Supporting Information

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SI Materials and Methods

Cell Lines. Hek293TREx cells (kindly provided by Jack Kaplan, University of Illinois, Chicago, IL) were maintained in DMEM supplemented with PenStrep, nonessential amino acids, 10% FBS (vol/vol), 15 μg/mL blasticidin, and 100 μg/mL zeocin (all from Invitrogen). For protein expression in HEK293TRex cells, doxycycline (20 ng/mL; Fisher) was added to the growth medium and maintained for 16 h. Menkes disease fibroblasts (YSTT cells) were maintained in DMEM supplemented with 10% FBS (vol/vol), 200 μg/mL G418, and 0.5 μg/mL Puromycin (Invitrogen). HepG2 cell lines were maintained in DMEM with 10% FBS (vol/vol) and PenStrep.

Cloning and Mutagenesis in ATP7B. An N-terminal FLAG tag (DYKDDDDK) was added to the N-terminal of ATP7B Arg^{875} after the start codon by PCR using ATP7B pcDNA 3.1(+) as a template. Flag-ATP7B cDNA was subcloned into the pcDNA 5 FRT/TO (Invitrogen) using BamHI and NotI. This construct was then used to introduce the 2623A→G replacement (R875G) by PCR using the Stratagene Quickchange XL mutagenesis kit and the oligonucleotides 5′-CCGG-AAGCACTGTAATTGCGGGG-TCTATAAATGCACATGGC-3′ and 5′-GCCATGTGCATTTA-TAGACCCCGCAATTACAGTGCTTCCGG-3′. The presence of the mutation and the fidelity of the cDNA sequence were verified by automated fluorescent DNA sequencing. Turbofect (Fermentas) was used for transfection according to the manufacturer's protocol. The pcTYR plasmid used for expression of tyrosinase was previously described (1, 2).

Cloning, Expression, and Purification of the A-Domain. The cDNA fragment encoding the ATP7B A-domain (residues 789–907) with either Arg or Gly at position 875 was amplified by PCR using primers 5′-CATATGTCAGAAGCCCTGGCTAAACTCATG and 5′-CTCGAGCTGAGCCTCTTCCACCAGTTTCAC and human ATP7B cDNAs in pCDNA5.1 as a template. Following cloning into pCDNA3.3 TOPO vector (Invitrogen), the fragment was subcloned into the pTXB1 (New England Biolabs) vector using restriction sites NdeI and XhoI. The plasmids were transformed into BL21 DE3. Protein expression was induced at an OD_{600} of 0.7 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For NMR experiments, protein was expressed at 16 °C for 16 h in minimal medium and then metabolically labeled with $^{15}NH_4Cl$ (0.82 g/L LB). Cells were then collected by centrifugation and disrupted using a French press with three passes at 1,500 psi, and recombinant proteins were purified on the chitin beads (NEB) and eluted with 50 mM 2-mercaptoethane sulfonate sodium (MESNA; Sigma) for 24 h at room temperature. Cleaved protein was checked by SDS/PAGE, concentrated using Ultracel-3K (Millipore), and dialyzed into 50 mM sodium phosphate buffer with 50 mM glutamate and 50 mM arginine (pH 7.0) (3). Purified protein was kept at 4 °C and analyzed by NMR 36 h later.

HSQC Analysis. The NMR samples contained 0.2 mM protein, 50 mM sodium phosphate (pH 7.0), 50 mM arginine, 50 mM glutamate, 5% (vol/vol) D_2O, and 0.3 mM 2,2-dimethyl-2-silapentane-5-sulfonic acid for chemical shift referencing. Thermal denaturation of the protein was monitored by recording a series of $^1H,^{15}N$-HSQC spectra in the range of 280–330 K.

Immunofluorescence Microscopy. Cells were treated with 2–10 μM $CuCl_2$ for 4 or 16 h, rinsed with PBS, and fixed with ice-cold acetone-methanol (50:50). Cells were blocked in blocking buffer [1% (wt/vol) gelatin, 1% (wt/vol) BSA, and 0.02% (wt/vol) sodium azide in PBS] and then incubated with appropriate primary and secondary antibodies in blocking solution (1:500 antibody dilution or stated otherwise) for 1 h at room temp. For Flag tag ATP7B, primary mouse anti-FlagAlexa (Sigma) and secondary Alexa Fluor 488 goat anti-mouse antibody (ANASPEC) were used. For TGN 38, the primary antibody was rabbit anti-TGN38 (Santa Cruz) and the secondary antibody was Alexa Fluor 633 goat anti-rabbit (Molecular Probes). For ER detection, the primary antibody was rabbit anti-calnexin (dilution 1:150; Santa Cruz). Coverslips were mounted onto slides using Vectashield w/ DAPI (Vector Laboratories). In immunofluorescence experiments involving CHX, it was used at a final concentration of 50 μg/mL. Cells were pretreated with CHX for 2 h and then incubated in copper (5 μM) in presence of 50 μg/mL CHX for 4 h. Cells were fixed, blocked, and incubated with antibodies as mentioned above.

Tyrosinase Activation Assay. YSTT cells grown on glass coverslips were transfected with either 2 μg of pcTYR or 2 μg each of pcTYR and Flag ATP7B (Arg^{875} or Gly^{875}) expression plasmids using Turbofect. Twenty-four hours later, cells were washed twice in PBS and fixed for 30 s in cold acetone-methanol (1:1). These fixation conditions do not destroy tyrosinase activity. The cells were then incubated for 4 h at 37 °C in 0.1 M Na-phosphate buffer (pH 6.8) containing 0.15% (wt/vol) levo-3,4-dihydroxy-L-phenylalanine (L-DOPA). Coverslips were mounted on slides, and formation of the black L-DOPA chrome pigment was detected by phase microscopy. Pigment color intensity was quantified using Image J software (National Institutes of Health).

Analysis of ATP7B Variants by Western Blot. HEK293TRex cells were seeded on 6-cm dishes and allowed to grow to 70% confluency in the growth medium described in the previous section. Cells were transfected with 2.5 μg of DNA using Turbofect, and protein expression was induced overnight with tetracycline (20 ng/mL; Fisher). Cells were washed with PBS and homogenized in 1.5 mL of lysis buffer [25 mM imidazole, 0.05 mM DTT, 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.25 M sucrose, and protease inhibitor tablets (Roche)] with 20 strokes of loose pestle and 20 strokes of tight pestle in a dounce under ice The homogenate was centrifuged at 600 × *g* for 10 min at 4 °C. The resulting supernatant was again centrifuged at 20,000 × *g* for 30 min, and the pellet was resuspended in 100 μL of lysis buffer. Total protein in the fractions was estimated by Lowry assay, and 20 μg of total protein for each variant was analyzed by 10% SDS/PAGE, transferred to a PVDF membrane (Millipore) using transfer buffer [10% (vol/vol) methanol, 10% (vol/vol) 100-mM CAPS (pH 11) in water], and detected using mouse anti-Flag antibody (1:5,000; Sigma) and mouse monoclonal Na,K-ATPase α-1 antibody (equal loading control, 1:10,000; Millipore). HRP-conjugated goat anti-mouse IgG was used as the secondary antibody. The intensity of protein bands from three independent experiments was quantified using densitometry (Alpha Imager 2200, Alpha Innotech Corp.) and normalized to Na,K-ATPase expression.

Domain-Domain Interactions. The A-domain intein fusion (Arg^{875} or Gly^{875}) or intein fusion alone was expressed in 500 mL of *Escherichia coli* BL21 culture medium as described above. *N*-ATP7B maltose-binding protein fusion (~105 kDa) was also expressed in 500 mL of culture medium as described previously (4). The copper-bound form of *N*-ATP7B was generated in vivo

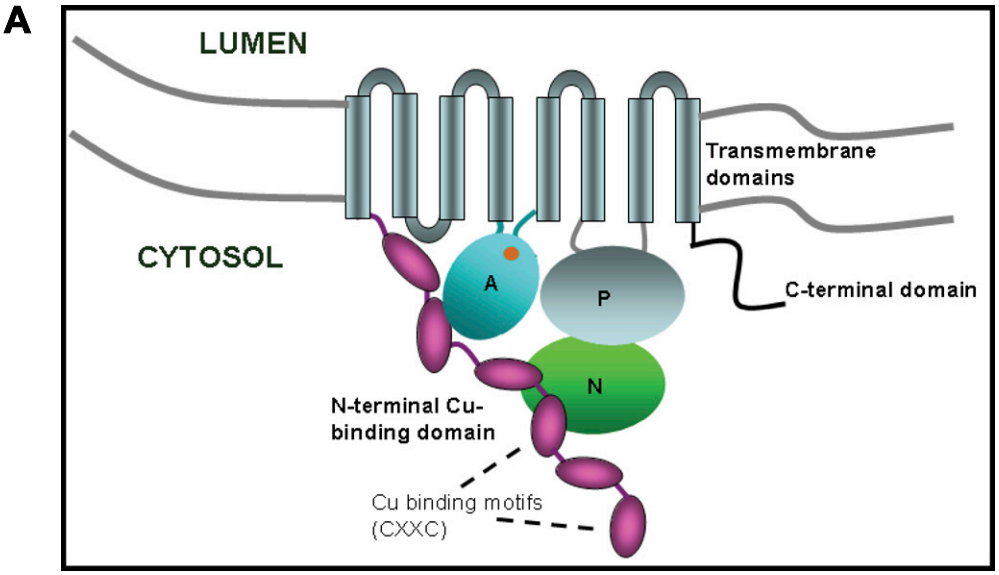
by adding 250 μM $CuCl_2$ during protein expression. Bacterial cells were spun down by centrifugation at 5,000 rpm at 4 °C, and cells lysed were with a French press (three passes), followed by centrifugation at 17,000 rpm (Beckman Coulter Optima L-100K centrifuge, rotor 70 Ti) at 4 °C to remove cell debris and collect supernatants. IPTG-induced and uninduced cell culture (100 μL) from each category was analyzed by 10% (wt/vol) SDS/PAGE to estimate the amount of expressed protein by densitometry. Supernatants were then mixed to have equimolecular amounts of *N*-ATP7B and the A-domain (or intein used as a control) and were incubated for 1 h in an automatic rotator at 4 °C.

Chitin beads were washed to remove ethanol and incubated for 1 h with cell lysates from uninduced bacteria to decrease nonspecific binding. Supernatants containing a mixture of *N*-ATP7B (apo- or holoform) and the A-domain (Arg^{875} or Gly^{875} variant) were applied to the chitin beads. As an additional control, supernatant with *N*-ATP7B (±copper) alone was applied to chitin beads to rule out nonspecific interactions with the resin. Proteins were incubated for 1 h in the automatic rotator at room temperature to allow for intein-chitin binding. Chitin beads were then washed with 16 column volumes of 50 mM sodium-phosphate buffer (pH 7.0) containing 500 mM NaCl and a complete EDTA-free protease inhibitor mixture (Roche). To cleave the A-domain and elute interacting proteins, the beads were incubated for 24 h with the same buffer containing 50 mM MESNA. The eluates (50 μL) were then precipitated with trichloroacetic acid; pellets were solubilized in Laemmli gel sample buffer and analyzed by 15% (wt/vol) SDS/PAGE, fixed, and stained with Coomassie blue. The samples were run to see both the A-domain (13 kDa) and intein fusion N-terminal domain (~105 kDa) in the same gel. Bands for both variants of the A-domain were quantified in gel by densitometry. Aliquots containing equal amounts of Arg^{875} and Gly^{875} A-domains were then run on an 8% (wt/vol) acrylamide gel and transferred to a PVDF membrane, and the presence of *N*-ATP7B was detected with rat polyclonal anti-*N*-ATP7B antibody.

Copper Measurements in Menkes Fibroblasts (YSTT Cells). The YSTT cells grown in 3-cm plates were maintained in basal medium, treated for 4 h with 5 μM $CuCl_2$, or loaded with high copper (50 or 100 μM $CuCl_2$) for 24 h. Cells were washed and collected, and protein concentration in cell lysates was measured using Bradford methods. Copper concentration in cell lysates was measured using an Atomic Absorption Spectrophotometer AA6650 (Shimadzu) and normalized per protein.

Localization of ATP7B-Arg^{875} in Copper-Overloaded Menkes Fibroblasts. To express ATP7B-Arg^{875} in copper-overloaded cells, the YSTT cells grown on glass coverslips were treated with 50 or 100 μM $CuCl_2$ for 24 h, washed twice with PBS, and then transfected with 2 μg Flag-ATP7BArg^{875}. Protein expression and immunodetection were carried out with the standard protocol used throughout this study. To make sure that no loss of copper occurs during transfection, cells were also cultured in 3-cm plates (as described above) and treated with the same reagents as cells on the coverslips. Copper measurements were carried out for each stage of transfection and protein expression. Copper remained significantly elevated at each step of the procedure.

1. Braiterman L, et al. (2009) Apical targeting and Golgi retention signals reside within a 9-amino acid sequence in the copper-ATPase, ATP7B. *Am J Physiol Gastrointest Liver Physiol* 296:G433–G444.
2. Guo Y, Nyasae L, Braiterman LT, Hubbard AL (2005) NH2-terminal signals in ATP7B Cu-ATPase mediate its Cu-dependent anterograde traffic in polarized hepatic cells. *Am J Physiol Gastrointest Liver Physiol* 289:G904–G916.
3. Banci L, et al. (2009) Solution structures of the actuator domain of ATP7A and ATP7B, the Menkes and Wilson disease proteins. *Biochemistry* 48:7849–7855.
4. Lutsenko S, et al. (1997) N-terminal domains of human copper-transporting adenosine triphosphatases (the Wilson's and Menkes disease proteins) bind copper selectively in vivo and in vitro with stoichiometry of one copper per metal-binding repeat. *J Biol Chem* 272:18939–18944.



B

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ATP7B_HUMAN	826	QRGDIVKVVPGGKFPVDGKVLEGNTMADESLITGEAMPVTKKPGSTVIAGSINAHGSVLIKATHVGNDTTLAQIVK	901
ATP7B_RAT	819	QRGDIIKVVPGGKFPVDGKVLEGNTMADESLITGEAMPVTKKPGSIVIAGSINAHGSVLIKATHVGNDTTLAQIVK	894
ATP7B_MOUSE	828	QRGDVIKVVPGGKFPVDGKVLEGNTMADESLITGEAMPVTKKPGSIVIAGSINAHGSVLLKATHVGNDTTLAQIVK	903
ATP7B_SHEEP	866	QRGDIIKVVPGGKFPVDGKVLEGNTMADESLITGEAMPVTKKPGSMVIAGSMNAHGSVLITATHVGNDTTLAQIVK	941
ATP7A_HUMAN	843	QRGDIIKVVPGGKFPVDGRVIEGHSMVDESLITGEAMPVAKKPGSTVIAGSINQNGSLLICATHVGADTTLSQIVK	918
CopA_BACSU	318	LVNDIVYVKPGERIPVDGEVVEGRSAVDESMITGESLPVDKNPGDSVTGSTVNANGFLKIKAVNVGKDTALSHIIK	393
CopA_STAEQ	313	KVGDTLLIKPGEKIPVDGKVTKGDTSIDESMLTGESIPVEKSSGDSVIGSTMNKNGSIMIEATQVGGDTALSHIIK	388
CopA1_HELPY	250	VVGDILKVLPGTLIAVDGEIIEGEGELDESMLSGEALPVYKKVGDKVFSGTFNSHTSFLMKATQNNKNSTLSQIVE	325
ATCS_SYNE7	255	QVEDWVRVRPGEKVPVDGEVIDGRSTVDESMVTGESLPVQKQVGDEVIGATLNKTGSLTIRATRVGRETFLAQIVQ	330
ATHM7_ARATH	468	QKNDVIKIVPGAKVASDGYVIWGQSHVNESMITGEARPVAKRKGDTVIGGTLNENGVLHVKVTRVGSESALAQIVR	543

Fig. S1. Domain organization of ATP7B (*A*) and a sequence alignment for the ATP7B orthologs in the region containing the Gly^{875}→Arg substitution (*B*). ATP7B is a 165-kDa membrane protein composed of multiple domains. The N-terminal copper-binding domain (*N*-ATP7B) consists of six subdomains (purple ovals), each with one CxxC copper-binding site. In this study, we show that *N*-ATP7B interacts with the A-domain (blue), which is also directly connected to the transmembrane domain. During the ATP7B transport cycle, the A-domain controls conformational transitions and transiently interacts with the ATP-binding domain composed of the nucleotide-binding N-domain (green) and phosphorylation P-domain (gray). The Gly^{875}→Arg substitution (orange circle) is located within the A-domain. (*B*) Gly (indicated by a star), at position 875, is conserved in mammalian and avian ATP7B as well as in human ATP7A, the paralog of ATP7B.

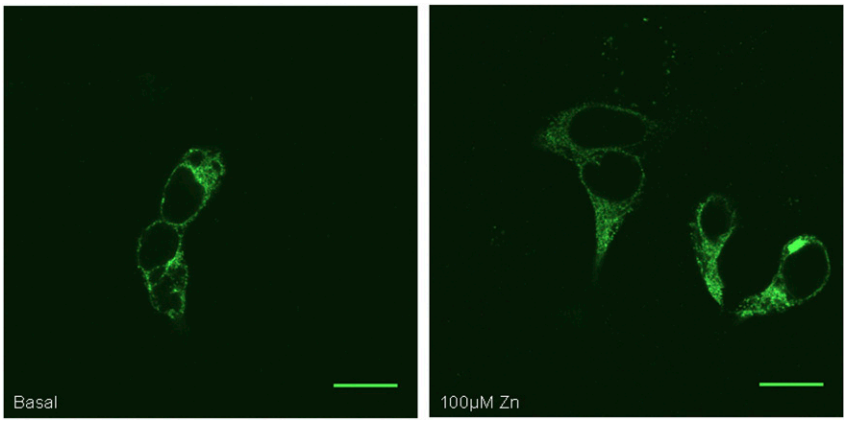

Fig. S2. Zinc does not alter the localization of ATP7B-Arg^{875}. HEK293Trex-expressing FlagATP7B-Arg^{875} cells were treated with 100 μM $ZnCl_2$ for 4 h or maintained at basal conditions. Staining with anti-Flag antibody (green) shows that the characteristic ER pattern of ATP7B-Arg^{875} localization remains unchanged. (Scale bar: 7 μm.)

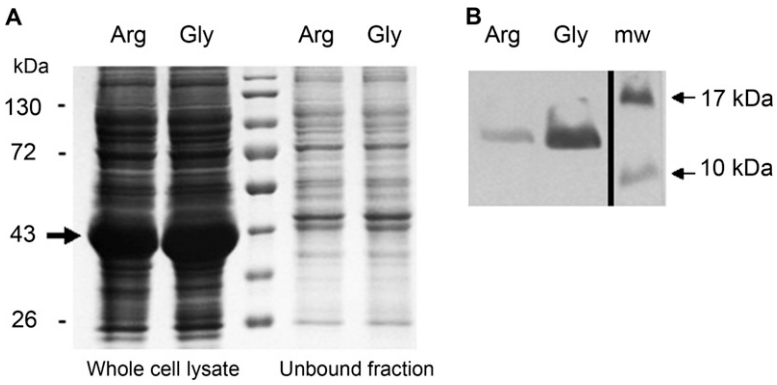

Fig. S3. Expression and purification of the Gly^{875} and Arg^{875} variants of the A-domain. (*A*) Whole-cell lysate: soluble fraction after lysis of *E. coli* cells overexpressing the intein fusion A-domains containing Gly^{875} or Arg^{875}. Unbound fraction: the fraction of the whole-cell lysate collected after passing it over to chitin beads (flow-through) shows that both variants were bound to the resin. (*B*) Protein eluate collected after cleavage with 50 mM MESNA.

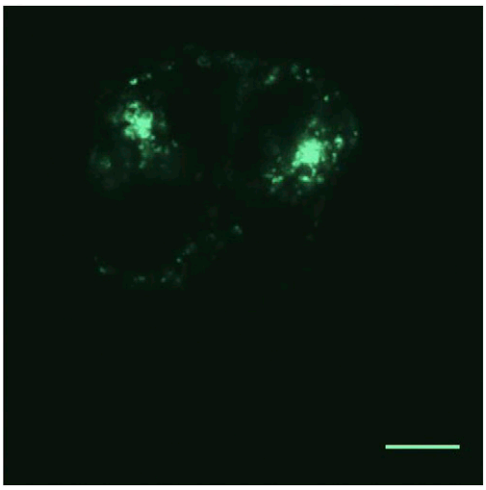

Fig. S4. Copper promotes TGN localization of ATP7B-Arg^{875} in the absence of de novo protein synthesis. HEK293Trex-expressing FlagATP7B-Arg^{875} cells were preincubated with 50 μg/mL CHX for 2 h, followed by treatment with 5 μM copper for 4 h in the presence of CHX. Staining with anti-Flag antibody (green) shows localization of ATP7B-Arg^{875} in the TGN. (Scale bar: 7 μm.)

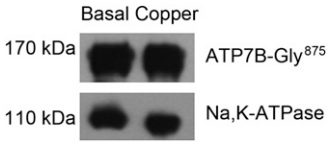

Fig. S5. Copper does not affect ATP7B-Gly^{875} levels. Western blots of membrane fractions from HEK293Trex cells transfected with Flag-ATP7B-Gly^{875} and treated overnight with or without 5 μM copper or maintained at basal conditions are shown. Immunostaining of Na,K-ATPase is used as a loading control.

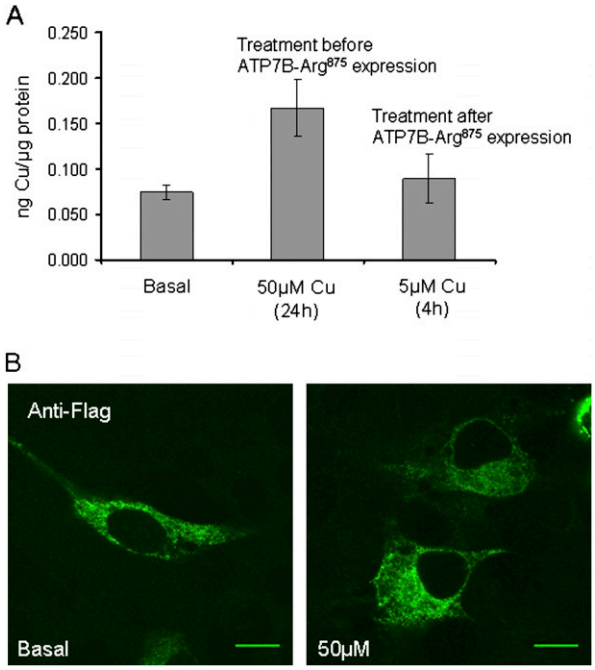

Fig. S6. Accumulated copper is not available to ATP7B-Arg^{875} for its ER exit. (*A*) Intracellular copper concentration was measured in Menkes fibroblasts (YSTT cells) treated with 50 μM (24 h) or 5 μM (4 h) $CuCl_2$ or in cells maintained in basal medium. Copper concentration was normalized per total protein. (*B*) YSTT cells were preincubated with copper for 24 h or maintained under basal conditions before transfecting them with ATP7B-Arg^{875}. Staining with anti-Flag antibody (green) shows localization of ATP7B-Arg^{875} in ER similar to ATP7B-Arg^{875} in basal medium. (Scale bar: 14 μm.)