Human copper transporter ATP7B (Wilson disease protein) forms stable dimers in vitro and in cells

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ATP7B is a copper-transporting P1B-type ATPase (Cu-ATPase) with an essential role in human physiology. Mutations in ATP7B cause the potentially fatal Wilson disease, and changes in ATP7B expression are observed in several cancers. Despite its physiologic importance, the biochemical information about ATP7B remains limited because of a complex multidomain organization of the protein. By analogy with the better characterized prokaryotic Cu-ATPases, ATP7B is assumed to be a single-chain monomer. We show that in eukaryotic cells, human ATP7B forms dimers that can be purified following solubilization. Deletion of the four N-terminal metal-binding domains, characteristic for human ATP7B, does not disrupt dimerization, i.e. the dimer interface is formed by the domains that are conserved among Cu-ATPases. Unlike the full-length ATP7B, which is targeted to the trans-Golgi network, 1-ΔMBD-7B is targeted primarily to vesicles. This result and the analysis of differentially tagged ATP7B variants indicate that the dimeric structure is retained during ATP7B trafficking between the intracellular compartments. Purified dimeric species of 1-ΔMBD-7B were characterized by a negative stain electron microscopy in the presence of ADP/MgCl2. Single-particle analysis yielded a low-resolution 3D model that provides the first insight into an overall architecture of a human Cu-ATPase, positions of the main domains, and a dimer interface.

The copper-transporting ATPases (Cu-ATPases) are major regulators of copper homeostasis (1). Together, they are responsible for transfer of dietary copper into the blood, activation of the secreted copper-dependent enzymes, balancing copper levels in the cytosol, and the removal of excess copper from the body. Inactivation of either ATP7A or ATP7B causes a systemic copper imbalance and a broad spectrum of pathologies, ranging from metabolic changes and inflammatory response to tissue degeneration and death. Mutations in ATP7A are associated with Menkes disease, distal spinal muscular atrophy, and occipital horn syndrome (2). Mutations in ATP7B cause Wilson disease, a hepatoneurologic disorder with a significant variability in the time of onset, rate of progression, and specific manifestations (3). In addition, certain ATP7B polymorphisms have been seen more frequently in patients with Alzheimer disease (4), and overexpression of ATP7B has been observed in cancers (5). Only a small fraction of disease-causing mutants, as well as polymorphisms, have been characterized so far.

The challenge in determining consequences of pathogenic mutations lies in a complex structure of Cu-ATPases; their still poorly understood copper-transport mechanism and a very sophisticated regulation. ATP7A and ATP7B are highly homologous (>50% sequence identity) membrane proteins, which belong to the P1B-type subfamily of ion-transporting ATPases. They use the energy of ATP hydrolysis to facilitate transport of copper from the cytosol into the lumen of secretory pathway. ATP7A and ATP7B (175- and 164-kDa proteins, respectively) consist of a core that is flanked by the regulatory N and C termini (Fig. 1A). The core is required for the ATP-dependent transport activity and is conserved from bacteria to humans. It includes the membrane domain with eight transmembrane segments and the cytosolic N, P, and A domains. The N domain forms a binding site for ATP/ADP; the P domain houses the invariant catalytic Asp that is transiently phosphorylated during catalysis; and the A domain is thought to facilitate dephosphorylation and conformational transitions. The transmembrane part has one or more copper-binding sites. Studies of bacterial orthologs suggest that one copper atom is being translocated for each hydrolyzed ATP (6, 7); the stoichiometry of copper transport for human Cu-ATPases remains unknown.

Significant progress has been made in determining the structure and mechanism of prokaryotic Cu-ATPases (8–14). A high-resolution structure of LpCopA in the ligand-free form has been solved and yielded first insights into the architecture of the protein core that is conserved between prokaryotic and human Cu-ATPases. Compared with their prokaryotic orthologs, the mammalian Cu-ATPases are about twice as large, because in addition to the core, they contain multiple regulatory domains involved in protein targeting, trafficking,
post-translational modification, and interactions with other proteins. Much of the regulatory functions reside in the N terminus, which has six metal-binding domains (MBD) connected by long and flexible linkers. The first four MBDs can be deleted without the loss of transport activity (15), whereas MBD5 or MBD6 must be present for the human Cu-ATPase to function (16). The C terminus of human Cu-ATPases is about 100 amino acid residues long and is required for regulation of protein trafficking and stability (15, 17).

A molecular model of ATP7B was generated based on the LpCopA structure to predict consequences of the Wilson disease–causing mutations (18). The model is useful but has limitations because it lacks the structural elements involved in regulation of ATP7B. It is also based on the assumption that the quaternary structures of prokaryotic and human Cu-ATPases are similar. Although there is little doubt that the core structure of the individual polypeptide chains is indeed the same, it remains unknown whether the quaternary architecture of human and bacterial Cu-ATPases is similar. The prokaryotic Cu-ATPases are constitutively targeted to the plasma membrane, and their primary function is to transport copper across this membrane. In contrast, ATP7B is targeted and functions intracellularly: within the trans-Golgi network and in various vesicles of the secretory pathway. The lipid and protein environment and the ion and redox gradients in these compartments differ significantly from those in the prokaryotic plasma membrane. How the human Cu-ATPases have adapted to their environment at the structural level is unknown. In addition, changes in copper levels and hormonal signaling regulate distribution of human Cu-ATPases between the compartments; yet the structural basis of ATP7B retention in compartments and trafficking remains poorly understood. Structural information on the full-length human Cu-ATPases is needed to understand their transport mechanism, regulation, and effects of disease-causing mutations. Toward this goal, we expressed human ATP7B in relevant human cells, optimized conditions for solubilization and purification, and generated initial structural information using negative staining and single-particle analysis. These experiments revealed that, unlike prokaryotic pumps, ATP7B forms a stable dimer in vitro and in cells. This unexpected finding and the presence of the dimer in both major intracellular locations (trans-Golgi network (TGN) and vesicles) raise a spectrum of new hypotheses about the mechanism of Cu translocation and intracellular trafficking. The developed system paves the way for a more extensive structural analysis of human ATP7B.

Results

Adenovirus-mediated expression of a Twin-Strep–tagged ATP7B (TST-ATP7B) in HEK293 cells produces a correctly targeted and active protein

Recent advances in EM suggested that a single-particle analysis could be a suitable way to establish the structure–function correlates for ATP7B. Generation of the recombinant ATP7B in insect cells and in human cells has been previously reported (19, 20). These earlier studies placed an emphasis on high quantities of the recombinant protein for enzymatic studies, whereas potential effects of overexpression on structural heterogeneity of ATP7B have not been carefully considered. Consequently, we first optimized the functional expression and purification of human ATP7B to produce protein in quantities sufficient for structural studies while minimizing potential sources of protein heterogeneity. To aid protein purification,
the Twin-Strep tag was added to the N terminus of ATP7B, and the resultant TST-ATP7B (Fig. 1A) was incorporated into an adenovirus, which was purified prior to infection (17). The copper transport activity of TST-ATP7B was verified using a tyrosinase assay (Fig. 1B). In this assay, copper-dependent activation of tyrosinase serves as a reporter for the ATP7B-mediated copper transfer into the secretory pathway, where tyrosinase undergoes functional maturation (21); the activity is detected through the formation of a brown-colored levo-3,4-dihydroxy-L-phenylalanine (L-DOPA) pigment in cells. The measurements were done in Menkes fibroblasts, which lack the endogenous Cu-ATPases and cannot produce active tyrosinase unless a recombinant Cu-ATPase is introduced (Fig. 1B, left panel). Expression of TST-ATP7B in Menkes fibroblasts activated tyrosinase, as evident from the pigment formation (Fig. 1B, right panel), and demonstrated that the N-terminal Twin-Strep tag did not disrupt the copper transport activity of TST-ATP7B.

To produce ATP7B for purification, we selected HEK293 cells. These human cells express ATP7B endogenously and, consequently, provide a native lipid milieu for the recombinant TST-ATP7B. Overexpression may result in ATP7B targeting to more than one intracellular compartment. This is likely to increase molecular heterogeneity because of a different kinase-mediated phosphorylation of ATP7B in the TGN and vesicles (22). Consequently, an adenovirus titer was optimized to avoid significant overexpression of TST-ATP7B. In addition, the copper chelator tetrathiomolybdate (TTM) was added to the culture medium to limit copper-dependent trafficking of TST-ATP7B within cells. Under these conditions, TST-ATP7B was found predominantly in the TGN (an expected location) as evidenced by colocalization with the TGN marker TGN46 (Fig. 1C). The activity of TST-ATP7B, expressed in HEK293, was additionally verified in vitro by measuring ATPase and pNPPase activities. The microsomal membranes isolated from cells expressing TST-ATP7B showed significantly higher ATPase (Fig. 1D) and pNPPase (Fig. 1E) activities compared with cells expressing the catalytically inactive mutant D1027A-ATP7B (used as a background control). Thus, in HEK293 cells the recombinant TST-ATP7B is targeted correctly and is active.

**Purification of the recombinant TST-ATP7B revealed presence of stable dimers**

TST-ATP7B was purified following solubilization with 1% DDM. The first affinity chromatography step yielded a significantly enriched TST-ATP7B (Fig. 2A, left panel). Analysis of the elution fractions by a non-denaturing gel showed two species. The apparent molecular masses (240 and 480 kDa) were consistent with the monomeric and a dimeric...
form of ATP7B in detergent micelles (Fig. 2A, right panel). Both species were further purified by size-exclusion chromatography and retained their monomeric and dimeric state, respectively (Fig. 2B, left panel). Silver staining of fractions analyzed by a standard SDS-PAGE demonstrated that the size-exclusion step produced a highly pure protein at quantities (150–200 μg/ml) sufficient for structural analysis by a negative stain electron microscopy, EM (Fig. 2B, right panel).

Presence of a persistent ATP7B dimer was unexpected, because the prokaryotic Cu-ATPases are monomeric (13), and it was assumed that the human Cu-ATPases are also monomers. To rule out the possible effect of the TST tag on the oligomerization state of ATP7B, we generated and expressed the ATP7B variants with either the His₆ tag (in Si9 cells) or His₆ tag-GFP (in HEK293A cells) or FLAG tag at the N terminus (in HEK293 cells) and analyzed their oligomerization state. In all conditions, following solubilization with DDM, we detected a mixture of a monomer and dimer (Fig. 2C). To test the effect of a detergent on oligomerization state, we solubilized the TST-ATP7B with 1% undecyl maltoside (UDM) and dodecyl maltose neopentyl glycol (DMNG). Solubilization with either UDM or DMNG yielded a mixture of a monomer and dimer (Fig. 2D). Altogether, these data suggested that the formation of ATP7B dimer is unlikely to be driven by the tag/solubilization and may naturally occur in cells.

**ATP7B forms dimers in cells**

To test the hypothesis that ATP7B dimers exist in cells, we first examined whether the endogenous ATP7B dimerizes similarly to the recombinant TST-ATP7B. To this end, the microsomal membranes from the uninfected HEK293 cells were resuspended in the presence of 1% DDM, the solubilized fraction was analyzed by a blue native gel, and ATP7B was detected by Western blotting. The endogenous ATP7B ran as a mixture of 230 and 480-kDa (Fig. 3A, left panel). Further analysis of the endogenous species by 2D denaturing gels confirmed that both species contained the same ATP7B protein (Fig. 3A, right panel). Thus, with respect to dimerization, the endogenous ATP7B and the recombinant TST-ATP7B behave similarly. This finding is significant because the concentration of endogenous ATP7B is considerably lower compared with the recombinant protein. Therefore, dimerization of the endogenous ATP7B strongly argues against the dimerization of TST-ATP7B being artificially induced by overexpression and/or solubilization.

To further verify dimerization of ATP7B in vivo, we took advantage of the previously characterized ATP7B variants that have different intracellular localization. HEK293T cells were transfected separately or together with the wild-type GFP-ATP7B (normally localized to the TGN) (Fig. 3B, top panels) and the FLAG-ATP7BR875G/S340G/S341G mutant (normally targeted to vesicles (23)) (Fig. 3B, middle panels), and the localization of both proteins was assessed by confocal microscopy. In the majority of cotransfected cells (60–70%), GFP-ATP7B and FLAG-ATP7BR875, S340/S341 were colocalized (Fig. 3B, bottom panels), strongly suggesting interactions between the two variants. Notably, the localization pattern included both TGN and vesicles, i.e. the two ATP7B variants not only interacted but the complex had the TGN-retention characteristics that were intermediate between the WT and mutant ATP7B (Fig. 3B, middle panels). Lack of colocalization in the remaining 30–40% of cells may reflect a significantly higher expression of one of the variants compared with the other in these cells.

**Dimerization does not require the regulatory N-terminal domains MBD1–4**

Unlike prokaryotic orthologs, ATP7B has a large regulatory N-terminal domain. Consequently, we wondered whether the additional metal-binding domains that are absent in the N terminus of prokaryotic Cu-ATPases are responsible for ATP7B dimerization. To test this hypothesis, we generated a truncated variant of TST-ATP7B, in which the first four N-terminal domains (MBD1–4) were deleted, whereas the TST tag, the MBD5 and MBD6 (essential for the transport activity), and the
63 N-terminal amino acid residues (required for the copper dependent trafficking of ATP7B (15, 17)) were retained (1–4ΔMBD-ATP7B) (Fig. 4A). The tyrosinase-based copper transport assay showed that the 1–4ΔMBD-ATP7B construct with the TST tag was active (Fig. 4B). Purification of this variant from HEK293 cells revealed presence of monomers and dimers (Fig. 4D) in proportion similar to that of the full-length ATP7B. Because the loss of the regulatory domains does not affect dimerization, the dimerization interface is located within the remaining, the core portion, of ATP7B.
The ATP7B dimer is formed at or prior to the TGN and is retained in vesicles

Although the first four metal-binding domains are not required for dimerization, these domains influence ATP7B trafficking behavior. In HEK293 cells, we observed significant differences in the intracellular localization of the full-length ATP7B, the 1–4ΔMBD-7B variant, and the catalytically inactive ATP7B-D1027A (DA-7B) mutant. Under basal copper conditions, DA-7B had tight perinuclear localization similar to the wild-type ATP7B (Fig. 4C). In contrast, the 1–4ΔMBD-7B variant was mostly vesicular, indicating that the first four MBDs may modulate the TGN targeting/retention (Fig. 4C). This conclusion was further verified by quantifying the number of ATP7B-containing vesicles in low and high copper conditions (Fig. 4, E and F). As previously shown, the wild-type ATP7B responded to copper elevation by leaving the TGN and trafficking to vesicles (Fig. 4E). In contrast, the catalytically inactive DA-7B and 1–4ΔMBD-7B were localized mostly to the TGN (DA-7B) or vesicles (1–4ΔMBD-7B), respectively, independently of copper concentration. Expression and purification of the DA-7B and 1–4ΔMBD-7B variants under identical conditions demonstrated that they had a very similar monomer: dimer ratio (Fig. 4D). This observation suggests that the ATP7B maturation occurs at or prior to ATP7B entry into the TGN, and the dimeric state is retained when ATP7B traffic to vesicles.

Sample optimization for the structural analysis of Δ1–4ΔMBD-ATP7B dimer

Although following solubilization the significant fraction of ATP7B presents as a monomer, the presence of ATP7B dimer in the functionally relevant cell compartments (the TGN and vesicles) suggested that the dimer could be a stable and physiologically relevant form of ATP7B. To test this hypothesis, we sought to directly visualize ATP7B using EM of negatively stained samples. We decided to focus on the structure of 1–4ΔMBD-7B for two reasons: first, at ~280 kDa, the size of the 1–4ΔMBD-7B dimer is sufficient for imaging in negative stain, and second, this shorter variant is structurally simpler than the full-length ATP7B, making initial characterization easier. To decrease conformational heterogeneity, 1–4ΔMBD-7B was stabilized in one conformation by adding ADP/MgCl₂ throughout the purification procedure. Free detergent micelles, which have similar hydrodynamic radii and obscure protein particles, were removed by gradient-dependent fixation using glutaraldehyde in accordance with the GraFix protocol (24–26) (Fig. 5A). These steps significantly improved the quality of micrographs (Fig. 5B) and allowed data collection for further analysis. Mass spectrometric analysis of the purified cross-linked 240-kDa band and other higher molecular mass species showed that they contained predominantly ATP7B (Fig. 5A, middle panel). Spectral counting revealed a negligible amount of contaminants, supporting the conclusion that the stably cross-linked species was a dimer of ATP7B (Fig. 5A, right panel). The dimer sample was also subjected to gradient dependent centrifugation in the absence of cross-linker (but in the presence of ADP/MgCl₂) (Fig. 5B, middle panel). A negative stain and a single-particle analysis showed that the 2D class averages from the non–cross-linked and cross-linked samples were similar in size and shape, indicating that the dimer is stable irrespective of the cross-linker. The 2D classes of Δ1–4ΔMBD-ATP7B treated with fixative and centrifuged (the GraFix protocol) were better aligned and resolved when compared with the samples not subjected to a gradient dependent centrifugation or fixative treatment (Fig. 5C). Consequently, the GraFix-treated samples were used for negative staining, which yielded well resolved 2D classes (Fig. 5C).

3D reconstructions of 1–63Δ1–4ΔMBD-7B dimer revealed overall architecture with elements of symmetry

The random conical tilt approach was used to generate an initial model for the 1–4ΔMBD-7B dimer. This method does not make any assumptions about the structure of the target molecule and therefore is likely to produce an unbiased initial model for 3D classification (Fig. 6). Additionally, we collected negative stain images of the 1–4ΔMBD-7B monomer and generated a 3D model to serve as a control when measuring the size of the dimer (Fig. 6). Fig. 6 illustrates representative 2D classes of the 1–4ΔMBD-7B monomer (Fig. 6A) and dimer (Fig. 6B). The 2D averages of the 1–4ΔMBD-7B dimer show clear elements of symmetry that are absent in the monomer. As expected, the dimer particles are larger than the monomer, and their cross-section fits the anticipated size (~150 × 130 Å) for a 1–4ΔMBD-7B dimer (Fig. 6B). 8273 dimer particles contributing to the 2D class averages (Fig. 5D) were combined to perform a reference-based 3D classification using the RCT initial model. The 3D classes (Fig. 6E, models 1–3 and 6 highlighted in red dashed boxes) show symmetric features and have the geometry necessary to accommodate the 1–4ΔMBD-7B dimer. Classes 5 and 6 appeared flattened (possibly because of uneven staining) and, when examined from different angles, lacked...
space to accommodate the dimeric species. The 3D classes of the monomer (Fig. 6C) and dimer (Fig. 6E) show clear differences in their respective shapes. The monomers are more elongated and cylindrical, whereas the dimer is larger and mostly triangular in shape (Fig. 6, C and E). The final 3D refinement was performed on the dimer particles without imposing a sym-
Figure 6. Generation of 3D models for 1–4ΔMBD-7B monomer and dimer. A, representative 2D classes generated using RELION 1.4 of monomeric 1–4ΔMBD-7B. B, representative 2D classes generated using RELION 1.4 of dimeric 1–4ΔMBD-7B (these eight most representative 2D classes are a subset of classes shown in Fig. 5C and reproduced here to aid comparison). C, RCT model of the 1–4ΔMBD-7B monomer was subjected to 3D classification yielded four classes (top panel); respective Euler angle distributions for each model are shown underneath. D, final 3D model of the monomer after refinement (middle panel) and the corresponding Euler angle distribution plot (left panel). Also shown is the Fourier shell correlation plot (right panel) with the 0.143 and 0.5 FSC criterion for resolution estimate. E, RCT model of the 1–4ΔMBD-7B dimer was subjected to 3D classification yielded six classes (top panel); respective Euler angle distributions for each model are shown below. 3D classes that were chosen for final refinement are boxed (in red). F, final 3D model after refinement (middle panel) and the corresponding Euler angle distribution plot (left panel) Fourier shell correlation plot with the 0.143 and 0.5 FSC criterion for resolution estimate (21 Å).
metry criterion to avoid bias in the final model. The resultant 3D model had a fairly complete angular coverage (Euler plot) and demonstrated that 1–4ΔMBD-7B is an asymmetric dimer with a potentially very significant dimer interface (Fig. 6F). The monomeric particles were also analyzed using an identical refinement approach. This analysis yielded a final model, which was approximately a half the size of the dimeric model. Both final models had a resolution of 21 Å based on the Fourier shell correlation cutoff of 0.143 (Fig. 6, D and F, far right).

**ATP7B dimer interface includes both membrane and cytosolic domains**

The 3D model was used to predict the orientation of 1–4ΔMBD-7B monomers within a dimer and the position of the interface. First, the previously generated homology model for ATP7B core (18) was fitted into the shell of the 3D EM model for the monomer (Fig. 7A, panel (II)). The ATP7B core was easily accommodated within the volume of the monomeric species while leaving sufficient space for two metal-binding domains and the C terminus that were present in the 1–4ΔMBD-7B construct. Compared with the 2D back projections of the homology model, the ATP7B monomer has an extra density, which could accommodate the fifth and sixth MBDs (Fig. 6A, panel (I) and (III), bottom panels). These MBDs were placed using rigid body fitting in the region proximal to the TM1 (Fig. 7A, panel (II)). To identify the dimerization interface, the two homology models for the ATP7B core (with their respective fifth and sixth MBDs shown in magenta) were placed adjacent to each other without any steric clashes in two possible configurations: with either the P-N domains or the A domains being proximal (Fig. 7B, panels (I) and (II)). The configuration with the proximal A domains yielded a splayed configuration, whereas the dimer with the interface adjoining the P-N domains rendered a more compact form. To evaluate the likelihood for either configuration, the 2D back projections were generated from the model and compared with the 2D back projections of the ATP7B monomer and dimer EM volumes. The 2D back projection of the dimer with the limited contact via the A domain has less similarity to the actual dimer (Fig. 7A, panels (I) and (III)), whereas the dimer with significant transmembrane contacts and proximal P-N domains was very similar to the experimental data (Fig. 7B, panel (II) and (III)). We conclude that this arrangement is likely to reflect the structure of human ATP7B.

**Discussion**

Human ATP7B maintains both systemic and hepatic copper homeostasis and is essential for human health. However, the mechanistic understanding of ATP7B function and regulation remains limited, largely because of experimental challenges working with this multidomain protein and the lack of a detailed structural framework for the full-length molecule. Our experiments pave the way for studies of the effect of Wilson disease mutations on the ATP7B quaternary structure. By opti-
mizing several steps in protein expression and purification, we
succeeded in generating human ATP7B in quantities sufficient
for structural studies by electron microscopy. As a proof of
principle, we carried out single-particle analysis of the nega-
tively stained ATP7B samples. The studies yielded the first
3D model for the ATP7B variant that contains all essential
domains, including the transmembrane part, catalytic N and
P domains, the transduction A domain, two metal-binding
domains, and the C terminus. The 3D model illustrates the
overall molecular architecture with a likely 2-fold symmetry
and a significant protein interface between the transmem-
brane regions of the pump (Fig. 7). We provide evidence
that ATP7B forms dimers in its native cellular environment
and retains dimeric organization while trafficking within the
cell.

Our data do not unambiguously establish whether the dimer
is the primary form of the protein or whether it exists in equi-
librium with a monomer. Regardless, the protein interface in
the dimer is likely to involve transmembrane segments and
some cytosolic domains. Even if the ATP7B monomer func-
tions independently, which remains to be established, such sig-
nificant interdomain contacts are bound to influence the cop-
per transport characteristics and increase the complexity of
ATP7B regulation.

The dimer envelope has a sufficient space for two ferre-
doxin-like MBDs in the region adjacent to the P and A
domains. This region also has enough space to accommodate
the C termini of two monomers. If the C termini and MBDS
interact or form a compact assembly, then it would provide a
structural foundation for the recent finding showing a func-
tional “communication” between the N- and C termini (15).
Future work with unstained samples in vitrified ice would
allow these structural elements to be resolved and to deter-
mine the precise way in which these elements interact with
each other.

Our current data show that the four N-terminal MBDS are
neither required for dimerization nor involved in interactions
with the cellular machinery that enables the TGN-to-vesicle
trafficking of ATP7B. However, the loss of these domains
strongly favors localization to vesicles. This observation sug-
gests that in the wild-type ATP7B the first four N-terminal
domains either directly contribute to protein retention
within the TGN or inhibit trafficking from the TGN indi-
directly, by masking other regions of the protein. An “unmask-
ing” scenario is supported by our previous study in which we
demonstrated that the S340A/S341A mutations within the
MBD1–4 region decreased the interaction between the N
terminus and the core of ATP7B and facilitated protein traf-
icking to vesicles (23).

Our reconstruction shows two additional features that may
prove to be functionally significant: 1) an angle of ~20° is evi-
dent between the transmembrane region and the cytosolic
domain regions in the dimer map (when viewed from the side).
This could be due to presence of the two C termini in the dorsal
region of the model or may reflect normal motions between the
cytosolic domains and the transmembrane region that were
captured by cross-linking and 2) in the current reconstruction,
the densities seen in the cytosolic region are not perfectly sym-
metric. This may reflect averaging populations of somewhat
differently cross-linked dimers of the very flexible ATP7B mol-
ecule. The lack of perfect symmetry could also be due to a gen-
unely “out-of-sync” arrangement of monomers. The latter
would have far reaching mechanistic implications, but fur-
ther improvements/higher resolution are needed to deter-
mine whether the observed pseudo symmetry is genuine.
Interestingly, our attempts to stabilize ATP7B in the E2 state
were not successful and, so far, resulted in protein precipi-
tation. This observation provides evidence that the E1 and
E2 conformations of ATP7B were different and highlights
the need for future work to understand ATP7B molecular
dynamics.

Beyond explicit observations related to ATP7B structure and
trafficking, our studies suggested potential formation of super-
complexes of ATP7B with other proteins. This hypothesis is
born out of observations that we made during extensive solubi-
ization trials of the pump. Specifically, we noticed that utiliza-
lation of various detergents at different detergent:protein ratios
did not result in a clear dimer-to-monomer transition. Instead,
we observed a transition from high molecular mass complexes
(which run as distinct bands on a blue native gel) to a mixture of
monomers and dimers. This result may mean that in the native
environment ATP7B dimer tightly associates with other pro-
teins and that disruption of these contacts during solubilization
also destabilizes dimerization. The protocols developed in this
study would help to test this hypothesis and enable further
characterization of ATP7B complexes with its intracellular
partners. Taken together, our studies provide information
about the quaternary architecture of the human Cu-ATPase
and serve as a springboard for structural and mechanistic studi-
es of ATP7B

Experimental procedures

**ATP7B constructs**

The full-length human ATP7B with the N-terminally tagged
green fluorescent protein GFP-ATP7B (27) was used to further
introduce a His6 tag in the N-terminal position using QuikChange II XL site-directed mutagenesis (Stratagene),
resulting in plasmid pSJ101. To generate ATP7B with the
N-terminal Twin-Strep tag, TST (28) followed by a TEV prote-
ase cleavage site (29), the plasmid pFVT80 encoding the full-
length human ATP7B with a FLAG tag and TEV protease cleav-
age site in tandem to the ATP7B coding region (gift of Dr.
Arnab Gupta), was used as a template for insertional mutagen-
esis. First, two primers were synthesized as follows (Table 1): a
5’ primer contained 27 bases complementary to the 5’ region
flanking the FLAG tag site of pFVT80 and 21 bases com-
plementary to the 5’ of the TWIN Strep sequence. The 3’
primer was complementary to 21 bases of the 3’ end of the
TWIN strep coding region and 24 bases complementary to
the TEV protease cleavage site. The primers were used to
amplify a plasmid pEXPR-IBA105 (IBA) containing coding
sequence for the TST tag. This step produced the PCR prod-
uct encoding the TST tag followed by the TEV cleavage site.
Second, this product was used to replace the FLAG tag with
the TST tag. pFVT80 served as the template, and the gel-
purified PCR product from the first step served as the primers, resulting in plasmids pSJ104 and pSJ106. pSJ106 was used to introduce catalytically inactive mutations D1027(A/N) (designated pSJ107 and pSJ108) in the P domain. Primers used in generation of the above constructs were purchased from Integrated DNA Technologies (Table 1). After sequence verification of all plasmids for presence of tag and lack of unwanted mutations, the constructs were packaged into adenoviruses and purified as described previously (30). The sequencing was performed by the Johns Hopkins University DNA Sequencing Facility.

**Tyrosinase assay for copper transport activity**

The ATP7A-null Menkes fibroblasts YST cells were cultured and seeded in 10-cm tissue culture dishes containing glass coverslips (22 × 22 mm), and plating densities were ∼1.5 × 10^6 or ∼7.5 × 10^5. The ATP7B constructs were tested for Cu(I) transport activity after in YST cells reached 95% confluency by cotransfection with apo-tyrosinase and incubation for 12 h at 37°C. After incubation, the cells were washed in 1× PBS, fixed in acetone:methanol mixture (1:1, v/v), and incubated for 4 h with 0.1 sodium phosphate (pH 6.8) containing 0.15% (w/v) L-DOPA at room temperature. The coverslips were then mounted onto slides, and the formation of dark colored DOPA-chrome was examined using phase contrast microscopy.

**Cell culture and adenoviral infection**

HEK293A cells were seeded in 10-cm dishes and further scaled to twenty 15-cm dishes (400 ml) with plating densities of ∼1.5 × 10^6 or 7.5 × 10^5. The cells were infected 2–3 days later, with ∼0.3–4 × 10^9 viral particles/dish in complete medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin) for 30 min at 37°C. Prior to infection with virus, the solution was filtered with 0.2-μm Millipore filter to remove any residual viral aggregates. To restrict ATP7B trafficking within the cells, protein expression was performed in the presence of 25 μM TTM in the medium, which retains ATP7B in the TGN. After incubation, the viral solution was removed, complete medium supplemented with 25 μM TTM was added, and cells were incubated at 37°C for 16–24 h overnight. At the end of the expression, the medium was removed, and the production phase was terminated by adding CO2 equilibrated complete medium containing 1 mM HEPES, 25 μM TTM, and 9 μg/ml of cycloheximide. Incubation for 3 h at 37°C allowed all of ATP7B to reach the TGN in the absence of new protein synthesis. The cells were then washed with Hanks’ balanced salt solution; Thermo Fisher Scientific, Inc.) with 1 mM EDTA, scraped, and gently rinsed. The cells were then pelleted by centrifugation, and the buffer was removed prior to flash freezing in liquid nitrogen and final storage at −80°C until further use.

**Testing ATP7B expression and localization**

Small scale protein expression was carried out in 6-well dishes with cells cultured and infected with adenovirus as described above. The coverslips were rinsed with 1× PBS followed by 1 ml of ice-cold 4% PFA/PBS (pH 7.2–7.4) for exactly 1 min. Following this, 1 ml of ice-cold methanol was added to each well and gently mixed after each addition. After aspiration, an additional 2.0 ml of methanol was aspirated again and removed. The methanol wash was repeated twice. After the final 2 ml of methanol was added, the cells were incubated for 10 min and then washed with PBS. The cells were blocked in 1% BSA/PBS at room temperature for 30 min. The primary and secondary antibodies were diluted in 1% BSA/PBS and incubated for 30 min at room temperature, followed by three 5-min washes with 0.2% BSA/PBS at room temperature. Primary antibodies for immunostaining of ATP7B and the Golgi marker TGN46 were from IBA (mouse anti-strep tag), Abcam (mouse anti-C-terminal ATP7B), GenTex (sheep, TGN46) and Sigma (mouse anti-FLAG). Secondary antibodies Alexa Fluor 568 (donkey anti-sheep), Alexa 555 (donkey anti-mouse), and Alexa 488 (donkey anti-rabbit) were from Life Technologies. The immunostained cells were analyzed using an oil immersion objective (Plan-Apo-chromat 63×/1.40 Oil DIC M27) on a LSM 510 META confocal microscope (Zeiss). Immunofluorescence assays were carried out at least three times, and more than 10 cells were evaluated in each experiment.

**ATPase assay**

Frozen HEK293A cell pellets (plating densities ∼1.5 × 10^6 or 7.5 × 10^5) containing overexpressed ATP7B were suspended in 10 ml of buffer containing 50 mM MOPS (pH 7.4), 150 mM NaCl, 20% glycerol, 100 μM ABSF 4-(2-aminophenyl) benzenesulfonyl fluoride hydrochloride (ABSF), 1 mM DTT, and protease inhibitors (MOPS buffer). The cells were homogenized (5–10 times) and centrifuged at 5,000 rpm for 15 min at 4°C. The supernatant was further subjected to centrifugation at 50,000 rpm in a Beckman rotor 70Ti at 1 h at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of MOPS buffer and transferred to a glass homogenizer. The pellet was homogenized, using five strokes, with a glass piston, aliquoted into 1.5-ml tubes, and flash frozen in liquid nitrogen for storage at −80°C until further use. A fresh aliquot was
slowly thawed on ice, and the ATPase assay was performed as described in Ref. 31. Protein concentration was measured using Pierce 660-nm protein assay reagent (Thermo Fisher Scientific, Inc) and the freshly thawed microsomes; 0.05 mg/ml of enzyme was added to the reaction mixture containing buffer, 1 mM DTT, 50 μM bathocuproinedisulfonic acid, and 2, 4, or 6 μM copper in a 96-well plate. ATPase activity was triggered by the addition of 3 mM freshly made ATP/MgCl₂ solution at 30 °C for 15 min. The reaction was stopped by the addition of 10% SDS, freshly prepared ascorbate, and ammonium molybdate solution and mixed thoroughly. After incubation for 15 min at room temperature, the absorption at 600 nm was measured. All reactions were performed in triplicate, with blank buffers or membranes from non-transfected cells used as a control.

**pNPPase assay**

The microsomes for the pNPPase assay were prepared as described above for the ATPase assay. Membrane protein fraction at 0.05 mg/ml was added to the reaction buffer (50 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 30 mM NaCl, and 1 mM DTT) containing 50 μM bathocuproinedisulfonic acid or 2, 4, or 6 μM copper in a 96-well plate format. The pNPPase reaction was started by adding 10 mM pNPP and allowed to proceed at room temperature for 20 min. The reaction plate was then transferred on ice, and 100 μl of 0.5 N NaOH was added and mixed thoroughly. The absorbance was measured at 410 nm (ε = 17,000 M⁻¹ cm⁻¹). All reactions were performed in triplicate, with controls using blank buffers and membranes from uninfected cells.

**Protein purification**

Frozen cell pellets containing recombinant affinity tagged ATP7B were placed on ice, followed by the addition of PBS (pH 7.4), 20% glycerol, 1 mM AEBSF, and complete protease inhibitor mixture (Roche). The cells were solubilized by adding 1% DDM (w/v) (or UDM or DMNG) to the cell pellet and homogenized 12 times using a motorized Dounce homogenizer. The homogenate was incubated with gentle rotation at 4 °C for 1.5 h and then centrifuged at 50,000 rpm for 1 h at 4 °C in a Ti70 fixed angle rotor (Beckman). The solubilized protein was passed over 0.5 ml of streptavidin resin (Strep-tactin; IBA), using a peristaltic pump. After protein loading, the column was washed with 10 column volumes of buffer. Bound protein was eluted with 5 mM desthiobiotin in dissolved in PBS (pH 7.4), 20% glycerol, 1 mM AEBSF. After analyzing the fractions on a SDS-PAGE and blue native gel, the peak fractions containing ATP7B were pooled and concentrated using a 50-kDa MWCO Vivaspin (GE Healthcare) concentrator. The concentrated protein sample (500 μl) was loaded onto a HiLoad 16/600 Superdex 200 prep grade size exclusion column pre-equilibrated with running buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, and 0.05% detergent). The peak fractions were examined by SDS-PAGE and blue native-PAGE. The maximum yields for purified ATP7B using this protocol is 0.5 mg/liter.

**Colocalization of GFP-ATP7B and FLAG-ATP7B**

Hek293TRex cells were maintained in MEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% non-essential amino acids, 12.5 μg/ml blasticidin, and 100 μg/ml Zeocin. During experiments, media without blasticidin and Zeocin were used. Hek293TRex cells on coverslips were transiently transfected with the WT GFP-ATP7B or mutant FLAG-ATP7B using Turbofect. Protein expression of FLAG-ATP7B was induced with 40 ng/ml doxycyclin for 16 h in basal conditions. The cells were rinsed with PBS and fixed with a cold acetone-methanol mixture (1:1) for 30 s. The cells were blocked in blocking buffer (1% gelatin, 1% BSA in PBS) and then incubated with appropriate primary and secondary antibodies diluted in blocking buffer (primary antibody: mouse anti-FLAG antibody 1:250 (Sigma) and rabbit anti-TGN 46 1:300 (Genetex); secondary antibody: Alexa Fluor 488 goat anti-mouse antibody 1:500 (Invitrogen) and Alexa Fluor 555 goat anti-sheep 1:500 (Invitrogen)). Coverslips were mounted onto glass slides using Vectashield with DAPI (Vector Laboratories). Images were taken using a Zeiss PALM confocal microscope and a 100× oil immersion objective.

**Generation of 1–4ΔMBD-ATP7B (1–4ΔMBD-7B)**

To generate a truncated construct of ATP7B, an ATP7B construct containing an N-terminal GFP followed by the first 63 amino acids and MBDS 1–4 deleted (designated pYG36, (17)) was used as a template. Primers with the overhangs containing restriction sites KasI and AgeI were used to amplify a ~1-kb region containing the first 63 residues and MBDS 5 and 6 to the site AgeI of pYG36. Restriction site KasI is located at the 3’ end of the TST of pSJ106, and AgeI is located within the sixth MBD of pSJ106. Both the PCR-amplified ~1-kb product and pSJ106 were sequentially digested using restriction enzymes AgeI and KasI then gel-purified and ligated using T4 DNA ligase overnight. The ligated DNA was transformed into XL10 gold super competent cells. The final construct contained a TST, 1–63, and MBD 5 and 6 of ATP7B and was designated 1–4ΔMBD-7B. The sequence was verified by DNA sequencing at the Johns Hopkins University DNA Sequencing Facility.

** Trafficking of WT, D1027A, and 1–4ΔMBD-ATP7B**

HEK293A cells were plated on coverslips in 6-well dishes and infected with adenovirus (as described above) expressing the WT or one of the variants. The proteins were expressed under basal conditions (regular growth medium), low copper (25 μM TTM added to the growth medium) or high copper (50 μM CuCl₂ added to the medium). Following a 16-h incubation, the medium was removed, and fresh medium containing 9 μg/ml cyclohexamide was added with the respective basal, TTM, and copper-containing medium for 3 h to arrest protein expression. The cells were fixed with 4% PFA/PBS for 1 min followed by several methanol washes before being rehydrated with PBS. Primary antibodies for the C terminus of ATP7B and the organelle TGN (TGN46; Santa Cruz) were used for incubation for 1 h,
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followed by several washes with PBST and incubation with secondary antibodies.

Negative stain EM of 1–4ΔMBD-7B dimer

Thin continuous carbon films were deposited on freshly cleaved mica using a Denton vacuum carbon evaporator (Integrated Imaging Center (IIC), Johns Hopkins University) and stored until used. To prepare grids, carbon was floated off the mica, lowered onto 400-mesh gold grids (EMS Inc.), and allowed to air dry overnight. Just prior to sample application, the grids were glow-discharged in air, followed by deposition of 10 μl of protein solution (~100 μg/ml). After allowing 2 min for molecules to stick to the carbon film, the grid was transferred to droplets of 2% uranyl-formate solution for negative staining. At the end of the staining process, excess stain was gently adsorbed using a filter paper, and the grids were air-dried for 20 min. The images were acquired using a FEI Tecnai T12 electron microscope operating at 100 kV with a spherical aberration (2.3 mm) and equipped with a FEI Eagle 4k × 4k camera (physical pixel size, 15 μm; IIC, Johns Hopkins University) at a calibrated magnification of 71,428× corresponding to 2.07 Å/pixel, respectively. The images were later down-sampled by a factor of 2 using the e2proc2d.py function within the EMAN software suite (32), rendering a magnification of 35,714× corresponding to 4.14 Å/pixel, respectively. The defocus value ranged between 0.5 and 1 μm, respectively.

Generation of initial RCT based consensus model for 1–4ΔMBD-ATP7B dimer

An initial model was generated using the random conical tilt (RCT) approach. Grids containing the 1–4ΔMBD-7B dimer were used to collect 20 tilt pair micrographs (0° and 50°). 2540 particle pairs were manually selected and boxed (box size, 60) using Xmipp (33) within the SCIPION (34) suite of integrated EM software programs. The particles were manually recentered within their boxes. After several rounds of 2D classification (35), 2440 particle pairs were isolated. CL2D classification of the untitled particles yielded 12 classes, each with 100–200 homogenous particles (as verified by Xmipp 2D Class display window). The particles from the 12 classes and their corresponding tilted pairs were subjected to 3D reconstruction using the RCT within Xmipp. The resulting 12 models were visually inspected using UCSF chimera (36, 37) for obvious size and shape anomalies. Models 2, 3, 5, 7, 9, and 10 were selected for further processing. The particles corresponding to these models were combined and aligned against volume 2 using the projection matching algorithm within SCIPION. The resulting volume was low pass filtered to 80 Å for further 3D classification and 2D auto refinement.

Image processing and generation of final model for 1–4ΔMBD-ATP7B dimer

The RCT initial model was used to perform 3D classification and auto refinement of a larger data set made of particles from an independent set of images taken from the same sample. Using RELION 1.4 (38, 39) 13,505 particles were manually picked using a box size of 60. The program ctffind3 (40) was used to estimate the contrast transfer function for all the micrographs. After several rounds of 2D classification, 8273 particles were isolated and subjected to 3D classification within RELION using the RCT-based consensus model as reference. The resulting six 3D classes were visualized using UCSF chimera. Models 1, 2, 3, and 6, which showed strong elements of symmetry and the geometry necessary to accommodate the 1–4ΔMBD dimer), were combined and subjected to maximum likelihood-based 3D auto refinement using the RCT model as a reference. The data converged and produced a final model at 28 Å (0.5 FSC cutoff). All maximum likelihood calculations were performed using compute nodes on a high performance computing facility, the Maryland Advanced Research Computing Core.

Density map analysis

The final 3D model for Δ1–4MBD-ATP7B dimer was visualized using UCSF chimera. A homology model of ATP7B core (18) was fitted into the model volume using the rigid body docking feature “fit map” within the volume viewer mode of UCSF chimera. Another identical copy of the core structure was used to make a symmetrical dimer partner. Metal-binding domains 5 and 6 (Protein Data Bank code 3EW9) from the NMR ensemble was used to complete the N-terminal portions of the core structures.

Negative stain EM of 1–4ΔMBD-7B monomer

Negative staining of amphiphile solubilized 1–4ΔMBD-7B monomer using 2% uranyl formation was carried out on 400-mesh gold grids (EMS, Inc.) coated with a layer of uniform carbon using a carbon evaporator (Northwestern University). The images were acquired using a JEOL 1400 electron microscope operating at 120 kV with a spherical aberration (3.4 mm) and equipped with a camera physical pixel size of 15 μm at a calibrated magnification of 32,609× corresponding to 4.6 Å/pixel, respectively. The defocus value ranges between 0.5 and 1.5 μm, respectively.

18,452 particles were manually picked using 50 pixels box size and e2boxer.py option within the EMAN2.07 suite. An initial model was generated using RCT of 1900 tilt pairs. Following 2D classification, the best models were selected (see text), and the particles were transferred to RELION 1.2. The RCT model was filtered to 40 Å and used to perform 3D classification. The 3D classes were examined carefully and were found to have the same dimensions overall. The particles from these classes were combined and were subjected to 3D auto refinement using RELION 1.2. The resulting final model was used for rigid body fitting using UCSF Chimera.

Preparation of Δ1–4MBD-ATP7B samples for mass spectrometry

The peak fractions (shown in the blue native gel in Fig. 5) were subjected to TCA/Acetone precipitation to remove detergents. After TCA precipitation, the sample was loaded onto a Novagen NuPAGE 4–16% gradient SDS/PAGE gel. Following protein separation, the gel was stained using Protean Superblue Ultra Coomassie dye and destained with 1% acetic acid. After sufficient detaining was achieved the gel bands were carefully excised and subjected to MS sample
identification (Johns Hopkins Mass Spectrometry and Proteomic Facility).

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