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PAPER

Mutation in the CPC motif-containing 6th transmembrane domain affects intracellular localization, trafficking and copper transport efficiency of ATP7A protein in *mosaic* mutant mice—an animal model of Menkes disease†

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Copper is an essential micronutrient for all living organisms. ATP7A protein is a copper-transporting ATPase which plays a vital role in the maintenance of cellular copper homeostasis in mammals. This protein is retained within the trans-Golgi network, but after binding copper it can be translocated to the cell membrane to participate in the efflux of excess Cu. Mutation of the *ATP7A* gene in humans results in the severe neurodegenerative disorder, Menkes disease. The mouse *ATP7A* homolog encodes a protein that plays the same role in copper transport. *Mosaic* mutant mice display a lethal phenotype which resembles Menkes disease, although the underlying molecular defect has not been characterized until now. In the present study we identified a G to C nucleotide exchange in exon 15 of the *Atp7a* gene in *mosaic* mutants, which resulted in an arginine to proline substitution in the highly conserved 6th transmembrane domain of the ATP7A protein. This mutated protein was mislocalized in kidney cells isolated from *mosaic* mutant mice, and following exposure of these cells to increased copper concentrations it was not translocated to the plasma membrane. Disturbance of ATP7A function in *mosaic* mice results in increased copper accumulation in the small intestine and kidneys, and in Cu deficiency in the brain, liver and heart. Mouse models of Menkes disease belong to the *mottled* mutant group. The *mosaic* mutant represents another interesting animal model for Menkes disease that will be of value in research on copper metabolism and transport in mammals.

Introduction

Copper (Cu) is a trace element essential for the normal growth and development of all living organisms. Many enzymes exploit the redox ability of a copper cofactor to catalyze reactions used in fundamental metabolic processes including respiratory oxidation, neurotransmitter synthesis, regulation of iron uptake, oxygen radical scavenging and connective tissue formation.¹ However, an excess of copper is highly toxic because copper ions are a potent generator of reactive free radicals that can cause oxidative

damage to proteins, lipids and nucleic acids.^{1–3} Therefore, living organisms have developed complex control mechanisms for maintaining optimal copper levels. Cu uptake, transport and utilization by cells require the activities of a closely integrated network of proteins. The first group is comprised of copper transporters belonging to the CTR family and chaperone proteins that are involved in copper uptake and intracellular transport, respectively. The second group consists of P-type ATPases (ATP7A and ATP7B), which are involved in Cu efflux and intracellular sequestration. P-type ATPases are localized in the trans-Golgi network (TGN) and catalyze the ATP-dependent transport of copper ions across the plasma or intracellular membranes.

The X-chromosome encoded human ATP7A protein is an eight transmembrane domain P-type ATPase with six copper binding motifs in its cytoplasmic N-terminal region. This protein contains a catalytic domain and functional motifs that govern its intracellular cycling between the TGN and the plasma membrane.^{1,4–6} ATP7A preserves cellular copper homeostasis by expelling Cu ions from the cell's interior to prevent toxic accumulation. Under normal conditions this protein is primarily localized to the TGN but also in some other post Golgi compartment. However, if cells are exposed to the

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increased copper concentrations, ATP7A is trafficked to the cytoplasm and plasma membrane.^{4,5,7} It has been proposed that this Cu-induced trafficking of ATP7A is fundamentally crucial for cellular copper homeostasis.^{8,9} Another important role of ATP7A is to deliver copper to the secretory pathway where this metal ion cofactor is incorporated into Cu-dependent enzymes such as lysyl oxidase, tyrosinase, dopamine- β -hydroxylase and peptidylglycine- α -amidating monooxygenase.^{1,10,11}

Mutations in the human ATP7A gene lead to a neurological disorder called Menkes disease (OMIM ID #309400,¹¹). There is considerable variability in the severity of this condition and based on the clinical symptoms, two forms of Menkes disease have been described: classical Menkes disease which is the most severe form and occipital horn syndrome (OHS) which is the mildest.^{12–17} Patients suffering from classical Menkes disease exhibit a wide range of symptoms including cerebellar degeneration, mental retardation, hypothermia, abnormal hair, bone and skin tissue defects, vascular aneurysms, and kidney and urinary tract disease. Most die in early childhood, usually at 3–5 years of age.^{5,12,14,18} Patients with OHS have less profound neurological manifestations, their clinical features are related to connective tissue defects and they have a longer lifespan.^{12,14,16,18,19} Certain missense mutations in the ATP7A gene lead to the recently described ATP7A-related distal motor neuropathy, which is a distinct form of Menkes disease characterized by a progressive distal motor neuropathy without overt signs of systemic copper-dependent enzyme dysfunction.²⁰

The mouse ATP7A protein, which is the subject of the present study, is encoded by the X-linked *Atp7a* gene. In the mouse, mutations in this gene result in the *mottled* phenotype. Many *mottled* mutants have arisen spontaneously or have been induced by chemical or radiation mutagenesis. Currently, about 35 different mutations in the *mottled* locus have been described¹⁵ and 13 of them have been characterized at the molecular level.^{21–23} *Mottled* mutants exhibit defects in copper metabolism and some develop a phenotype closely mimicking the Menkes syndrome. As in humans, the severity of the phenotype in *Atp7a* mutant mice is dependent on the *mottled* allele and varies between *mottled* mutations. The *brindled* (*Atp7a*^{mo-br}) and *macular* (*Atp7a*^{mo-mf}) mouse *mottled* mutants have been extensively studied and are widely acknowledged as good animal models of the severe form of Menkes disease.^{21,24–27}

The *mosaic* mutation (*Atp7a*^{mo-ms}) arose spontaneously in the outbred mouse colony at the Department of Genetics and Evolution of the Jagiellonian University in Kraków.²⁸ The phenotype of the *mosaic* mutant closely resembles those described in *brindled* and *macular* mice.²⁹ *Mosaic* mutant males exhibit many clinical features characteristic of defective copper metabolism, including defects in pigmentation and hair structure, a decrease in body weight, poor viability, progressive paresis of the hind limbs and death at about day 16. Similar to other *mottled* mutants, *mosaic* mice show disturbances in copper transport and absorption. In mutant males, copper is accumulated in the small intestine and kidneys, but Cu deficiency was found in the brain, liver and heart.^{28,30,31} The accumulation of copper in the kidneys often leads to kidney disease in *mosaic* mutants.^{31–33}

Although *mosaic* mice exhibit disturbances in copper metabolism, the molecular defect underlying the observed phenotype

has yet to be identified. However, we previously demonstrated linkage between the *mosaic* phenotype and a polymorphic form of the 4th exon of the *Atp7a* gene, resulting from alternative splicing,³⁴ which strongly indicated that a mutation in this gene is responsible for the observed abnormalities. The aim of the present study was to analyze the sequence of the *Atp7a* gene to identify the critical mutation and to examine its effect on protein expression, intracellular localization and trafficking in *mosaic* mutant cells.

Experimental

Animals

All mice used in this study were bred in the Department of Genetics and Evolution at Jagiellonian University and were derived from a closed, outbred colony. The animals were obtained by mating heterozygous *ms*/+ females with normal +/– males. The experimental material consisted of one group of 14-day-old normal male (+/–) mice and one group of *mosaic* mutant males (*ms*/–) of the same age. These mice were housed at constant temperature (22 °C) under artificial light (12 h photoperiod) and fed a standard MuriGran diet (Motycz, Poland).

All experiments were performed in accordance with Polish legal requirements with the consent of the Commission of Bioethics of Jagiellonian University.

DNA extraction and mutation analysis of the *Atp7a* gene

Mouse genomic DNA was isolated from tail biopsies from three unrelated (derived from different litters of different breeding pairs) *mosaic* mutants and three unrelated wild-type (WT) control males using a standard phenol–chloroform extraction procedure. The oligonucleotide primers listed in Table S1 (ESI†) were used for PCR and sequence analysis. PCR products were examined by agarose gel electrophoresis and then purified using an MSB Spin PCRapace kit (Invitex). These DNA fragments were sequenced in the forward and reverse directions using a DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare Life Sciences) and a MegaBACE 1000 sequencer (GE Healthcare Life Sciences). The obtained sequences were compared with the reference sequence of the mouse *Atp7a* gene (NC_000086.6) using BLAST2 software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).³⁵

Western Blot analysis

Fifty micrograms of total kidney protein extracts were separated by electrophoresis on 6% and 10% SDS-polyacrylamide gels and the resolved proteins were electroblotted onto PVDF membranes (Amersham Life Sciences, Little Chalfont, UK). The membranes were initially blocked by gentle agitation in TBST (Tris-buffered saline +0.15% Tween 20) containing 5% fat-free powdered milk for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibody: mouse IgG1 raised against the C-terminus of mouse ATP7A (BD Transduction Laboratories Cat No. 611770) diluted 1:500 in TBST. After washing three times, the membranes were then incubated for 1 h at room temperature with peroxidase-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted

1 : 20 000 in TBST. Immunoreactive bands were detected using the ECL Plus enhanced chemiluminescence western blotting detection system (GE Healthcare Life Science). Equal loading and the quality of the protein extracts were confirmed by reacting the same blot with a specific antibody to β -actin (goat polyclonal IgG against human actin; Santa Cruz Biotechnology, Santa Cruz, CA, SC-1615).

Immunohistochemical analysis of kidney sections

The experimental animals were sacrificed by cervical dislocation and their kidneys were excised immediately and fixed in 4% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS) (Sigma) at 4 °C for 24 h. After washing three times for 30 min in PBS, the kidneys were soaked in 12.5% sucrose for 1.5 h and then in 25% sucrose for 12 h at 4 °C. Next, the kidneys were embedded in Tissue-Tek compound, frozen in liquid nitrogen and sectioned into 20 μ m slices using a cryostat (Leica). The sections were washed in PBS and permeabilized by bathing in PBS + 0.1% Triton X-100 (Sigma) for 10 min. Non-specific antibody binding was blocked by incubation in PBS + 3% BSA for 1 h, and the sections were incubated overnight at room temperature with primary rabbit polyclonal anti-ATP7A antibody (Genway Biotech) diluted 1 : 250 in PBS + 3% BSA. The sections were washed 3 times with PBS and then incubated with Cy3-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch) diluted 1 : 500 in PBS + 3% BSA. Finally, the sections were washed three times for 10 min in PBS at room temperature and mounted using Vectashield with 4',6-diamidino-2-phenylindole (DAPI; Vector Labs). As a negative control, some sections were prepared without incubating with the primary antibody.

The immunohistochemical analysis of kidney sections was performed for the *mosaic* males and untreated WT controls, plus a second group consisting of *mosaic* and control males that had been injected subcutaneously with CuCl₂ (10 μ g Cu per g of body weight) in 0.9% NaCl and then sacrificed 4 h later. Five animals were analyzed from each group. The prepared sections were examined using a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Jena, Germany).

Results and discussion

Identification of the critical mutation in the *Atp7a* gene in the *mosaic* mutant

In a previous study we demonstrated linkage between the polymorphism in the 4th exon of the *Atp7a* gene and the *mosaic* phenotype.³⁴ However, we were unable to detect any mutation within the coding region of the *Atp7a* gene using a reverse transcriptase-PCR based approach. In the present study, we analyzed the genomic sequence of the *Atp7a* gene in *mosaic* mutant males and WT males with primers listed in Table S1 (ESI†). DNA sequence analysis of all exons and parts of the flanking introns revealed a G to C nucleotide exchange within exon 15 of *Atp7a* in the *mosaic* mutant, corresponding to position 3009 of the cDNA (Fig. 1A), which produces the amino acid substitution R978P (Fig. 1B). The nucleotide and amino acid numbering was assigned according to the OTTMUST0000044157 sequence from the Ensembl database (<http://www.ensembl.org>). Extensive searches of the Ensembl and NCBI databases failed to

reveal any known polymorphisms in this position of the *Atp7a* gene. Moreover, this mutation was not found in any of the three analyzed WT control animals, but was present in all three unrelated *mosaic* males. This strongly indicated that this missense mutation is responsible for the *mosaic* phenotype. As shown in Fig. 1C, the R978P mutation is located in the luminal end of the 6th transmembrane domain, which contains the highly conserved CPC (Cys-Pro-Cys) motif.

Western blot analysis of the ATP7A protein

The identification of a missense mutation in the sequence of the *Atp7a* gene in *mosaic* mutants prompted us to examine the expression of the ATP7A protein. We decided to analyze the kidney because ATP7A is highly expressed in this organ.^{36,37} Using an antibody directed against the C-terminal region of ATP7A, Western blot analysis was performed on total protein extracts from kidneys. The expected 190 kDa ATP7A band was detected in the kidney of 14-day-old *mosaic* mutants and WT control males (Fig. 2, upper panel). Equal loading and the quality of the protein extracts were confirmed by reacting the same blot with an anti- β -actin antibody (Fig. 2, lower panel). This result demonstrated that the mutation in exon 15 of the *Atp7a* gene did not affect the expression of the ATP7A protein.

Immunohistochemical localization of ATP7A in the kidney

Although the expression of ATP7A protein in the kidney of the *mosaic* mutants was apparently unchanged, these animals suffer from kidney degeneration caused by copper accumulation.^{31–33} Therefore, we examined the localization of the ATP7A protein in the kidneys of 14-day-old *mosaic* mutant and WT mice using immunohistochemistry and confocal microscopy. ATP7A was detected in the renal cortex but not in the renal medulla in both groups of mice (data not shown). Renal cortex is composed mainly of the renal tubules and glomeruli (Fig. S1, ESI†). In the renal cortex the expression was restricted to the renal tubules and ATP7A was absent from the glomeruli. Different levels of expression were observed in the renal tubules, from very intense to almost negligible (Fig. 3). We noticed that tubules with bright staining were mainly localized in the outer part of the renal cortex, although ATP7A was also identified in some tubules of the inner cortex.

To analyze the impact of increased copper concentration on the localization of ATP7A, 14-day-old *mosaic* mutant and WT males were injected with CuCl₂. The ATP7A protein pattern was similar in the kidneys of copper-treated and untreated males of both groups, but more tubules in the cortex of the treated animals exhibited bright staining. In the untreated WT mice, ATP7A protein was localized mainly near the basolateral membrane in the majority of cortical tubule cells (Fig. 3A), and immunostaining was rarely seen in the intracellular compartments and the perinuclear region (data not shown). In contrast, ATP7A was observed throughout the cytoplasm and around the nucleus in the cortical tubules of the untreated *mosaic* mutants (Fig. 3B). After copper injection, the kidney cells of WT males exhibited clear localization of ATP7A close to and at the basolateral membrane (Fig. 3C). In *mosaic* mutants injected with copper, a diffuse pattern of ATP7A protein distribution was observed in the cytoplasm (Fig. 3D). No staining was detected in

Fig. 1 Mutation analysis of the *Atp7a* gene of *mosaic* mutant male mice. (A) Sequencing of *Atp7a* PCR products revealed a point mutation in exon 15: a G to C nucleotide exchange at position 3009 of the cDNA. (B) This mutation resulted in an arginine to proline substitution at position 978 of the *mosaic* ATP7A protein. (C) Schematic presentation of the ATP7A protein secondary structure with the location of the catalytic domain indicated. The positions of the critical mutations in previously characterized *mottled* mutants (*brindled*, *11H* and *macular*), representing animal models for severe Menkes disease, are marked by light green boxes, the previously described polymorphism (P445_D446insA) is given in dark green box and the *mosaic* mutation R978P, identified in this study, is marked by an orange box.

Fig. 2 Western blot analysis of the ATP7A protein in the kidneys of 14-day-old WT and *mosaic* mutant male mice. Total kidney protein extracts (50 μ g lane⁻¹) were separated by SDS-polyacrylamide gel electrophoresis and the blot reacted with an anti-mouse ATP7A antibody. The presence of the 190 kDa band representing ATP7A demonstrated the expression of this protein in all analyzed samples. The membrane was re-probed with an antibody to β -actin (42 kDa band) to confirm equal loading of proteins from each tissue extract and also the quality of the extracts. Lane 1—WT mouse kidney, lane 2—*mosaic* mutant mouse kidney, lane 3—WT mouse kidney, lane 4—*mosaic* mutant mouse kidney.

the kidney sections from WT and *mosaic* males in the absence of the primary antibody (Fig. 3E). This result clearly indicated that the missense mutation in the *Atp7a* gene affects intracellular localization and trafficking of the ATP7A protein.

Due to the absence of the functional ATP7A protein in *mottled* mutant mice, cellular Cu uptake is unaltered, but efflux of copper ions to the extracellular space is greatly reduced. *Mosaic* male mice exhibit disturbances in Cu transport and metabolism characteristic of *mottled* mutants and they die within the third week of life.^{30,33,34} Previously, we found that *mosaic* males exhibit disturbances in copper transport and metabolism which appeared to be related to a lack of ATP7A protein activity. In the *mosaic* mutants copper was trapped in the small intestine and renal tubule cells but in the brain the copper level was significantly decreased.^{30,32} ATP7A is the key protein mediating copper transport across epithelial and endothelial cell barriers, and therefore it is crucial for the processes of intestinal absorption, renal reabsorption as well as for the transport across the blood–brain barrier.^{3,38} The X-linked inheritance pattern of the *mosaic* phenotype also

Fig. 3 Expression of ATP7A protein in the epithelial cells of the renal tubules of 14-day-old WT and *mosaic* mutant male mice. Excised kidneys were fixed with formaldehyde, infused with sucrose, then sections cut with a cryostat were permeabilized using Triton X-100, blocked with BSA and incubated with rabbit anti-ATP7A antibody followed by Cy3 anti-rabbit antibody (red staining). Slides were mounted using Vectashield with DAPI. (A) Intracellular localization of ATP7A protein in the cells of the renal tubules of the WT control. The protein is localized in close proximity to the basolateral membrane. The corresponding region of kidney stained with hematoxylin and eosin is shown in Fig. S1 (ESI[†]). (B) Dispersed pattern of ATP7A expression in the cells of the renal tubules of the *mosaic* mutant. (C) Basolateral localization of the ATP7A protein in the cells of the renal tubules of the WT following injection of cupric chloride. (D) ATP7A protein expression in the cytoplasm and around the nucleus in cells of the renal tubules of the *mosaic* mutant following injection of cupric chloride. (E) To confirm the specificity of the ATP7A antibody, kidney sections of WT and *mosaic* males were incubated without the primary antibody. No staining was detected in these negative controls (scale bar = 20 μ m).

suggested linkage to the *Atp7a* gene.²⁷ Moreover, the phenotype of *mosaic* mutants closely resembles that of *brindled* mice.²⁹ Previous analysis of the coding region of the *Atp7a* gene in *mosaic* mutants revealed the presence of an additional 3 nucleotides (CAG) at position 1417 at the 3' end of exon 4. This 3 bp insertion is associated with alternative splicing of exon 4 and results in the addition of an alanine residue within the interval between the 4th and 5th copper binding domains

of the ATP7A protein.³⁴ This transcript variant was found in *mosaic* mutants and not in control males, but the same insertion was also found in other inbred strains of mice and is recognized as a polymorphism²² (transcript ID ENSMUST00000055941 in Ensembl database). Multiple alignment of mouse ATP7A protein with other species revealed that an additional alanine was not present in humans or other mammals but was found in rat ATP7A (Fig. S2, ESI[†]). It is therefore unlikely that the

presence of this additional amino acid could be the cause of the *mosaic* phenotype. However, the linkage between this transcript variant and the *mosaic* phenotype strongly indicated that mutation in the *Atp7a* gene is the underlying cause of this phenotype.³⁴ In the present study we identified a G to C nucleotide exchange in exon 15 of the *Atp7a* gene resulting in a substitution of arginine by proline in the ATP7A protein of *mosaic* mutants. Exon 15 encodes the highly conserved 6th transmembrane domain containing a CPC amino acid motif, which serves as a channel for cupric ion transport^{19,39} and is critical for the protein's function.^{2,6} Moreover, this domain is characteristic of and highly conserved in all P-type ATPases.

The membrane-spanning regions of proteins are comprised of hydrophobic amino acids in the lipid bilayer with positively-charged residues at the points of membrane entry–exit.^{2,3,39} The substitution of an alkaline, positively-charged arginine residue for a neutral and hydrophobic proline which can also produce a bend in the polypeptide chain, in the 6th transmembrane domain may result in incorrect folding, which can lead to a change in the tertiary structure of the protein. In addition, such a substitution might affect the anchoring of ATP7A in the cellular membrane system and as a consequence alter protein trafficking, because comparison with the recently published CopA structure indicated that R978 is located at the luminal end of the 6th transmembrane domain at the border of the membrane.⁴⁰ Furthermore, the efficiency of copper binding and delivery may also be affected. In Cu-transport ATPases the transmembrane domains undergo conformational changes during cation translocation across membranes.⁴¹ The altered cellular localization and trafficking of the mutated ATP7A in *mosaic* mutant kidney cells strongly support the hypothesis that the R978P substitution affects the anchoring of this protein to the sub-cellular membrane system. Copper accumulation in the small intestine and kidney, and copper deficiency in other organs of *mosaic* mutants^{32,33} might be a result of a compromised transport function of the mutated ATP7A protein.

We postulate that the *mosaic* mutant represents a valuable animal model for the severe form of human Menkes syndrome. So far, 183 mutations in the *ATP7A* gene have been deposited in the human gene mutation database (HGMD, <https://portal.biobase-international.com/hgmd>): 171 are associated with Menkes disease, 10 with occipital horn syndrome (OHS) and 2 with distal hereditary motor neuropathy.²⁰ A wide range of different types of mutations in this gene have been described, including complex chromosome rearrangements, large deletions, small deletions/insertions/duplications, single nucleotide substitutions leading to nonsense or missense mutations, and splice site mutations.^{13,16,39,42,43} Missense mutations in the human *ATP7A* gene are relatively frequent (33% of the total) and they are almost exclusively located between the region encoding the first transmembrane domain of the ATP7A protein and the stop codon.^{13,16} In humans as in mice, the 6th transmembrane domain containing the CPC motif is encoded by exon 15. In this exon, the majority of known mutations are missense mutations resulting in the classical form of Menkes disease with long survival (>6 year).^{18,42} It was also previously reported that in the cultured CHO cells mutation, which affects the first cysteine in the CPC motif (C1000R), resulted in the disturbances in copper transport. In the mutant cells, cultured under both copper basal and copper

elevated conditions, ATP7A protein was detected in the perinuclear region.⁴⁴ It indicates that mutation in the conserved 6th transmembrane domain affects the copper transport.⁴⁴

In the *brindled* mice, which of all *mottled* mutants have a phenotype closest to that of Menkes disease,²⁵ the lethal phenotype is caused by the deletion of 6 nucleotides (CTCTTG) at position 2478–2483 in exon 11.^{23,24} The deleted nucleotides encode leucine and alanine residues within the cytoplasmic region of the protein located between the 4th transmembrane domain and the transduction domain.²¹ Hemizygous *brindled* males die in the second week of life.^{21,23,24} *Macular*, another mouse *mottled* mutant, also exhibits a lethal phenotype.^{27,45,46} Analysis of the coding region of the *Atp7a* gene in *macular* mutants revealed a T to C exchange at position 1382 which produces a serine to proline substitution in the conservative 8th transmembrane domain. *Macular* males also die in the second week of life.^{27,45}

In the present study we analyzed the expression, intracellular localization and Cu-stimulated trafficking of the ATP7A protein in epithelial cells of the renal tubules (polarized cells) of the WT and *mosaic* mutant mice. ATP7A protein is highly expressed in the renal tubules,^{1,36,37} because in the healthy kidney, the proximal tubules are the first site of copper reabsorption from urine. The reclaimed copper ions are then transferred back to the blood circulation via the ATP7A protein.^{1,37,47} In the aim to test the specificity of the antibody used in immunohistochemistry by performing Western blot analysis, which aims at the comparison of ATP7A level in the kidney and liver of wild-type mice, the two tissues showing an opposite pattern of ATP7A protein expression are the kidney and liver (high and low level, respectively) at least in adult mice. Therefore, if the antibody is specific, a clear difference in the signal between these two organs should be observed. Indeed, a strong band was detected in the kidney whereas in the liver only a weak band was observed (data not shown). Our results perfectly reproduce those obtained in our previous study⁴⁸ where we analysed expression of ATP7A protein. Our results are in agreement with previous findings demonstrating that the ATP7A expression in the renal cortex is restricted to the renal tubules.^{8,27,37} Furthermore, the observed differences in the level of expression in the cortical tubules and ATP7A localization near to the basolateral membrane have been described previously,³⁷ which is a strong indication that the antibody used for immunostaining was specific. In the present study we found that in the untreated WT mice, ATP7A protein was localized mainly close to the basolateral membrane in the majority of renal tubule cells. Our finding is in agreement with the previous one showing that ATP7A localization in the cells of the renal tubules in mice is age-dependent. It has been reported that in 2 weeks old mice ATP7A is located in the basolateral membrane, whereas in the 8-week-old mice the protein exhibit basolateral staining only in some cells, in the majority of tubules ATP7A localization was mostly vesicular and perinuclear. In the kidney of adult animals (20-weeks-old) ATP7A protein showed perinuclear expression.³⁷

It has previously been demonstrated that when the copper concentration increases in the cell, ATP7A is transported in vesicles via the cytoplasm to the plasma membrane in order to carry out its primary role in the efflux of excess copper.^{1,5,7} In contrast, the exposure of kidney cells of the *mosaic* mutants to

a higher copper concentration did not stimulate protein trafficking to the basolateral membrane. Thus, it may be concluded that the absence of ATP7A activity hinders the removal of excess copper in the kidneys of *mosaic* mice and results in the accumulation of this element to toxic levels, as previously demonstrated.^{30,31,33} This is also in agreement with the finding that the uptake of copper from urine is undisturbed in *mottled* mutants, but the mechanism of its transfer from the cell into the circulation is impaired due to the low/lack of activity of ATP7A protein, so that Cu ions are trapped in the epithelial cells of the kidney tubules.^{32,33,47,49}

A similar pattern of ATP7A expression was found in another *mottled* mutant, *11H* (*Atp7a*^{mo-11H}).⁵⁰ The embryonic lethal *11H* mutation is caused by a single C to A nucleotide substitution at position 4137 of the *Atp7a* gene, which results in a change from alanine to a negatively charged aspartic acid residue at position 1364 within the 7th transmembrane domain of ATP7A protein.²² Immunohistochemical analysis revealed that expression of ATP7A in *11H* mutants exhibits reticular distribution around the nucleus and throughout the cytoplasm.⁵⁰ Western blotting showed that this mutation leads not only to mislocalization, but also causes impaired glycosylation of ATP7A.⁵⁰ Our analysis of the ATP7A in *mosaic* males using the same procedure indicated that the molecular weights of the mutant and WT proteins are identical. This suggests that the *mosaic* mutation results in mislocalization, but does not affect the glycosylation of ATP7A. It is noteworthy that a similar expression pattern of ATP7A in the *11H* and *mosaic* mutants does not correlate with the phenotypic severity. *Mosaic* mutants survive gestation and die about 16 days after birth, but *11H* mutants die in the uterus.

In *brindled* mice, which exhibit a phenotype closely resembling that of the *mosaic* mutant, ATP7A protein is correctly localized in the TGN but fails to relocate to the cytoplasm and the plasma membrane under elevated copper conditions.²¹ In the *macular* mutants, ATP7A is only partially retained in the TGN and this protein appears to be redistributed to post-Golgi compartments and the plasma membrane.⁵⁰

Conclusions

The results of the present study indicate that mutation in the mouse *mottled* locus leads to great variation in the expression pattern of the ATP7A protein. It may be concluded that *mottled* mutants (including *mosaic*) represent a valuable model for studying the phenotypic effects of different mutations within the *Atp7a* gene. In *mosaic* mutants we identified a G to C nucleotide exchange in exon 15 of the *Atp7a* gene which resulted in an arginine to proline substitution in the highly conserved 6th transmembrane domain of the ATP7A protein. Results of the immunohistochemical analysis of the ATP7A in the kidney cells isolated from *mosaic* mutant mice exhibit changes in intracellular localization of the protein in comparison to the wild-genotype mice. We also found that in the mutants copper transport efficiency of ATP7A protein is decreased because, following exposure of these cells to increased copper concentrations it was not translocated to the plasma membrane. Disturbance of ATP7A function in *mosaic* mice results in increased copper accumulation in the

small intestine and kidneys, and in Cu deficiency in the brain, liver and heart.

Disclosure

The authors report no conflict of interest.

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