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Hepatic copper-transporting ATPase ATP7B: function and inactivation at the molecular and cellular level

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Abstract Copper-transporting ATPase ATP7B (Wilson disease protein) is a member of the P-type ATPase family with characteristic domain structure and distinct ATP-binding site. ATP7B plays a central role in the regulation of copper homeostasis in the liver by delivering copper to the secretory pathway and mediating export of excess copper into the bile. The dual function of ATP7B in hepatocytes is coupled with copper-dependent intracellular relocalization of the transporter. The final destination of ATP7B in hepatocytes during the copper-induced trafficking process is still under debate. We show the results of immunocytochemistry experiments in polarized HepG2 cells that support the model in which elevated copper induces trafficking of ATP7B to sub-apical vesicles, and transiently to the canalicular membrane. In *Atp7b*$^{-/-}$ mice, an animal model of Wilson disease, both copper delivery to the *trans*-Golgi network and copper export into the bile are disrupted despite large accumulation of copper in the cytosol. We review the biochemical and physiological changes associated with Atp7b inactivation in mouse liver and discuss the pleiotropic consequences of the common Wilson disease mutation, His1069Gln.

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Introduction

In most cellular processes, a fine balance of micronutrients must be maintained to avoid either excess or deficiency. Copper is a required cofactor for the function of several essential metabolic enzymes. An insufficient copper supply leads to disturbances of pathways that involve enzymatic reactions dependent on copper in proteins such as Cu, Zn-superoxide dismutase (SOD1), ceruloplasmin, tyrosinase, and many others. The ability of copper to participate in redox reactions also requires tight regulation of metal availability. Such tight regulation ensures that copper is channeled to the right compartment where it is utilized, while excess copper is promptly excreted. On a whole body level, the central role in regulation of copper homeostasis belongs to the liver. Liver utilizes 50% of the copper that crosses the intestinal epithelium [1] and also mediates copper excretion. Under normal physiological conditions about 98% of all copper excretion occurs via the bile [2].

In the liver, several proteins are involved in the uptake, distribution, and export of copper (for recent reviews see [3, 4]). Although functions of the individual proteins are mostly known, how

these proteins are orchestrated to maintain cellular copper homeostasis is yet to be fully understood. The high affinity copper transporter, hCtr1, is likely to be responsible for the uptake of copper into hepatocytes. Once copper is translocated across the plasma membrane into the cell, it is transferred to the cytosolic copper chaperones [5]. The mechanism of copper transfer from hCtr1 to the metallochaperones and subsequent distribution among the chaperones is unknown, although direct contact between the transporter and the chaperone was demonstrated for the yeast Ctr1/Atx1 pair in vitro [6]. Copper is then delivered to the mitochondria for incorporation into cytochrome C oxidase in a complex process that involves several copper binding proteins [7–9]. Copper is also shuttled to cytosolic SOD1, an essential enzyme involved in radical detoxification [10–12]. Finally, the copper requirements of the secretory pathway are satisfied through coordinated action of two proteins. Atox1 is a copper chaperone that delivers copper to the copper transporting ATPase, ATP7B [13, 14]. ATP7B then translocates copper across the membrane so the metal can be biosynthetically incorporated into ceruloplasmin, a secreted copper-dependent ferroxidase synthesized in the liver [15–17].

In addition to its important biosynthetic function, ATP7B plays an essential role in export of excess copper into the bile. The precise mechanism of copper excretion into the bile remains controversial, however it is clear that intracellular trafficking of ATP7B in response to elevated copper represents an important step to this process (see below for details). Disruption of ATP7B function has dramatic consequences for liver function and overall copper metabolism. In patients with Wilson disease (WD) both copper delivery to the secretory pathway and biliary excretion are disrupted due to mutations in *ATP7B* [18]. As a result, copper accumulates in the liver and other tissues, which lead to a spectrum of liver pathologies, neurological abnormalities, and, if left untreated, death. In recent years, significant progress has been made in understanding structure, function, and regulation of ATP7B (for review see [19]). The present review describes the biochemical and cellular properties of hepatic ATP7B and the major WD-causing mutant H1069Q and highlights consequences of ATP7B inactivation in the liver of *Atp7b*$^{-/-}$ mice, an animal model of WD.

Molecular architecture and enzymatic properties of ATP7B (Wilson disease protein)

The copper-transporting ATPase ATP7B is a large membrane transporter with distinct functional properties that are reflected in the architecture of the protein (Fig. 1). ATP7B binds and hydrolyzes ATP; copper binding to the transporter stimulates this reaction [20]. The energy released during ATP hydrolysis is utilized to bring about conformational changes within the ATP7B structure and release bound copper at the opposite side of the membrane. Five functional domains can be identified in the structure of ATP7B (Fig. 1). The transmembrane domain consists of eight transmembrane segments (TMS) and forms the copper translocation pathway. Recent studies identified several residues in this portion of the protein which are critical for the function of ATP7B or other copper-transporting ATPases [21–23], however the detailed structure of the copper-permeation pathway remains to be determined. TMS6 contains the characteristic CysProCys motif, in which Cys residues are thought to be involved in copper coordination during metal translocation (Fig. 1). By analogy with bacterial copper-transporting ATPases, other copper coordinating residues could be provided by TMSs 7, 8 [22, 24, 25]

The cytosolic N-terminal domain (N-ATP7B) has six metal-binding sites (MBS) and accepts copper from the copper chaperone Atox1 [26, 27]. The N-terminal MBS are similar in sequence and structurally [28] and each binds one copper in the conserved sequence GMT/HCxxC via cysteines [29, 30]. Binding of copper to MBS is ordered [31] and although structural changes within individual MBS are small [32], the entire N-ATP7B undergoes conformational transitions as evidenced by circular dichroism and proteolysis [29, 31]. Large body of mutagenesis work indicates that the MBSs closest to the membrane (MBS5 and MBS6) are required for the transport function while more distal MBSs play a regulatory role [33–36]. Binding of copper to MBS 5 and 6

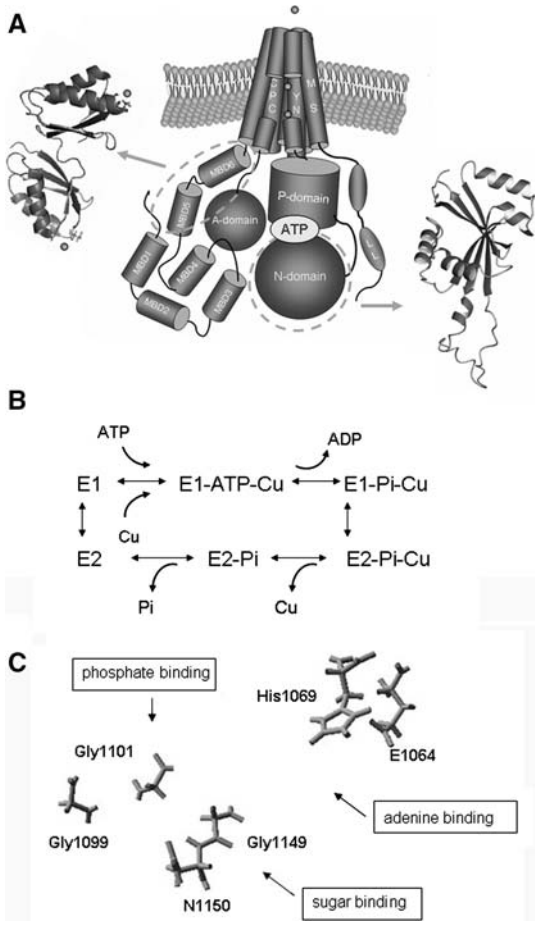

Fig. 1 Functional domains (**A**), catalytic cycle (**B**), and ATP-binding site (**C**) of ATP7B. Five functional domains of ATP7B are shown. The N-terminal domain contains six metal-binding sites (MBS1-6); the crystal structure of MBS5,6 according to [36] are shown on the left. The transmembrane portion consists of eight transmembrane segments (TMS); the characteristic CPC motif is located in TMS6; other residues in TMS7 and TMS8 that are conserved in copper-transporting ATPases and likely contribute to copper coordination are indicated by letters. Three other domains are cytosolic and include the actuator domain (A-domain); the ATP-binding domain composed of phosphorylation (P-domain) and nucleotide-binding domain (N-domain); and the C-terminus. The solution structure of the N-domain according to [43] is shown on the right. The Leu residues in the C-terminus required for protein return to TGN are indicated by LL

appears to alter the affinity of the intramembrane site(s) for copper [33] and thus control the catalytic activity of ATP7B [37]. MBS1–4, which are separated from MBS5,6 by a long linker, interact with the ATP-binding domain in the apo form and down-regulate catalytic activity; binding of copper or deletion of these domains relieve the interaction and accelerates catalysis [33, 37].

The N-terminal domain is also involved in directing ATP7B to the appropriate intracellular compartments during copper-induced relocalization [35] (see below) and is the site of interaction with various proteins, implicated in regulation of protein activity and trafficking. These include copper-chaperone Atox1 [26], Murr1/Commd1 [38], dynactin subunit p62 [39] and glutaredoxin [40]. Although the functional consequences for most of these interactions remain to be characterized, the diversity of interacting proteins emphasizes the complexity of regulatory processes mediated by the N-terminal domain.

The ATP-binding domain (ABD) is central for the activity of ATP7B. It is the harnessing of the energy of ATP hydrolysis by this domain that fuels the transport of copper across the membrane by ATP7B. ATP7B functions as a P-type ATPase, i.e. it forms a transient acyl-phosphorylated intermediate during ATP hydrolysis [37] (Fig. 1B). The phosphorylated residue Asp1027 belongs to a DKTG motif that is a signature sequence of the P-type ATPase family and is located within the ABD. The ABD is divided into two parts: the phosphorylation domain (P-domain), which houses Asp1027 and is structurally similar in all P-type ATPases, including copper-transporting ATPases [41, 42] and the nucleotide-binding domain [N-domain). The high-resolution structure of the N-domain of ATP7B in the presence of ATP has been recently solved by multidimensional NMR [43]. The structure revealed unique nucleotide-binding environment of ATP7B and other copper-ATPases compared to other P-type ATPases and provided structural framework for the analysis of various disease-causing mutations located in this domain (see also below). Five residues in the N-domain are conserved in all copper-transporting ATPases (in ATP7B, these are E1064, H1069, Gly1099, G1101, Gly1149); these residues and the less conserved N1150 form the ATP-binding site (Fig. 1C).

No direct structural information is available for the other two cytosolic domains of ATP7B. The A-domain is located between TMS5 and TMS6 and contains invariant sequence TGE. Recent

structure of the A-domain of bacterial thermophilic copper-transporting ATPase CopA suggests that this domain is very similar in its structure to the A-domain of Ca^{2+}-ATPase [42, 44]. In Ca^{2+}-ATPase and other P-type ATPases the A-domain is essential for dephosphorylation of the catalytic intermediate, with the invariant TGE motif playing a central role in this process [45]. The A-domain undergoes significant movements during catalysis coordinating conformational changes in the cytosolic and transmembrane portion of the pump [46, 47]. It seems likely that in ATP7B the A-domain can be involved in a cross-talk between the N-terminal MBS5 and MBS6 and the transmembrane portion via copper-dependent protein–protein interactions. In this scenario, MBS5 and/or MBS6 may interact with the A-domain (see Fig. 1A), as previously suggested in [48], and copper binding to MBS5 and/or MBS6 would alter the interaction resulting in the A-domain movement and coupled conformational changes in the transmembrane portion of the transporter. This hypothesis is supported by the observation that the Cys> Ala mutations within MBS6 or MBS1-5 of ATP7B affect the apparent affinity of the intramembrane sites for copper [33].

The C-terminal tail of ATP7B is not required for catalytic function but is important for protein stability [49, 50] and for regulation of the intracellular localization of ATP7B [51]. Recent studies from Cater and colleagues demonstrated that the C-terminal leucine based sequence $L^{1454}LL^{1456}$ is necessary for the TGN localization of ATP7B in basal medium and therefore may have a role in the recycling of trafficked ATP7B molecules back to the TGN [51] and/or TGN retention.

Copper dependent regulation of ATP7B function in hepatocytes.

The dual biosynthetic and homeostatic role of ATP7B in the liver is performed in distinct cell compartments. Current data indicate that several factors including copper concentration, catalytic activity, post-translational modification and interactions with other proteins affect the intracellular localization of ATP7B (for example [22, 52, 53]). It is also clear that certain structural elements may profoundly affect copper-dependent localization of ATP7B [34, 35, 51]. Studies from several laboratories provided convincing evidence that under basal copper conditions ATP7B is present in the Golgi, particularly the TGN [35, 52, 54, 55]. In this compartment ATP7B delivers copper to ceruloplasmin (Fig. 2), which is also found in vesicles in the secretory pathway, presumably in the copper-bound form. When copper is elevated, ATP7B leaves the TGN and traffics to a vesicular compartment (Fig. 2) (35, 52, 55]. This re-localization serves to remove excess copper from the cell. Interestingly, in hepatocytes, not all of the ATP7B leaves the TGN, even at high copper (our observation). Some protein remains behind, presumably for biosynthetic purposes. (The molecular basis for this apparent heterogeneity in ATP7B is currently unknown). As the cellular

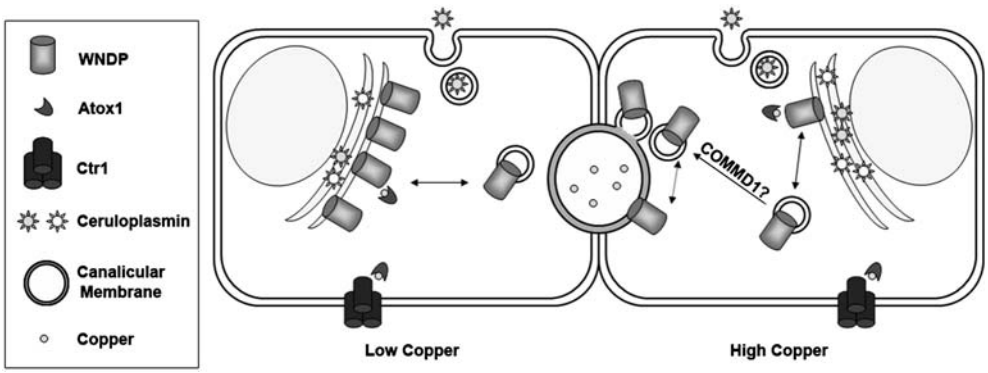

Fig. 2 Cartoon of ATP7B localization under low and high copper in hepatocytes

copper levels decrease, ATP7B returns to the TGN.

The final destination of ATP7B during copper-induced trafficking process has been a matter of debate. In the human liver, immunoreactivity for hepatic ATP7B was observed close to the plasma membrane [56]. However, the results from several laboratories [51, 52] agree that ATP7B does not traffic preferentially to the plasma membrane to directly export copper into the bile, as most of the localization of ATP7B in high copper is intracellular (vesicular). Consequently, two scenarios explaining the role of ATP7B in copper export have been proposed. In the first model, upon copper elevation ATP7B traffics to the vesicles, the copper chaperone Atox1 delivers copper to ATP7B, and the copper accumulates in the vesicular lumen. Then, in a process that has not been defined, ATP7B and the copper containing vesicles part ways, ATP7B returning to the TGN while the copper is taken to the canalicular membrane (the liver equivalent of the apical membrane), where vesicles fuse with the membrane and release the copper [2, 52]. The copper exported into the bile is then cleared into the stool. Data supporting this model include studies in primary and cultured hepatocytes, as well as transfected fibroblasts, all of which showed predominantly vesicular localization of ATP7B in high copper [34, 52].

The second model is similar except ATP7B reaches the canalicular membrane with the copper containing vesicles. Following vesicle fusion and copper release, ATP7B is quickly retrieved from the membrane via endocytosis and returns to the TGN when the concentration of copper returns to basal levels. This model is supported by data from Roelofsen and co-workers, who studied localization of ATP7B in partially polarized HepG2 cells under various copper conditions [55]. These authors demonstrated localization of ATP7B at the canalicular membrane by co-staining with a canalicular membrane marker for the multidrug resistant protein 2 (MRP2). More recently, the high resolution confocal microscopy and fluorescence imaging performed by Guo et al. also revealed partial localization of ATP7B at the apical membrane of WIF-B cells, a rat-human polarized hepatic cell line, after treatment with copper [35]. By contrast, the recent studies in fibroblasts [51] are inconsistent with the plasma membrane delivery of ATP7B. However, it is currently unclear how well fibroblasts, non-polarized cells that do not express ATP7B endogenously, recapitulate ATP7B trafficking in hepatocytes.

Since some of the trafficking studies employed the same hepatic cell types, methods, and similar reagents, the discrepancy in the results suggest quantitative rather than qualitative differences. For example, in contrast to Roloefson et al., Cater and colleagues detected very little ATP7B in the canalicular membrane of partially polarized HepG2 cells, while vesicles surrounding the canalicular membrane were very apparent [51, 55]. There are two possible reasons for the apparent discrepancy in the results. The staining of bile canaliculi markers is typically intense, since the local concentration of proteins in this very small region is relatively high. Thus, the signal from the marker protein can easily mask the overlapping staining of ATP7B, which is not very abundant and distributed between several membrane compartments. Secondly, for continuously cycled vesicles/protein only a certain percent of ATP7B-containing vesicles fuses with the membranes at every given time and thus, by definition, more intense staining is observed in the sub-canalicular vesicles and not at the membrane. Consequently, the detection of ATP7B at the plasma membrane may depend significantly on the sensitivity of anti-ATP7B antibodies.

To provide quantitative evaluation of localization of ATP7B in hepatocytes we measured co-localization between ATP7B and MRP2, a canalicular membrane marker, in partially polarized HepG2 cells treated with either BCS, a copper chelator to deplete copper, or in the presence of 50 μM $CuCl_2$. Z-sectioning (0.27–0.38 μM section height) was performed to decrease the possibility of vesicles overlapping in the XY plane due to vertical ''stacking''. Overlap between ATP7B and MRP2 was quantitated using the Zeiss LSM software. Figure 3A and B illustrates that in low copper the vast majority of canalicular membrane is devoid of ATP7B, in agreement with the previously published reports. Small amount of ATP7B can be detected in/at some canalicular

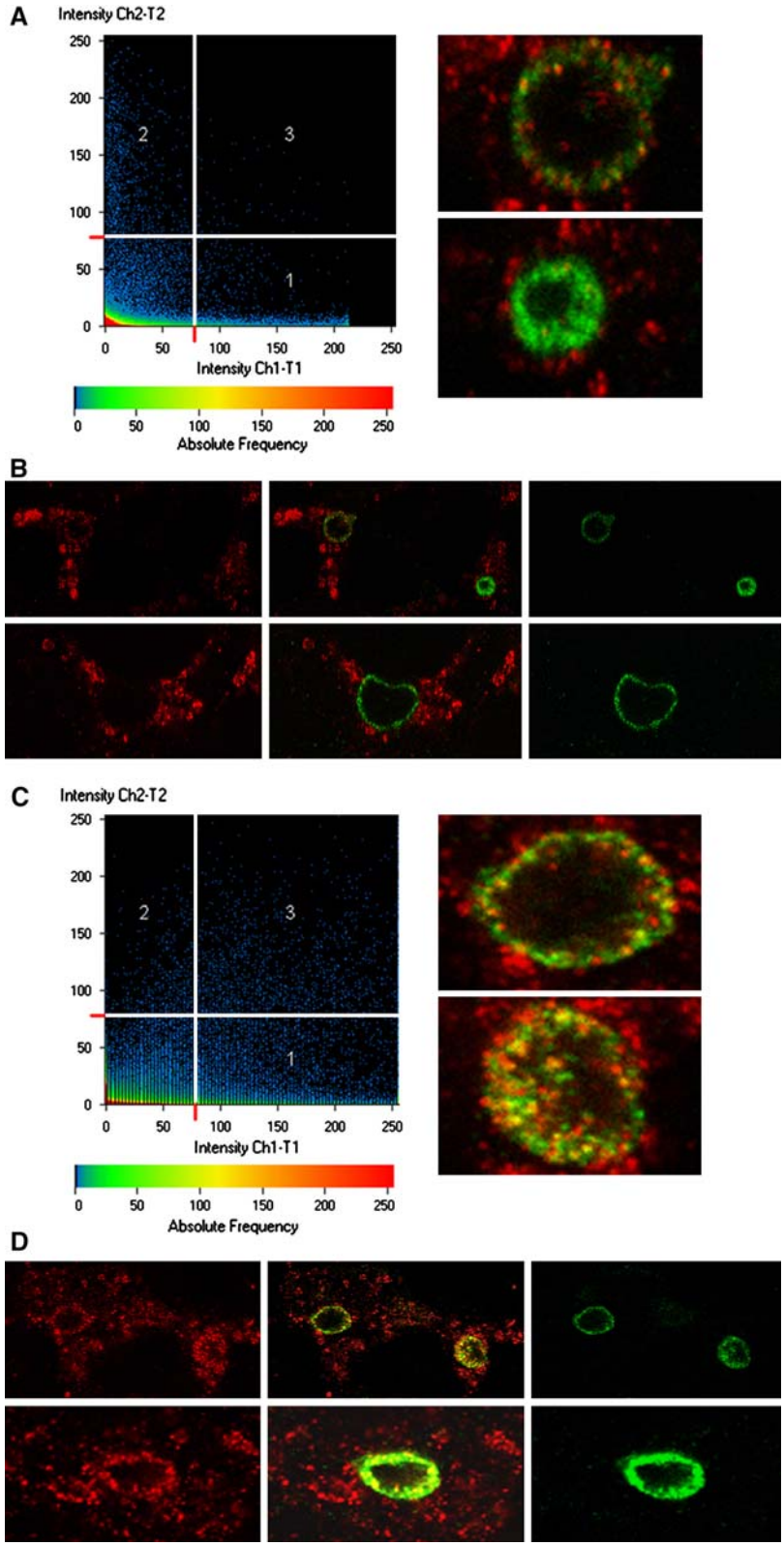

Fig. 3 Co-localization of ATP7B and MRP2 in partially polarized HepG2 cells in low and elevated copper. HepG2 cells are treated with 50 μM bathocuproinesulfonate, BCS, (**A**, **B**) or with 50 μM $CuCl_2$ (**C**, **D**) 3 h before immunostaining with anti-ATP7B Ab and anti-MRP2 Ab (Kamiya Biomedical Company). Alexa Fluor 555 Donkey anti Rat and Alexa Fluor 488 Donkey anti Mouse (Molecular Probes) were used as secondary antibodies. Cells were visualized on a Zeiss LSM 5 Pascal confocal microscope with a 100× objective lens. Z-sections of HepG2 cells were taken at 0.27–0.38 μM intervals, exciting and scanning separately for each wavelength. LSM software was used to compare regions of overlap between MRP2 and ATP7B. (**A**, **C**) Quantitation of co-localization (plot on the left) for typical images, shown on the right (1—ATP7B staining, 2—MRP2 staining, 3—overlapping pixel density). In either A or C, the top image illustrates staining less frequently observed pattern under corresponding conditions (15–20%); the bottom image illustrates the most common pattern (80–85%) Panels (B, D) Illustrate typical images for copper treated (**B**) and BCS treated (**D**) cells at lower magnification. (Left—ATP7B, Center—ATP7B and MRP2, Right—MRP2 alone)

membranes (Fig. 3A, right top panel) consistent with the ability of this protein to traffic constitutively. In high copper, there is a considerable increase in the staining of ATP7B in close proximity to canalicular membrane as well as within the canalicular membrane itself (Fig. 3C, D). When the laser intensity for acquiring MRP2 image is decreased from 10% to 5%, the overlap with ATP7B is even more apparent. At the same time, the detailed inspection of images (Fig. 3C, right panel) demonstrates that within the canalicular membrane, the co-localization with MRP2 is not extensive, suggesting that rather than staying in membrane for considerable period of time, ATP7B is continuously delivered (via fusing vesicles) and retrieved (via budding vesicles). This scenario may also explain the ''vesicular'' rather than smooth staining of ATP7B at the canalicular membrane, which was also observed by Guo and colleagues [35]. Overall, our results appear more consistent with the model shown in Fig. 2, in which copper- and ATP7B-containing vesicles transiently fuse with the canalicular membrane resulting is copper release and subsequent endocytosis of ATP7B.

The structural and functional consequences of the common Wilson disease mutation H1069Q

More than 300 mutations that result in Wilson disease phenotype have been identified in *ATP7B* (for details see: http://www.medicalgenetics.med.ualberta.ca/wilson/index.php). One of the most common and best-characterized mutations, H1069Q, is located in the nucleotide-binding portion of the ATP binding domain (the N-domain). In cultured cells, the expression of recombinant ATP7B with the H1069Q substitution results in the entrapment of the mutant in the ER and decreased protein stability [57]. Similarly, mis-localization of H1069Q mutant was observed in the liver of Wilson disease patient using immunohistochemistry and electron microscopy [58]. These results suggested an effect of His replacement on ATP7B folding. However, subsequent biochemical characterization of recombinant ATP7B as well as the isolated N-domain of ATP7B showed that the His1069 mutation does not significantly alter protein folding as indicated by unaltered proteolysis pattern [59] or CD spectroscopy measurements [60]. Further studies revealed marked effect of His1069 mutation on protein function. The substitution of His for either Gln, Ala, or Cys residues completely abolished the ability of ATP7B to form a phosphorylated intermediate upon addition of ATP [59]. Significantly, phosphorylation from inorganic phosphate (the reaction reverse to ATP-hydrolysis) is unaffected in the H1069C mutant indicating that the major effect of the mutation is on binding of ATP.

This conclusion has been confirmed by recent structural studies of the N-domain of ATP7B. The solution structure of the N-domain of ATP7B shows that in the ATP binding site H1069 is located near the adenine moiety (Fig. 1C), although whether it makes direct contacts with ATP remains to be determined [42, 43]. The mutation does not change the folding of the apo protein neither does it significantly alter the configuration of the binding site [43]. At the same time, the affinity of the N-domain for ATP is markedly decreased, by approximately 20-fold [60]. A similar effect (decrease in the affinity for ATP) was reported when the residue equivalent to H1069 was mutagenized in copper-transporting ATPase ATP7A, which is homologous to ATP7B [61].

How the lack of mutation on protein structure can be reconciled with mislocalization and decreased stability of mutant ATP7B in cells? Recent structural studies provide some clues. The NMR experiments demonstrated that the binding of ATP to the N-domain is accompanied by conformational changes that markedly increase protein stability. The apo-protein quickly aggregates at room temperature, while the ATP-bound N-domain is stable for weeks [43]. This observation suggests that ATP-binding markedly decreases protein dynamics offering insight into the cellular behavior of the H1069Q mutant. It seems that the conformational flexibility due to inefficient binding of ATP by ATP7B with the H1069Q mutation is recognized by the cell quality control apparatus resulting in entrapment of H1069Q mutant in ER. Binding of ATP (in WT protein) or decrease in cell temperature [57] decreases protein dynamics and allows ER exit. The ATP hydrolysis function of ATP7B H1069Q

mutant however remains impaired. Altogether, one can conclude that the major effect of the Wilson disease mutation H1069Q is on protein's ability to bind ATP and on protein dynamics associated with this event. The overall structural organization of the mutant remains preserved, however catalysis is impaired. The relatively mild phenotype of disease in patients with H1069Q mutations [62] and residual activity of the ATP7B homologue ATP7A with the equivalent mutation [61] suggest that efforts towards finding ATP analogues that may enhance the nucleotide-binding function of mutant protein could be a worthwhile direction in a search for new and more effective Wilson disease treatments.

Consequences of ATP7B inactivation in *Atp7b*$^{-/-}$ mice

Inactivation of ATP7B is associated with significant copper overload and severe and diverse abnormalities in the liver function [18, 63, 64]. In addition, the delivery of copper to the secretory pathway is disrupted resulting in production of inactive apo-cerulopasmin [15, 16, 58]. It is thought that accumulated copper facilitates formation of reactive oxygen species, which in turn induce wide-spread damage of proteins, lipids, and DNA. This mechanism has been supported by numerous reports documenting elevated lipid peroxidation, increased frequency of DNA mutations and decrease enzymatic function in Wilson disease patients and in the animal model of Wilson disease (for example, [65–67]). However, recent studies of *Atp7b*$^{-/-}$ mice indicate that these pathological changes are likely to reflect the later stages of the disease while at the early stages of copper accumulation, the effects of copper are more specific and restricted to specific cellular compartments and pathways. Specifically, in *Atp7b*$^{-/-}$ livers copper raises quickly and reaches its highest level in the 5–6 weeks-old animals [68, 69]. At this time, histopathology is not apparent, although detailed examination demonstrates alterations in nuclear size or morphology and mild inflammation, in some animals. Subsequently, after 12–20 weeks, the levels of copper decline and at the 7–11 months-old animals, the typical copper excess is about 4–5-fold [68]. During this period of time (from 3 to 11 months of age) dramatic changes occur in the liver. Marked increase of inflammation, necrosis, and fibrosis at 12–26 weeks are followed by regeneration of a large portion of the liver, while bile ducts proliferate resulting in the development of cholangiocarcinoma [68]. The mechanisms through which accumulated copper triggers the development of this complex phenotype are poorly understood and the elucidation of copper-induced changes in *Atp7b*$^{-/-}$ liver is likely to uncover new and fascinating chapters in human copper biology.

First experiments on biochemical characterization of livers from control and *Atp7b*$^{-/-}$ animals yielded interesting and somewhat unexpected results, which complement and extend previous studies on other animal models, such as LEC rat and toxic milk mice (for review, see [70]). The studies demonstrated that the vast majority of copper accumulates in the cytosol, where it is found associated with the low-molecular weight proteins, most likely metallothioneins, which are highly upregulated in response to copper [68, Huster et al. submitted]. The efficient copper sequestration by metallothioneins may explain why at the early stages of copper accumulation there are no apparent changes either at the tissue [68] or protein level (Huster et al. submitted). Among the membrane compartments copper is significantly elevated in the nuclei [68], which is consistent with the changes in the nuclear structure and function. It has also become clear that copper accumulation has very distinct effects on liver transcriptome (Huster et al. submitted). Most interesting and unexpected are effects on cholesterol metabolism (markedly down-regulated) and on the cell machinery involved in cell cycle and chromatin structure (up-regulated). Whether or not copper alters gene transcription or modulates mRNA stability remains to be established.

In summary, the copper-transporting ATPase ATP7B (Wilson disease protein) plays a central role in copper homeostasis in the liver. It is essential for the delivery of copper to ceruloplasmin in the biosynthetic pathway and is required for copper export into the bile. The dual function of ATP7B is mediated via copper-dependent

relocalization of the transporter from TGN to canalicular vesicles and possibly to the canalicular membrane. The common disease causing mutation, H1069Q, disrupts protein ability to bind ATP and undergo appropriate conformational transition leading to protein mislocalization and misfunction. The consequences of ATP7B inactivation on liver are severe and include targeted alteration of specific metabolic pathways, particularly at the early stages of the disease.

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