netherlands) electron microscope equipped with a Veleta CCD camera for
digital image acquisition.

Genome-wide association study

DNA samples from 235 Labrador retrievers were genotyped using
Illumina’s Canine HD BeadChip, containing ~170,000 SNPs. Data were
analysed in the R-package (R Development Core Team, 2011) ‘GenABEL’
version 1.6-6 (Aulchenko et al., 2007). Quality-control thresholds were set
using the function ‘check.marker’. A threshold of 98% successful genotypes
per individual and 98% successful genotypes per SNP was used. SNPs for
which at least 20 heterozygotes were typed were included in the analysis.
Furthermore, a check for sex mismatch (based on genotypes at the X
chromosome), duplicate sample checks and checks for heterozygosity
outliers were performed. The phenotype copper score was analysed as a
quantitative trait, and sex was modelled as a fixed factor. Stratification
and cryptic relatedness were adjusted for by mixed models implemented in the
function ‘mpgcore’ (Chen and Abecasis, 2007). A correction for residual
genomic control was applied. The heritability of hepatic copper score with
regression for sex was calculated based on genotype kinship matrix with the
function ‘polygenic_hglm’ based on the packages ‘hglm’ (Ronnegard et al.,
2010) and ‘GenABEL’. The autosomes and pseudo-autosomal region of the
X chromosome were analysed in male and female dogs together. An
additional stratified analysis for males and females was performed to
facilitate inspection of the X chromosome.
Genome-wide suggestive association criteria were set to an uncorrected P-value of \( P \leq 5 \times 10^{-8} \). Boundaries for the crucial intervals were determined for a significance level of \( P \leq 5 \times 10^{-4} \). Crucial intervals were screened for the presence of possible candidate genes.

**DNA sequence analysis and validation**

Exons and intron-exon boundaries from positional candidate genes were selected for Sanger sequencing in 96 individual dogs randomly selected from the 235 Labrador retrievers used in the initial GWAS. Variants that were significantly associated with hepatic copper level were genotyped in an independent replication cohort of 59 Labrador retrievers and in all 294 dogs. Statistical analysis was performed with R version 2.11 (R Development Core Team, 2011). A linear regression model with sex as a covariate was used to test the association of the mutations identified with Sanger sequencing in the candidate genes with hepatic copper score. The mutations were modelled in an additive way.

Genotypes for the mutations that were significant after statistical testing in the replication cohort were genotyped by Sanger sequencing in the total cohort of 294 dogs. Differences in hepatic copper scores with respect to combined \( \text{ATP7A} \) and \( \text{ATP7B} \) genotype were determined using a Wilcoxon rank-sum test within males and females separately.

**Functional evaluation of ATP7B**

Alignment of human and canine ATP7B protein sequence confirmed that the Arg at position 1453 in the canine protein corresponds to the Arg at position 1415 in the human ATP7B. The human pAdlox-ATP7B-GFP construct was used, and site-directed mutagenesis was performed to make pAdloxATP7B-p:Arg1415Gln-GFP. Both constructs were used for cell transfection and subsequent immunofluorescence and luciferase assays.

**DNAs and site-directed mutagenesis**

To prepare ATP7B-p:Arg1415Gln, the pAdlox-ATP7B-GFP construct (provided by S. Lutsenko, Baltimore, MD, USA) was used as the template, and site-directed mutagenesis was performed according to the manufacturer’s instructions for point mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), using the following oligonucleotides: 5′-GGGACTCCCCCCAGGCCACACCATG-GGTCCCATGGTGTGGCCTGGGGGGAGTCCC-3′ and 5′-GGGACCCATGGTGCTCGGCGTGGGCGGAGTCGCC-3′.

Given that \( \text{ATP7A} \) sequences are unstable in high-copy plasmids (reference PMID: 9571140), we used commercial DNA synthesis to generate silent mutations in the mouse \( \text{Atp7a} \) open reading frame in the hope that such changes would permit stable propagation in high-copy plasmids. Overlapping PCR was used to generate more than 1000 silent mutations as covariates/heritability without mutations as a covariate. In all heritability calculations, sex was added as a covariate.

**Luciferase assay**

For the luciferase assay, the pAdloxATP7B-H1069Q-GFP construct was used as a positive control, because ATP7B-H1069Q is the most common mutation causing Wilson disease in the Caucasian population and proved to be functionally active. Human cells were plated in a 12-well plate and transfected with pGL3-E1β-TATA-4MRE reporter (van den Berghe et al., 2007; provided by Bart van de Sluis, Groningen, The Netherlands) and then co-transfected with either empty vector (pEGFP-C1) or pAdloxATP7B-GFP or pAdloxATP7B-H1069Q-GFP or pAdloxATP7B-p:Arg1415Gln-GFP expression plasmids using the TransIT®-LT1 transfection protocol. After 24 h, cells were treated with 200 μM CuSO\(_4\) for 24 h. Cells were subsequently harvested in a Passive Lysis Buffer (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Firefly luciferase and Renilla luciferase activities were measured with a Dual-Luciferase® reporter assay system (Promega) on a GloMax® 96 Microplate Luminometer (Promega) according to the manufacturer’s instructions. Relative light units were calculated by dividing firefly measurements by Renilla measurements. All values were normalized to the ATP7B protein expression levels determined by ImageJ (Collins, 2007) analysis of western blot in the respective specimens. Western blot analysis was performed on lysates obtained by transfection of the cells with pEGFP-C1, pAdlox-ATP7B-GFP, pAdlox-ATP7B-H1069Q-GFP and pAdlox-ATP7B-p:Arg1415Gln-GFP expression plasmids. Lysates were prepared via the addition of lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8), 0.5% NP-40, 10% glycerol and 1× protease inhibitor cocktail (Sigma S88201)) for 10 min at room temperature. The mixture was placed into a microfuge tube, kept on ice for 10 min, and then spun at 17,000 g for 1 min. The supernatant was then mixed with 0.5 μg of anti-ATP7A antibody (1:1000) in 0.5× Laemmli sample buffer containing 5% β-mercaptoethanol heated at 95°C for 5 min. The mixture was electrophoresed on a 12% SDS-PAGE gel and transferred to nitrocellulose. After blocking with 5% non-fat milk in PBS-Tween 0.1% for 1 h, the blots were probed overnight at 4°C with each of the antibodies. The following primary antibodies were used: anti-ATP7A (Hycult Biotech, Plymouth Meeting, PA, USA), anti-Human Golgin-97 Mouse Monoclonal CDF4 (Molecular Probes, Paisley, UK), anti-GFP and anti-VAP-A from M.A. De Matteis (TIGEM, Naples, Italy), anti-α-tubulin (Sigma-Aldrich, St Louis, MO, USA), anti-TGN46 (Abd Serotec, Oxford, UK), anti-Na+/K+-ATPase (Abcam, Cambridge, UK), anti-MRP2 (Enzo Life Sciences, Lausanne, Switzerland), anti-occludin and secondary Alexa Fluor 568-conjugated antibodies (Invitrogen-Life Technologies, Grand Island, NY, USA). Dual-Luciferase® reporter assay kit was from Promega (Madison, WI, USA).

**Functional evaluation of ATP7A**

Dermal fibroblasts were derived from Labrador retrievers selected for the presence or absence of an \( \text{ATP7A}:c.980C>T \) substitution resulting in the \( \text{ATP7A:T327I} \) mutant. The substitutions were genotypically analyzed by DNA sequence analysis and validation. A PCR fragment containing the EGFP sequence flanked by MfeI sites was amplified in the 5′ region of \( \text{ATP7A} \) to generate the pAtp7a-GFP plasmid. GFP-tagged ATP7A\_T327I mutant was generated using standard mutagenesis service by GenScript.

**Reagents**

Antibodies were obtained from the following sources: anti-ATP7A (Hycult Biotech, Plymouth Meeting, PA, USA), anti-Human Golgin-97 Mouse Monoclonal CDF4 (Molecular Probes, Paisley, UK), anti-GFP and anti-VAP-A from M.A. De Matteis (TIGEM, Naples, Italy), anti-α-tubulin (Sigma-Aldrich, St Louis, MO, USA), anti-TGN46 (Abd Serotec, Oxford, UK), anti-Na+/K+-ATPase (Abcam, Cambridge, UK), anti-MRP2 (Enzo Life Sciences, Lausanne, Switzerland), anti-occludin and secondary Alexa Fluor 568-conjugated antibodies (Invitrogen-Life Technologies, Grand Island, NY, USA). Dual-Luciferase® reporter assay kit was from Promega (Madison, WI, USA).

**Cells and transfection**

Hela and HepG2 cells were routinely grown at 37°C in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% FBS (fetal bovine serum with inactivated complement for HepG2) on coverslips and transfected with pAdlox-ATP7B-GFP, pAdlox-ATP7B-H1069Q-GFP and pAdlox-ATP7B-R1415Q-GFP using TransIT®-LT1 and jetPEI® polylipid transfection protocols for Hela and HepG2, respectively, according to the manufacturer’s instructions. ATP7B-H1069Q was used as a control because it is one of the most common Wilson disease mutations in the Caucasian population and impairs ATP7B function. To reach polarization, HepG2 cells were grown according to the published protocol for 72 h (Sliman et al., 2003).

MDCK cells were grown on Transwell polycarbonate filters (Corning Costar, Lowell, MA, USA) in DMEM containing 10% FBS for 4-5 days to achieve sufficient polarization. GFP-tagged ATP7A-WT or ATP7B-T327I were transfected using TransIT®-LT1 2 days before the experiment.
The human dermal fibroblast cells HB156 from an individual with clinical Menkes disease (cell line: DD0355, no. 91071704) were purchased from the European Collection of Cell Cultures (ECACC). ECACC is managed as part of the Health and Safety Executive (HSE) in the UK. The human HDFn dermal fibroblast line (Invitrogen, Bleiswijk, The Netherlands) was used as a control cell line.

Fibroblast cultures were routinely maintained in DMEM (Invitrogen) supplemented with 1-glutamine (2 mM), 10% FBS, MEM non-essential amino acids (100 µM) and penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2 in air. For experiments, canine cells were used between passage 4 and 9.

Radioisotope experiments

Methods for radioisotope experiments were based on those described previously (Spee et al., 2007). In short, 64Cu was prepared using metallic copper wire (1 mg) irradiated overnight to provide an induced activity of approximately 16.6 Ci/g (Reactor Institute, Delft, The Netherlands). Four hours after irradiation and 30 min before the start of the study, the copper wire was dissolved in 100 µl 10.3 M HNO3 and subsequently neutralized with 0.5 M NaOH. DMEM was added to give a final concentration of 0.2% SDS was added to lyse the cells. Cellular copper loading was determined from counts of the cell lysate (450 and 800 keV for 1 min; Packard B5003 gamma counter; Packard BioScience Benelux, Groningen, The Netherlands). For efflux studies, after copper treatment for 22 h as described above, cells were washed four times with Hanks solution containing 3 µM CuCl2 before incubation in fresh medium containing no additional copper for 8 h. Cellular copper loading was assessed at 0, 0.5, 1.5, 3, 6 and 8 h as described in the previous paragraph. Copper loading was normalized to the protein concentration of the cell lysate (Bio-Rad Protein Assay Kit; Bio-Rad, The Netherlands).

Immunofluorescence

The intracellular distribution of GFP-tagged ATP7B or ATP7A proteins was observed 24 or 48 h after transfection. Cells were treated with 500 µM BCS overnight and fixed directly or washed and incubated for 2 h with 200 µM CuSO4 before fixation in 4% paraformaldehyde. Then the cells were incubated with blocking/permeabilizing solution (0.5% bovine serum albumin, 0.1% saponin and 50 mM NH4Cl in PBS) for 20-30 min. Primary and secondary antibodies were diluted in blocking/permeabilizing solution and added to the cells overnight and for 45 min, respectively. Co-localization between ATP7B variants and organelle markers was visualized using anti-TGN46 antibody (Golgi marker), VAP-A antibody (ER marker), Na+/K−-ATPase antibody (plasma membrane marker) and LAMP1 antibody (late endosome/lysosome marker) followed by the Alexa Fluor 568 secondary antibody. Antibodies against the tight junction protein occludin were used to label the border between apical and basolateral domains in MDCK cells. To visualize canalicular domains, polarized HepG2 cells expressing ATP7BWT or ATP7BR1415Q were labelled with anti-MRP2 antibody followed by the secondary anti-mouse Alexa Fluor 568. Canine dermal fibroblasts were treated with BCS or CuSO4 as described above, fixed and stained for endogenous ATP7A with anti-ATP7A antibodies and with anti-Golgin97 to reveal TGN46. The images were acquired using a 63×1.4 NA oil immersion objective at an LSM710 or LSM700 confocal microscope with appropriate filter sets (Carl Zeiss, Jena, Germany). The intracellular distribution of GFP-tagged ATP7B protein was analysed in 10 randomly selected fields, and the percentage of the cells exhibiting ATP7B in ER, Golgi and/or plasma membrane/vesicles was quantified. A Wilcoxon rank-sum test was used to test differences in percentages of GFP positivity within the ER, Golgi and/or plasma membrane. A P-value <0.05 was considered significant. For polarized MDCK cells, confocal z-stacks through the entire cell monolayer were taken, and x-z sections were generated using Zeiss Zen software.

Acknowledgements

We acknowledge the Dutch Labrador retriever breed club (Nederlandse Labrador Vereniging), collaborating specialists and owners of Labrador retrievers for their dedication to this study. We acknowledge Elena Polischuk and Advanced Microscopy and Imaging Core (TIGEM) for support with electron microscopy. We acknowledge Monique van Wolferen for assistance with western blotting. We would like to thank Svetlana Lutsenko for kindly providing the ATP7B construct.

Competing interests

Permutations for mutations described in this manuscript are filed by Mars Inc. under publication numbers WO2009044152A2 and WO2013083888A3. Y.G., A.J.M. and A.L.W. were employed by the WALTHAM Centre for Pet Nutrition.

Author contributions

H.F., Y.S.A., K.D., P.A.J.L. and A.J.M. designed genetic analysis methods, performed and interpreted GWAS analysis, sequencing and association analysis. T.S.G.A.M.v.d.I. phenotyped all dogs by scoring histological slide for clinical features of Menkes disease. M.C., F.P., G.C. and R.S.P. designed and executed the experiments for the functional evaluation of ATP7B (preparation of the construct, cell transfections, immunofluorescence studies and luciferase assay) and prepared the figures in consultation with B.J.A.v.d.S. M.J.P., V.L.H. and S.Z. developed the ATP7A construct and supported experiments in MDCKs. Y.G., A.L.W., B.S. and E.C.C.P.M. performed immunofluorescence on dermal fibroblasts and with ATP7A constructs in polarized MDCKs. H.F. performed statistical analyses and wrote the paper with input from Y.G., B.J.A.v.d.S.
K.D., F.G.v.S., T.S.G.A.M.v.d.I., R.H.J.H.H., A.L.W., Y.S.A., R.S.P., P.A.J.L. and J.R. All authors read and approved the final manuscript.

**Funding**

This work was funded by the WALTHAM Centre for Pet Nutrition, a part of Mars Petcare Inc. Part of the study was supported by Telethon Italy [grant no. TGM11C84].

**Supplementary information**

Supplementary information available online at http://dmm.biologists.org/lookup/ suppl/doi:10.1242/dmm.020263/-DC1

**References**

Aulchenko, Y. S., Ripke, S., Isaacs, A. and van Duijn, C. M. (2007). GenABEL: an R library for genome-wide association analysis. Bioinformatics 23, 1294-1296.

Banci, L., Bertini, I., Cloifi-Baffioni, S., Kozyreva, T., Zovo, K. and Palumaa, P. (2010). Affinity gradients drive copper to cellular destinations. Nature 465, 545-548.

Barnes, N., Bartee, M. Y., Braiterman, L., Gupta, A., Usbeyn, V., Zuzel, V., Kaplan, J. H., Hubbard, A. L. and Lutsenko, S. (2009). Cell-specific trafficking suggests a new role for renal ATP7B in intracellular copper storage. Traffic 10, 767-779.

Boyko, A. R. (2011). The domestic dog: man’s best friend in the genomic era. Genome Biol. 12, 216.

Braiterman, L., Niasae, L., Leves, F. and Hubbard, A. L. (2011). Critical roles for the COOH terminus of the cu-ATPase ATP7B in protein stability, trans-golgi network retention, copper sensing, and retrograde trafficking. Am. J. Physiol. Gastrointest. Liver Physiol. 301, G69-G81.

Bulakova, O. I., Xu, J., Lutsenko, S., Zeitzl, S., Das, K., Das, S., Ross, B. M., Meikins, C., Scheinberg, I. H. and Gilliam, T. C. (1999). Null mutation of the murine ATP7B (wilson disease) gene results in intracellular copper accumulation and late-onset hepatic nodular transformation. Hum. Mol. Genet. 8, 1665-1671.

Bull, P. C., Thomas, G. R., Rommens, J. M., Forbes, J. R. and Cox, D. W. (1993). The Wilson disease gene is a putative copper transporter P-type ATPase similar to the menkes gene. Am. J. Hum. Genet. 53, 327-337.

Chen, W.-M. and Abeasis, G. R. (2007). Family-based association studies for genomewide association scans. Am J. Hum. Genet. 81, 913-926.

Coffey, A. J., Durkie, M., Hague, S., Mclay, K., Emmerson, J., Lo, C., Klaffke, S., Joyce, C. J., Dhawan, A., Hadzic, N. et al. (2013). A genetic study of Wilson's disease in the united kingdom. Brain 136, 1476-1487.

Collins, T. J. (2007). ImageJ for microscopy. BioTechniques 43, S25-S30.

Coronado, V. A., Bonneville, J. A., Nazer, H., Roberts, E. A. and Cox, D. W. (2005). COMMD1 (MURR1) as a candidate in patients with copper storage disease and copper toxicosis. Hepatocyte. Genet. 21, 5390-5395.

Cui, J., Godding, K. K., Dyer, J. A. and Kellar, S. G. (2016). Copper biochemistry and molecular biology. Am. J. Clin. Nutr. 63, 797S-811S.

Loudianos, G., Dessi, V., Lovicu, M., Angius, A., Nurchi, A., Sturniolo, G. C., Marcellini, M., Zancan, L., Bragetti, P., Akar, N. et al. (1998). Further delineation of the molecular pathology of Wilson disease in the Mediterranean population. Hum. Genet. 102, 90-94.

Lovicu, M., Dessi, V., Lepori, M. B., Zappu, A., Zancan, L., Giacchino, R., Marazzi, M. G., Iorio, R., Vagnente, A., Vajro, P. et al. (2006). The canine copper toxicosis gene MURR1 is not implicated in the pathogenesis of Wilson disease. J. Nutr. Environ. 41, 592-598.

Miller, S. A., Dykes, D. D. and Polakoff, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215.

Moller, L. B., Hicks, J. D., Holmes, C. S., Goldstein, D. S., Brendli, C., Huppek, P. and Kaler, S. G. (2011). Diagnosis of copper transport disorders. Curr. Protoc. Hum. Genet. Chapter 17, Unit17 B.

Müller, T., Feichtinger, H., Berger, H. and Müller, W. (1996). Endemic tyrolean infantile cirrhosis: an ecogenetic disorder. Lancet 347, 877-880.

Müller, T., van de Sluis, B., Zernakova, A., van Binsbergen, J., Janecke, A. R., Bavdekov, A., Pandit, A., Weinrich-Schwaiger, H., Witt, H., Ellemunter, H. et al. (2003). The canine copper toxicosis gene MURR1 does not cause non-wilsonian hepatic copper toxicosis. J. Hepatol. 38, 164-168.

Polischuk, E. V., Concilli, M., Iacobacci, S., Chesi, G., Pastore, N., Piccolo, P., Paladino, S., Baldantoni, D., van Uzendoom, S. C. D., Chan, J. et al. (2014). Wilson disease protein ATP7B utilizes lysosomal exocytosis to maintain copper homeostasis. Dev. Cell. 29, 686-700.

Przybyłkowski, A., Gromadzka, G. and Czlonkowski, A. (2014). Polymorphisms of metal transporter genes DMT1 and ATP7A in wilson’s disease. J. Trace Elem. Med. Biol. 28, 8-12.

Development Core Team (2011). R: A Language and Environment for Statistical Computing. Vienna, Austria.

Riordan, S. M. and Williams, R. (2001). The Wilson’s disease gene and phenotypic diversity. J. Hepatol. 34, 165-171.

Ronnegard, L., Shen, X. and Alam, M. (2010). Fitting hierarchical generalized linear models. R J. 2, 20-28.

Rothuizen, J., Desmet, V. J., Van Den Ingh, T. S. G. A. M., Twedt, D. C., Ronnegard, L., Shen, X. and Alam, M. (2006). Sampling and handling of liver tissue. In WSAVA Standards for Clinical and Histological Diagnosis Fo Canine and Feline Liver Disease - WSAVA Liver Standardization Group (ed. J. R. Rothuizen), pp. 5-14, Philadelphia, Elsevier.

Schafer, M., Hopkins, R. G., Failla, M. L. and Giltnan, J. D. (1999). Hepatocyte-specific localization and copper-dependent trafficking of the Wilson’s disease protein in the liver. Am. J. Physiol. 276, G639-G646.

Shah, A. B., Chernov, I., Zhang, H. T., Ross, B. M., Das, K., Lutsenko, S., Parano, E., Pavone, L., Evgrafov, O., Ivanova-Smolenskaya, I. A. et al. (1997). Identification and analysis of mutations in the Wilson disease gene (ATP7B): population frequencies, genotype-phenotype correlation, and functional analyses. Am. J. Hum. Genet. 61, 317-328.

Slomian, T. A., Trugnan, G., Van Uzendoorn, S. C. D. and Hoeckstra, D. (2003). Raft-mediated trafficking of apical protein occurs in both direct and transcytotic pathways in polarized hepatic cells: role of distinct lipid microdomains. Mol. Biol. Cell. 14, 611-624.

Spee, B., Arends, B., van Wees, A. M. T. C., Bode, P., Penning, L. C. and Rothuizen, J. (2007). Functional consequences of RNA interference targeting COMMD1 in a canine hepatic cell line in relation to copper toxicosis. Anim. Genet. 38, 168-170.

Strausak, D., Howie, M. K., Firth, S. D., Schlicksupp, A., Pipkorn, R., Multhaupt, G. and Mercer, J. F. B. (2003). Kinetic analysis of the interaction of the copper chaperone Atox1 with the metal binding sites of the menkes protein. J. Biol. Chem. 278, 20821-20827.

Tanner, M. S. (1998). Role of copper in Indian childhood cirrhosis. Am. J. Clin. Nutr. 67, 1074S-1081S.

Theophilis, M. B., Cox, D. W. and Mercer, J. F. B. (1996). The toxic milk mouse is a murine model of Wilson disease. Hum. Genet. 56, 1619-1624.

Uzman, L. L. (1956). Histochromic localization of copper with rubric acid. Lab. Invest. 5, 299-305.
Van de Sluis, B., Rothuizen, J., Pearson, P. L., van Oost, B. A. and Wijmenga, C. (2002). Identification of a new copper metabolism gene by positional cloning in a purebred dog population. *Hum. Mol. Genet.* 11, 165-173.

den Berghe, P. V. E., Folmer, D. E., Malingrè, H. E., van Beurden, E., Klomp, A. E. M., van de Sluis, B., Merkx, M., Berger, R. and Klomp, L. W. J. (2007). Human copper transporter 2 is localized in late endosomes and lysosomes and facilitates cellular copper uptake. *Biochem. J.* 407, 49-59.

den Berghe, P. V. E., Stapelbroek, J. M., Krieger, E., de Bie, P., van de Graaf, S. F. J., de Groot, R. E. A., van Beurden, E., Spilker, E., Houwen, R. H. J., Berger, R. et al. (2009). Reduced expression of ATP7B affected by Wilson disease-causing mutations is rescued by pharmacological folding chaperones 4-phenylbutyrate and curcumin. *Hepatology* 50, 1783-1795.

Van Den Ingh, T. S. G. A. M., Van Winkle, T., Cullen, J. M., Charles, J. A. and Desmet, V. J. (2006). Morphological classification of parenchymal disorders of the canine and feline liver, 2. hepatocellular death, hepatitis and cirrhosis. In *WSAVA Standards for Clinical and Histological Diagnosis of Canine Liver Diseases* (ed. *WSAVA Liver Standardization Group*), pp. 85-101. Philadelphia: Elsevier.

Veldhuis, N. A., Valova, V. A., Gaeth, A. P., Palstra, N., Hannan, K. M., Michell, B. J., Kelly, L. E., Jennings, I., Kemp, B. E., Pearson, R. B. et al. (2009). Phosphorylation regulates copper-responsive trafficking of the Menkes copper transporting P-type ATPase. *Int. J. Biochem. Cell Biol.* 41, 2403-2412.

Veldhuis, N. A., Kuiper, M. J., Dobson, R. C. J., Pearson, R. B. and Camakaris, J. (2011). In silico modeling of the Menkes copper-translocating P-type ATPase 3rd metal binding domain predicts that phosphorylation regulates copper-binding. *Biometals* 24, 477-487.

Voskoboinik, I., Camakaris, J. and Mercer, J. F. B. (2002). Understanding the mechanism and function of copper P-type ATPases. *Adv. Protein Chem.* 60, 123-150.

Vulpe, C., Levinson, B., Whitney, S., Packman, S. and Gitschier, J. (1993). Isolation of a candidate gene for menkes disease and evidence that it encodes a copper-transporting ATPase. *Nat. Genet.* 3, 7-13.

Wang, Y., Zhu, S., Weisman, G. A., Gilin, J. D. and Petris, M. J. (2012). Conditional knockout of the Menkes disease copper transporter demonstrates its critical role in embryogenesis. *PLoS ONE* 7, e43039.
Supplementary material

Table S1: Study sample of Labrador retrievers

Liver biopsies were obtained from 294 Labrador retrievers and histological sections were scored for the level of hepatic copper on a scale from 0-5. The initial GWAS cohort consisted of 235 Labrador retrievers and the replication set consisted of 59 Labrador retrievers. Sex was not selected for in the recruitment process, however in total more females than males were included in the study. The majority of dogs were of middle age. Data are grouped by dataset (GWAS and independent replication set), sex (Male, Female) and hepatic copper score: normal hepatic copper (score <2), increased hepatic copper (score 2-3) and high hepatic copper (score ≥ 3). Age is reported as average ± standard deviation.

| Dataset       | Number of dogs | Age (years) | Copper score |
|---------------|----------------|-------------|--------------|
|               | Female dogs    |             |              |
| GWAS n=235    | 71             | 6.7±2.8     | < 2          |
|               | 45             | 5.3±2.5     | 2-3          |
|               | 38             | 5.7±2.4     | ≥ 3          |
|               | Male dogs      |             |              |
| n=81          | 46             | 5.6±2.3     | < 2          |
|               | 23             | 5.7±2.3     | 2-3          |
|               | 12             | 5.6±2.8     | ≥ 3          |
| Replication set n=59 | Female dogs | 21           | 7.2±3.6     | < 2 |
|               | 11             | 8.2±3.4     | 2-3         |
|               | 5              | 6.3±3.8     | ≥ 3         |
|               | Male dogs      |             |              |
| n=22          | 15             | 7.9±2.3     | < 2         |
|               | 7              | 7.0±3.7     | 2-3        |
Figure S1 Multidimensional scaling plot

Multidimensional scaling plot prior to correction. Population stratification was visualized by plotting the components C1 and C2 from the multidimensional scaling analysis in 235 Labrador retrievers from the genome wide association study. Labrador retrievers with hepatic copper score < 2 (white), copper scores between 2 and 3 (grey) and copper scores > 3 (black) are depicted.
Figure S2 QQ-plot

QQ-plot for the “mmscore” analysis for hepatic copper score in 235 Labrador retrievers.
Figure S3 Co-localization between organelle markers and ATP7B-WT and R1415Q in high and low copper conditions.

HeLa cells were stained with ER marker VAP-A, PM marker Na'/K+-ATPase and late endosome/lysosome marker LAMP1. Arrows indicate co-localization of ATP7B mutant with VAP-A (in both low and high Cu conditions) and overlap of ATP7B-WT with Na'/K+-ATPase or LAMP1 (in high Cu conditions). White bars represent 5 µm.
Figure S4 Co-localization between ATP7B\textsuperscript{WT} and ATP7B\textsuperscript{R1415Q} in polarized HepG2 cells in low and high copper conditions.

Polarized HepG2 were exposed to BCS or CuSO\textsubscript{4} for 4h and stained with canalicular marker MRP2 and nuclear marker DAPI. Arrows indicate canalicular vacuole (cyst), which does not contain either WT or mutant ATP7B in chelator-treated cells. In contrast canalicular vacuole receives ATP7B-WT upon Cu stimulation, while the mutant fails to reach canalicular surface domain. White bars represent 5 µm.
Figure S5 Western blot of ATP7B-WT, -R1415Q and –H1069Q

Duplicates of lysates from cells expressing GFP-tagged ATP7B\textsuperscript{WT}, ATP7B\textsuperscript{R1415Q} or ATP7B\textsuperscript{H1069Q} were subjected to Western blot with anti-GFP antibody. Tubulin is shown to reveal the protein load for each specimen. Plot shows densities of the ATP7B bands for corresponding specimens (normalized to WT-1). While ATP7B\textsuperscript{WT} and ATP7B\textsuperscript{R1415Q} signals appear to be similar, ATP7B\textsuperscript{H1069Q} exhibits reduced expression.
Figure S6 Western blot of canine dermal fibroblasts

Lysates from dermal fibroblasts treated with 3 μM copper for 6, 22 and 30 hours and untreated cells were subjected to Western blot with anti-ATP7A antibody. Representative bands of ATP7A and Tubulin (protein load) is shown in (A). Bar graphs show the average of the normalized densities of the ATP7A bands (B).
Figure S7 Zinc accumulation and efflux in canine dermal fibroblasts

Dermal fibroblasts derived from ATP7A<sup>T327I</sup> dogs show similar zinc uptake (A) and efflux (B) compared to dermal fibroblasts derived from dogs with ATP7A<sup>WT</sup>. Dots represent mean values and standard deviations were indicated by error bars.
Figure S8 Immunofluorescence in canine dermal fibroblasts shows normal trafficking to the plasma membrane in ATP7A\textsuperscript{T327I} upon copper treatment.

Dermal fibroblasts were incubated with 200 µM BCS overnight and fixed directly (upper row) or after additional incubation with 100µM CuSO\textsubscript{4} for 2h (lower row). Then fixed cells were stained with antibodies against ATP7A and TGN marker Golgin 97. Both ATP7A\textsuperscript{WT} and ATP7A\textsuperscript{T327I} co-localized with TGN marker Golgin97 in BCS-treated cells (upper row). Incubation with Cu induced redistribution of both ATP7A\textsuperscript{WT} and ATP7A\textsuperscript{T327I} from the TGN to peripheral post-Golgi vesicles and plasma membrane. White bars represent 12 µm.
Figure S9 Immunofluorescence in polarized MDCKs shows correct targeting of ATP7A\textsuperscript{T327I} to the basolateral membrane.

Both ATP7A\textsuperscript{WT} and ATP7A\textsuperscript{T327I} traffic to the basolateral surface of polarized MDCK cells. MDCK cells were transfected with GFP-tagged versions of either ATP7A\textsuperscript{WT} (A) or ATP7A\textsuperscript{T327I} (B) and grown of transwell filters for 4 days to achieve polarization of monolayer. The cells were then treated with 200 \( \mu \text{M} \) CuSO\textsubscript{4} for 4h (to stimulate ATP7A trafficking to the cell surface), fixed and stained for tight junction marker occludin. DAPI-labeled nuclei are shown in blue. Z-stacks of MDCK monolayers were grabbed using confocal microscope. Arrows indicate ATP7A\textsuperscript{WT} (A) or ATP7A\textsuperscript{T327I} (B) at the lateral walls of the polarized MDCK cells just beneath the level of tight junctions labeled with occludin. White bars represent 16 \( \mu \text{m} \).