

# Copper binding to the N-terminal metal-binding sites or the CPC motif is not essential for copper-induced trafficking of the human Wilson protein (ATP7B)

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The Wilson protein (ATP7B) is a copper-translocating P-type ATPase that mediates the excretion of excess copper from hepatocytes into bile. Excess copper causes the protein to traffic from the TGN (*trans*-Golgi network) to subapical vesicles. Using site-directed mutagenesis, mutations known or predicted to abrogate catalytic activity (copper translocation) were introduced into ATP7B and the effect of these mutations on the intracellular trafficking of the protein was investigated. Mutation of the critical aspartic acid residue in the phosphorylation domain (DKTGTIT) blocked copper-induced redistribution of ATP7B from the TGN, whereas mutation of the phosphatase domain [TGE (Thr-Gly-Glu)] trapped ATP7B at cytosolic vesicular compartments. Our findings demonstrate that ATP7B trafficking is regulated with its copper-translocation cycle, with cytosolic vesicular localization associated with the acyl-phosphate intermediate. In addition, mut-

ation of the six N-terminal metal-binding sites and/or the transmembrane CPC (Cys-Pro-Cys) motif did not suppress the constitutive vesicular localization of the ATP7B phosphatase domain mutant. These results suggested that copper co-ordination by these sites is not essential for trafficking. Importantly, copper-chelation studies with these mutants clearly demonstrated a requirement for copper in ATP7B trafficking, suggesting the presence of an additional copper-binding site(s) within the protein. The results presented in this report significantly advance our understanding of the regulatory mechanism that links copper-translocation activity with copper-induced intracellular trafficking of ATP7B, which is central to hepatic and hence systemic copper homeostasis.

**Key words:** ATP7B, copper translocation, metal-binding site, P-type ATPase, Wilson disease, WND.

## INTRODUCTION

The P-type ATPase ATP7B plays a pivotal role in mammalian copper homeostasis and defects in this protein cause the copper-toxicity disorder Wilson disease [1]. P-type ATPases are an extensive family of transmembrane proteins that mediate the translocation of various cations, including Mg<sup>2+</sup>, Ca<sup>2+</sup>, Na<sup>+</sup>, K<sup>+</sup>, Zn<sup>2+</sup>, Ag<sup>+</sup> and Cu<sup>+</sup>, across cellular membranes and often against concentration gradients [2]. Mammals have two copper-translocating P-type ATPases: ATP7B and its paralogue ATP7A. Both proteins contain the characteristic features of P-type ATPases which include an ATP-binding site (GDGIND), a phosphorylation domain [DKTGT(I,L)T], a phosphatase domain [TGE (Thr-Gly-Glu)] and eight transmembrane regions (Figure 1) [1–3]. In addition, heavy-metal-transporting P-type ATPases, such as ATP7A and ATP7B, form a subfamily termed CPX-type ATPases [4]. Members of the subfamily are characterized by a variable number of heavy MBSs (metal-binding sites) (GMXCXXC) in the N-terminal region and a conserved Cys-Pro-Xaa motif (Xaa=cysteine, histidine or serine) in the proposed cation transduction channel (Figure 1) [4]. Both ATP7A and ATP7B have six N-terminal GMXCXXC motifs, which have been shown to preferentially bind copper with a stoichiometry of one copper atom per binding motif [5]. The CPC (Cys-Pro-Cys) motif situated in the sixth transmembrane domain has also been shown to bind copper, which may facilitate translocation of the cation through the membrane [6,7].

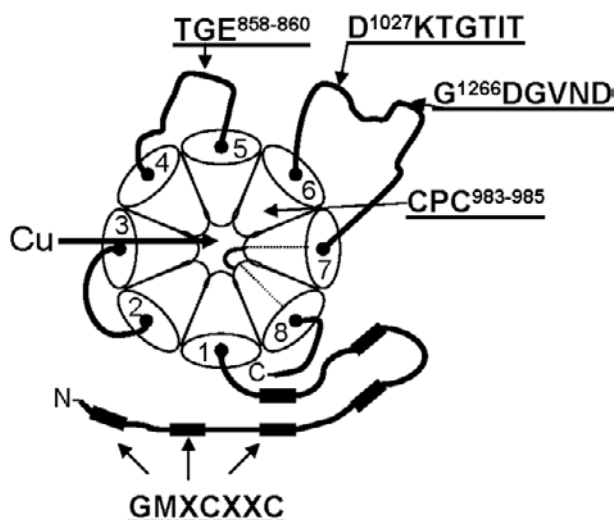
The enzymatic properties of both copper-ATPases conform to the classical P-type ATPase model [2,8]. Phosphorylation of the aspartic acid residue in the phosphorylation domain is required

for copper translocation and is dependent on the presence of cation (copper) and ATP [9,10]. The transiently phosphorylated intermediate (acyl-phosphate) is sensitive to basic pH, ADP and treatment with hydroxylamine and the enzymes are inhibited by the structural homologue of P<sub>i</sub>, orthovanadate [9,10]. Acyl-phosphorylation drives protein conformational changes leading to the translocation of the bound cations across the lipid bilayer [2]. The reaction cycle is completed with hydrolysis of the acyl-phosphate (dephosphorylation) by an intrinsic phosphatase activity (via TGE motif) and the pump is returned back to its original conformation to allow further cation binding and translocation [2,8,11].

ATP7A and ATP7B are likely to have evolved from a single ancestral copper-ATPase to perform specific functions in different cell types. ATP7B is expressed predominantly in liver hepatocytes [12] but also in regions of the brain [13], breast [14] and placenta [15], while ATP7A is expressed in most extra-hepatic tissues [16]. Studies on the subcellular localization of the copper-ATPases have shown that both proteins reside at the final compartment of the Golgi apparatus, the TGN (*trans*-Golgi network) [17–19]. At this location, copper is incorporated into various cuproenzymes such as lysyl oxidase in fibroblasts (mediated by ATP7A) [20] or ceruloplasmin in hepatocytes (mediated by ATP7B) [21]. In hepatocytes, an increase in copper levels stimulates trafficking of ATP7B to pericanalicular vesicles and ATP7B recycles back to the TGN when copper concentrations are decreased [22,23]. The target vesicles for ATP7B have not been characterized and are possibly novel compartments that sequester excess copper and undergo a mechanism similar to lysosomal exocytosis to expel

Abbreviations used: BCS, bathocuproinedisulfonic acid; CHO-K1, Chinese-hamster ovary K1; CPC motif, Cys-Pro-Cys motif; MBS, metal-binding site; TGE, Thr-Gly-Glu; TGN, *trans*-Golgi network; wt, wild-type.

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**Figure 1** Schematic representation of ATP7B

Transmembrane domains are numbered 1 to 8. Filled rectangles represent the six N-terminal MBSs (GMXCXXC). The amino acids mutated in the present study and their positions in ATP7B are shown. The conserved domains are as follows: phosphorylation domain, D<sup>1027</sup>KTGTIT; phosphatase domain, TGE<sup>858–860</sup>; ATP-binding domain, G<sup>1266</sup>DGVND; CPC motif, CPC<sup>983–985</sup>. Protein orientation is from cytosolic side facing to luminal side, the direction in which ATP7B translocates copper across the membrane.

their luminal contents into bile [23–25]. ATP7A displays similar copper-induced trafficking to ATP7B, but relocates from the TGN to the basolateral membrane in polarized cells [22], where it can efflux copper directly from the cell [26]. In non-polarized cell types, ATP7A traffics to the plasma membrane [19], while ATP7B traffics to a dispersed population of cytosolic vesicles [17,18]. The predominant subcellular localization of each copper-ATPase depends on copper levels, although recycling between the compartments is likely to be constitutive in the presence of either high or low concentrations of copper [26].

We have previously shown that the copper-induced trafficking of ATP7A is regulated with its catalytic cycle [11]. Formation of the acyl-phosphorylated intermediate (catalytic activation) is required for ATP7A redistribution to the plasma membrane, while hydrolysis of the acyl-phosphate (dephosphorylation) is essential for the retrieval of trafficked ATP7A molecules back to the TGN [11]. In the present study, we show that the trafficking of ATP7B is also coupled with its catalytic activity, with the vesicular localization associated with its acyl-phosphate intermediate. Surprisingly, copper co-ordination by either the six N-terminal MBSs or the CPC motif is not essential for ATP7B trafficking even though the trafficking process is dependent on copper. This observation suggests the presence of an additional copper-binding site(s) in the molecule. The results presented in this report represent significant progress towards understanding the mechanism by which copper regulates the subcellular localization of its own transporter.

## EXPERIMENTAL

### Cells, reagents and antibodies

CHO-K1 (Chinese-hamster ovary K1) cells were cultured as monolayers in Eagle's Basal Medium (Trace BioSciences, Nobel Park, VIC, Australia) supplemented with 0.2 mM proline, 10% (v/v) foetal calf serum, 2 mM L-glutamine, 1.2 mM NaHCO<sub>3</sub> and 20 mM Hepes (Commonwealth Serum Laboratories,

Broadmeadows, VIC, Australia). Transfection of CHO-K1 cells with plasmid DNA was performed using Lipofectamine™ (Life Technologies, Mount Waverley, VIC, Australia) and the manufacturer's recommendations. Transfected cells were selected using 500 µg/ml G418 (Life Technologies) for 14 days. The *Saccharomyces cerevisiae* mutant strain, *ccc2Δ* (*MATα*, *his3-200*, *trp-1-101*, *ura3-52*, *leu2*, *ade5*, *ccc2Δ::LEU2*) [27], was cultured on YPD solid medium containing 2% (w/v) trypticase peptone, 1% (w/v) yeast extract, 2% (w/v) glucose and 2% (w/v) agar. This strain was transformed with plasmid DNA using a lithium acetate method as previously described [28] and transformants were selected on DOB-Ura medium composed of 0.66% (w/v) yeast nitrogen base without amino acids, 2% glucose and 0.08% (w/v) CSM (complete supplement mixture minus uracil). The sheep polyclonal anti-ATP7B antibody (NC36) was raised against amino acids 1–199 (N-terminal), 1309–1315 and 1376–1465 (C-terminal) of human ATP7B as previously described [29]. All reagents used in the present study were purchased from Sigma (Castle Hill, NSW, Australia) unless otherwise stated.

### Generation of ATP7B key domain mutants

The Asp<sup>1027</sup> residue in the phosphorylation domain was changed to glutamate (D-E) by PCR-based mutagenesis of the *ATP7B* cDNA as previously described [25]. PCR-based mutagenesis was also used to change the Thr<sup>858</sup>, Gly<sup>859</sup> and Glu<sup>860</sup> residues in the phosphatase domain to alanine residues (TGE-AAA). In this case, the oligonucleotides 5'-CAATACCATGGCTGATGAGTCCCTCATCGCGGCCGCTGCCA-3' (hWND#20) and 5'-TCTGTTCC-AAGTTCCTCTT-3' (hWND#19) were used to amplify nt 2544–3259 of *ATP7B*. The oligonucleotide hWND no. 20 introduced the desired codon changes, with the codons encoding the alanine residues chosen to form a NotI restriction endonuclease site. The PCR product was digested with *Hinf*I and *Pin*AI to produce a 193 bp fragment (nt 2561–2753), which together with an *Stu*I/*Hinf*I fragment isolated from pCMB278 (nt 2516–2753 of *ATP7B*) was then ligated into *Pin*AI/*Stu*I-digested pCMB278. The resultant plasmid was designated pCMB515. The ATP7B mutant containing both the phosphorylation- and phosphatase-domain changes (D-E + TGE-AAA) was generated by isolating the TGE-AAA mutation from the pCMB515 plasmid using *Stu*I/*Pin*AI and using this fragment to replace the corresponding wt (wild-type) region in the D-E cDNA in pCMB514. The resultant plasmid was designated pCMB516.

The ATP7B construct with the cysteine residues of the six N-terminal MBSs mutated to serine residues (MBS1-6c/s) was created as previously described [29]. To generate ATP7B with the cysteine residues in the CPC<sup>983–985</sup> motif mutated to serine residues (CPC-SPS), the oligonucleotide 5'-CGGTTTGCTTTC-CAGACGTCCATCACGGTGCTGTCATTGCCTCACCCCTC-GTCCCTGGGGCTG-3' (hWND#22) introduced the codon changes when used in combination with 5'-GCTGACATCGCTA-GAAAT-3' (hWND#14) to amplify nt 2905–3499 of *ATP7B*. The PCR product was digested with *Aat*II and *Aoc*I to produce a 234 bp fragment (nt 2924–3159), which together with an *Aoc*I/*Sal*I fragment isolated from pCMB411 (nt 3160–4395 of *ATP7B*) was then ligated into *Aat*II/*Sal*I-digested pCMB411. The resulting plasmid was designated pCMB517. To create the final expression construct, the entire CPC-SPS cDNA was isolated from pCMB517 using *Xba*I/*Sal*I and cloned into the *Nhe*I/*Sal*I sites of pCMB77, generating pCMB518.

The following strategy was employed to create two ATP7B double mutants, one containing the six N-terminal MBS mutations and the phosphatase domain mutation (MBS1-6c/s + TGE-AAA) and another in which the CPC motif and the phosphatase

**Table 1** Plasmids generated and used in the present study

Plasmid	Characteristics	Reference
pWSK29	Bacterial propagation vector	[45]
pCMB77	Mammalian expression vector	[19]
pVT-103U	Yeast expression vector	[30]
pCMB278	wtATP7B in pCMB77	[29]
pCMB514	D-E in pCMB77	[25]
pCMB515	TGE-AAA in pCMB77	Present study
pCMB516	D-E + TGE-AAA in pCMB77	Present study
pCMB517	CPC-SPS in pCMB411	Present study
pCMB518	CPC-SPS in pCMB77	Present study
pCMB519	CPC-SPS + TGE-AAA in pCMB77	Present study
pCMB398	MBS1-6c/s in pCMB77	[29]
pCMB520	MBS1-6c/s + TGE-AAA in pCMB77	Present study
pCMB521	MBS1-6c/s + CPC-SPS in pCMB77	Present study
pCMB522	MBS1-6c/s + CPC-SPS + TGE-AAA in pCMB77	Present study
pCMB554	wtATP7B in pVT-103U	[29]
pCMB555	MBS1-2c/s in pVT-103U	[29]
pCMB594	D-E in pVT-103U	Present study
pCMB595	TGE-AAA in pVT-103U	Present study
pCMB596	D-E + TGE-AAA in pVT-103U	Present study
pCMB557	MBS1-6c/s in pVT-103U	[29]
pCMB597	MBS1-6c/s + TGE-AAA in pVT-103U	Present study
pCMB598	CPC-SPS in pVT-103U	Present study
pCMB599	CPC-SPS + TGE-AAA in pVT-103U	Present study
pCMB600	MBS1-6c/s + CPC-SPS in pVT-103U	Present study
pCMB601	MBS1-6c/s + CPC-SPS + TGE-AAA in pVT-103U	Present study

domain are mutated (CPC-SPS + TGE-AAA). The TGE-AAA mutation (nt 2754–4395) was isolated from pCMB515 using PinAI/SalI. This fragment was used to replace the corresponding wt region in pCMB398 (resulting plasmid designated pCMB520) and in pCMB515 (resulting plasmid designated pCMB519) respectively. The ATP7B construct with the six N-terminal MBSs, the CPC motif and the phosphatase domain mutated (MBS1-6c/s + CPC-SPS + TGE-AAA) was engineered by isolating the CPC-SPS and TGE-AAA mutations together from pCMB519 (nt 2153–4395) using StuI and SalI. This fragment was then cloned into the StuI/SalI sites of the *MBS1-6c/s* cDNA in pCMB398, replacing the corresponding wt region (resulting plasmid designated pCMB522). The ATP7B construct with the six N-terminal MBSs and the CPC motif mutated (MBS1-6c/s + CPC-SPS) was generated by using StuI/PinAI to drop out the region in pCMB522 containing the TGE-AAA mutation and by replacing this fragment with an StuI/PinAI fragment isolated from wtATP7B (from pCMB278). The resultant plasmid was designated pCMB521. The PCR-derived inserts and ligation sites in all constructs generated were sequenced to confirm integrity. The plasmid constructs generated and used in the present study are outlined in Table 1.

The ATP7B mutant constructs described above were incorporated into the yeast expression vector pVT-103U. This vector contains the *ADHI* (alcohol dehydrogenase 1) gene promoter, the  $2\mu$  origin of replication for propagation in yeast, the *URA3* marker for plasmid selection in yeast, and the *ori* and intergenic region of the phage f1 (f1 *ori*). In addition, pVT-103U also contains an origin of replication and the  $\beta$ -lactamase gene for propagation and selection (ampicillin resistance) in *Escherichia coli* [30]. ATP7B cDNA constructs can be unstable in high copy number vectors. Although pVT-103U is only an intermediate copy number vector [30], ATP7B constructs generated using this vector were still propagated in the Able<sup>®</sup>-K *E. coli* strain (Stratagene, La Jolla, CA, U.S.A.) to reduce the copy number 10-fold to ensure stable propagation of ATP7B.

The ATP7B constructs were cloned into pVT-103U using the following strategy. Nucleotides 494–4395 were removed from

wtATP7B in pVT-103U (pCMB554) or from MBS 1 + 2c/s in pVT-103U (pCMB555) (previously described in [29]) using BseAI and XhoI. These fragments were replaced with the corresponding region from each ATP7B mutant cDNA, which was isolated from their respective expression plasmids (shown in Table 1) using BseAI and SalI. Constructs with MBS 1–2 mutated were inserted into pCMB555. Digestion profiles, exploiting endonuclease sites introduced during the generation of each mutant, were used to verify that the mutant cDNAs replaced the wtATP7B segment of pCMB554. Final plasmid constructs were designated as shown in Table 1.

### Protein preparation and Western-blot analysis

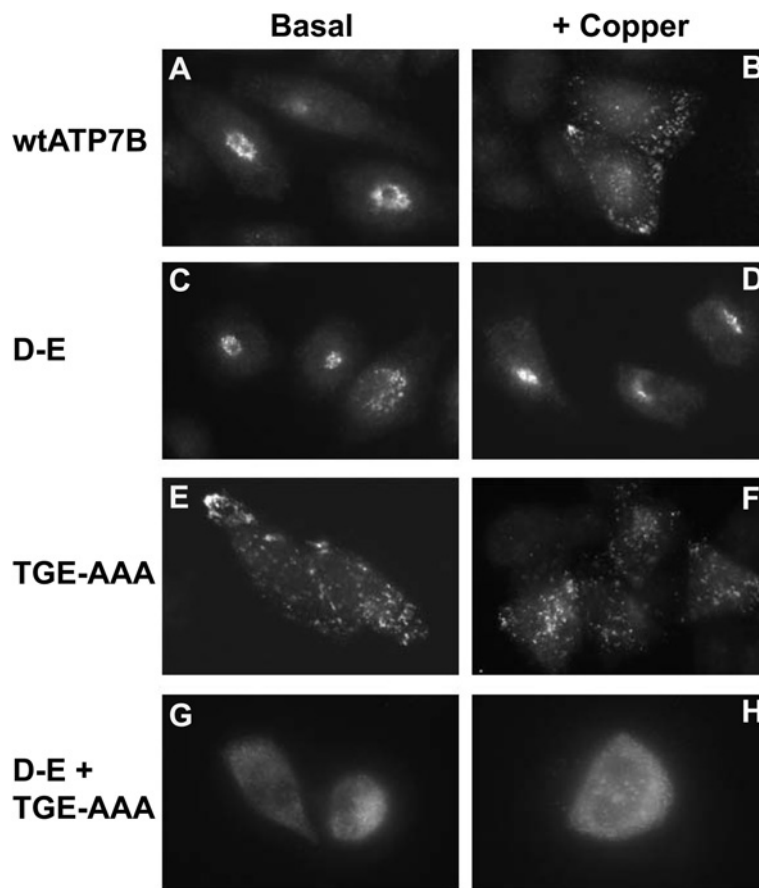
Yeast protein extracts were prepared using a trichloroacetic acid method as described in Clontech's Yeast Protocols Handbook (PT3024-1). Whole cell protein extracts (30  $\mu$ g) were fractionated by SDS/PAGE (7.5% polyacrylamide). Following electrophoretic separation, proteins were transferred to nitrocellulose (Amersham, Rydalmere, NSW, Australia) using a Trans-Blot SD semi-dry electrophoretic transfer apparatus (Bio-Rad, Regents Park, NSW, Australia) and immunoblot analysis was carried out using the Lumi-Light chemiluminescence blotting kit (Roche, Castle Hill, NSW, Australia) according to the manufacturer's protocol. ATP7B was detected using the ammonium sulfate-precipitated NC36 antibody (diluted 1:1000) [29], followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (Chemicon, Boronia, VIC, Australia) (1:4000). Exposed Hyperfilm<sup>™</sup> (Amersham) was fixed and developed using a Fuji medical film processor (Fuji Photo Film).

### Indirect immunofluorescence microscopy

Transfected CHO-K1 cells were seeded on to 13 mm glass coverslips in a 24-well tray and cultured for 16–72 h to approx. 50–70% confluence. The growth medium was replaced with basal medium (0.5–1  $\mu$ M Cu), 200  $\mu$ M CuCl<sub>2</sub>-supplemented medium or a medium containing the copper chelators BCS (bathocuproinedisulfonic acid; 200  $\mu$ M) and D-penicillamine (200  $\mu$ M) as indicated in the results. Following treatment, cells were fixed using 4% (w/v) paraformaldehyde in PBS for 10 min and then permeabilized with 0.1% (v/v) Triton X-100 in PBS for 10 min. Cells were blocked in 1% (w/v) BSA and 1% (w/v) gelatin in PBS at 4°C overnight. For the detection of ATP7B, coverslips were incubated with ammonium sulfate-precipitated NC36, diluted 1:10000 in blocking solution for 1 h. Following four PBS washes (15 min each) coverslips were incubated (room temperature for 1 h) with the secondary antibody, donkey anti-sheep IgG Alexa Fluor<sup>®</sup> 488 (1:4000; Chemicon) and again washed with PBS as above. Coverslips were mounted on glass slides using 2.6% (w/v) Dabco (Sigma) in 90% (v/v) glycerol. Immunolabelled cells were analysed using a  $\times 60$  oil objective with an Olympus Provis AX70 microscope.

### Complementation of *S. cerevisiae ccc2* $\Delta$

The *S. cerevisiae ccc2* $\Delta$  yeast strains that expressed wt and mutant ATP7B proteins were each inoculated into 5 ml of DOB-Ura and grown at 30°C overnight to saturation. Serial 10-fold dilutions in sterile distilled water were made in a 96-well plate starting from 10<sup>-1</sup>. For each dilution, 5  $\mu$ l was applied in triplicate on to iron-limited DOB-Ura agar plates containing 50 mM Mes and 250  $\mu$ M ferrozine and on to normal DOB-Ura plates to verify cell viability and then incubated at 30°C for 3 days. Images were taken using a Canon digital IXUS V camera.



**Figure 2** Effect of mutating the phosphorylation and phosphatase domains on the trafficking of ATP7B

CHO-K1 cells transfected with wt or mutant *ATP7B* constructs [amino acid substitution(s) indicated on the left] were cultured in basal medium ( $0.5\text{--}1\ \mu\text{M}$  Cu) or a medium supplemented with  $200\ \mu\text{M}$   $\text{CuCl}_2$  (+ Copper). Detection of ATP7B was performed by immunofluorescence analysis using ammonium sulfate-precipitated NC36 (diluted 1:10000) and donkey anti-sheep IgG Alexa Fluor<sup>®</sup> 488 (diluted 1:4000) (Chemicon). Photographs were taken using a  $\times 60$  oil objective with an Olympus Provis AX70 microscope.

## RESULTS

### Effect of mutations in the phosphorylation and phosphatase domains on the subcellular localization and trafficking of ATP7B

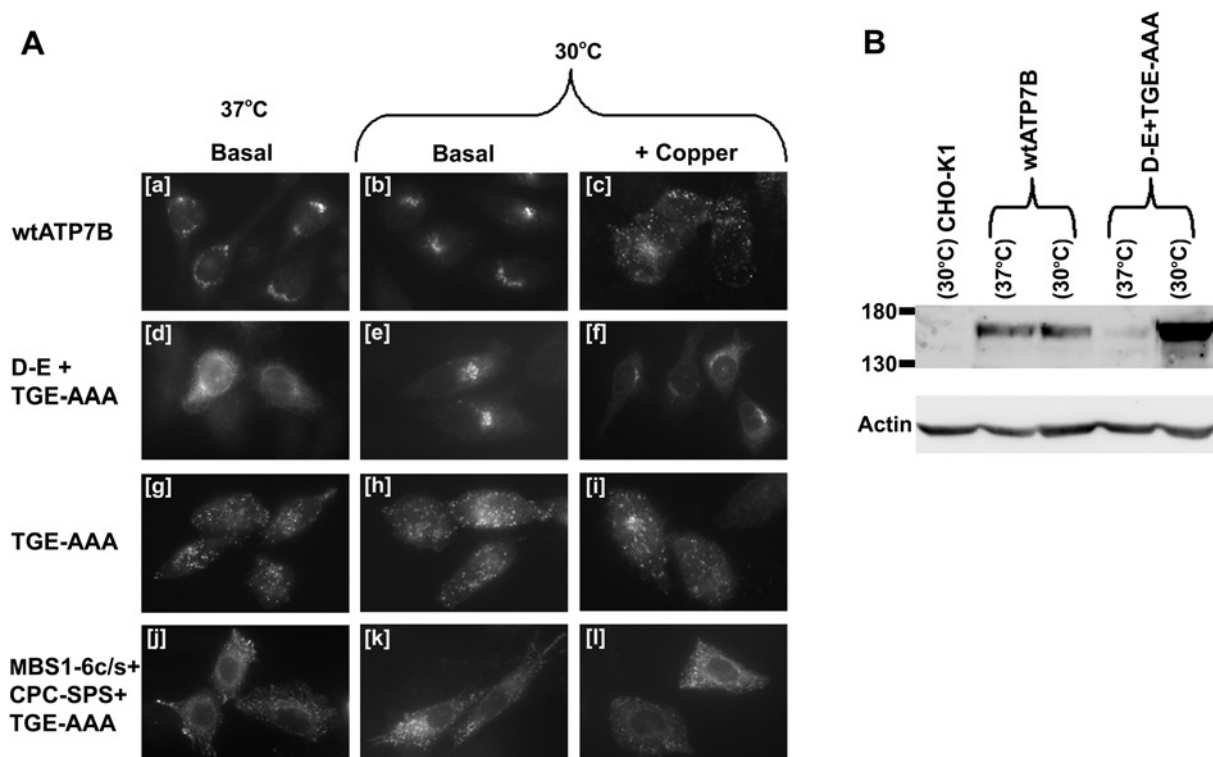
Under basal copper conditions, the localization of ATP7B to the TGN has been well established [17,18,25]. Despite previous attempts [18,31], the cytosolic vesicular compartments to which ATP7B traffics in elevated copper concentrations remain undefined. In the present study, the effect of various mutations within ATP7B on the ability of the protein to traffic from the perinuclear region to cytosolic vesicles was assessed.

The invariant aspartate ( $\text{Asp}^{1027}$ ) in the phosphorylation domain of ATP7B (DKTGTIT) has been shown to be essential for catalytic activity [29]. To determine if this aspartate is also required for the copper-induced trafficking of the protein, it was mutated to glutamate (D-E) and the ability of the mutant protein to traffic was studied in CHO-K1 cells (Figure 2). In cells cultured under basal copper conditions ( $0.5\text{--}1\ \mu\text{M}$  Cu), the D-E protein was detected in the perinuclear region (Figure 2C), similar to wtATP7B under the same conditions (Figure 2A). However, in cells exposed to  $200\ \mu\text{M}$  copper for 2 h, the D-E protein remained perinuclear (Figure 2D), while most of the wtATP7B molecules redistributed to cytosolic vesicles (Figure 2B). Increasing copper concentration to  $400\ \mu\text{M}$  and extending the exposure time to 4 h had no observable effect on the localization of the D-E protein (results not shown). These results demonstrate that  $\text{Asp}^{1027}$  is

required for the trafficking of ATP7B and support the hypothesis that the copper-induced trafficking process is dependent on acyl-phosphate formation [11].

Mutation of the phosphatase domain (TGE) in several P-type ATPases, including both  $\text{H}^+$ - and  $\text{Ca}^{2+}$ -ATPase, has been shown to prevent cation translocation and resulted in the accumulation of the acyl-phosphorylated intermediate [32,33]. Mutation of the  $\text{TGE}^{858\text{--}860}$  sequence in ATP7B to alanine residues (TGE-AAA) caused constitutive vesicular localization of the protein (Figure 2E), resembling the distribution of wtATP7B in elevated levels of copper (Figure 2B). There was no distinguishable difference in the vesicular staining of the TGE-AAA protein when cells were cultured in either basal medium or in a medium supplemented with  $200\ \mu\text{M}$   $\text{CuCl}_2$  (Figures 2E and 2F). This observation suggested that even in basal copper concentrations acyl-phosphate formation occurs, and in the absence of the intrinsic phosphatase activity, most of the ATP7B molecules remain phosphorylated and consequently are trapped at vesicular compartments.

To investigate whether the trafficking and vesicular localization of the TGE-AAA protein was dependent on the presence of the invariant aspartate ( $\text{Asp}^{1027}$ ), an ATP7B double mutant containing the D-E and TGE-AAA mutations was generated. The D-E + TGE-AAA protein localized abnormally in a hazy punctate pattern throughout the cytosol when expressed in CHO-K1 cells (Figure 2G). This phenotype was observed in all transfected cells in the absence and in the presence of  $200\ \mu\text{M}$   $\text{CuCl}_2$  (Figure 2H). In



**Figure 3** Effect of temperature reduction on the subcellular localization and stability of ATP7B mutants

(A) Immunofluorescence analysis of CHO-K1 cells expressing wt or mutant ATP7B constructs [amino acid substitution(s) indicated on the left] cultured at 30°C. Cells were cultured at 30°C for 3 days and then were incubated for 8 h (at 30°C) with basal medium or a medium supplemented with 200  $\mu$ M CuCl<sub>2</sub> (+Copper) before being analysed by immunofluorescence microscopy. Detection of ATP7B was performed as described in Figure 2. (B) Western-blot analysis comparing the amount of wtATP7B and D-E + TGE-AAA expression in CHO-K1 cells. Cells were cultured for 3 days at either 37 or 30°C before total cellular protein (~50  $\mu$ g) was subjected to SDS/PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. The membrane was incubated with ammonium sulfate-precipitated NC36 (diluted 1:1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1:4000) and analysed by chemiluminescence. The positions of the molecular mass markers (Bio-Rad) are indicated on the left in kDa. The nitrocellulose was then stripped using Re-Blot Plus-Mild (Chemicon) and reblotted with mouse anti- $\beta$ -actin monoclonal antibody (diluted 1:5000) and horseradish peroxidase-conjugated sheep anti-mouse IgG (diluted 1:4000).

addition, there were clearly fewer cells in which the D-E + TGE-AAA protein was detected in comparison with all other transfected cell lines and the cells that expressed the mutant protein displayed weak immunofluorescence staining (results not shown).

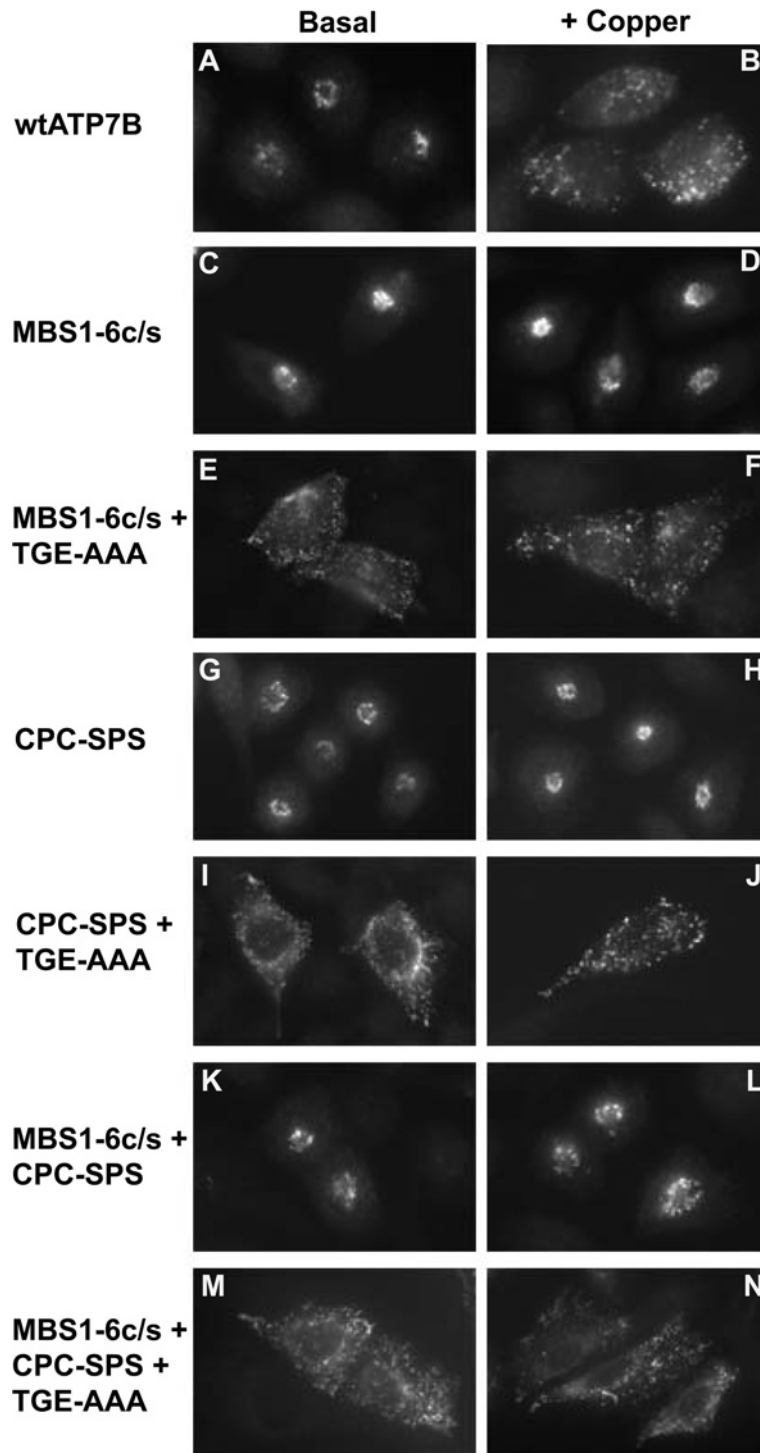
#### The D-E + TGE-AAA mutant protein is temperature-sensitive

Temperature-sensitive proteins that contain *tsf* (temperature-sensitive folding) mutations, which destabilize an intermediate in the folding pathway, often can fold correctly and maintain normal function when biosynthesized at a lower temperature [34]. To determine if the aberrant localization of the D-E + TGE-AAA protein was due to temperature-sensitive misfolding, CHO-K1 cells that express this mutant protein were incubated at 30°C for 3 days before being evaluated by immunofluorescence microscopy (Figure 3A) and Western-blot analysis (Figure 3B). The decrease in temperature resulted in the perinuclear staining of the D-E + TGE-AAA protein [Figure 3A (e)], which was clearly distinct from the hazy punctate pattern observed at 37°C [Figure 3A (d)]. Elevated copper (200  $\mu$ M) had no effect on the perinuclear localization of D-E + TGE-AAA [Figure 3A (f)], whereas under the same conditions most of the wtATP7B molecules trafficked to cytosolic vesicles [Figure 3A (c)]. The reduction in temperature also resulted in an increase in the number of cells in which the D-E + TGE-AAA protein was detected (results not shown), consistent with an increase in the stability of the D-E + TGE-AAA protein at 30°C. Western-blot analysis confirmed that culturing

the cells for 3 days at 30°C increased the amount of intact, stable D-E + TGE-AAA protein, while having no obvious effect on the amount of wtATP7B (Figure 3B). Allowing the D-E + TGE-AAA protein to assemble at a permissive temperature (30°C) not only corrected the subcellular localization of the protein [Figure 3A (e)], but also revealed that the D-E substitution suppressed the effect of the TGE-AAA mutation as the double mutant was incapable of trafficking from the perinuclear region [Figure 3A (f)]. To demonstrate that the vesicular localization of the TGE-AAA protein did not arise from a similar temperature (sensitive) effect, the effect of protein biosynthesis and assembly at 30°C on the subcellular localization of TGE-AAA was assessed [Figure 3A (h and i)]. In cells cultured at 30°C in either basal medium or a medium supplemented with 200  $\mu$ M copper, the TGE-AAA protein retained its cytosolic vesicular localization. Therefore the vesicular localization of TGE-AAA is unlikely to have arisen from protein misfolding.

#### Copper binding to the GMXCXXC and CPC motifs is not essential for ATP7B trafficking

Mutation of the cysteine residues in all six N-terminal copper-binding sites (GMXCXXC) to serine residues inhibited the trafficking of ATP7B [29]. This observation was consistent with copper binding to these sites being required for catalytic activation (formation of the acyl-phosphate) leading to trafficking (Figures 4C and 4D). However, the failure of this and other ATP7B



**Figure 4** Effect of mutating copper-binding sites on the trafficking of ATP7B

CHO-K1 cells transfected with wt or mutant *ATP7B* constructs [amino acid substitution(s) indicated on the left] were cultured in basal medium (0.5–1  $\mu\text{M}$  Cu) or a medium supplemented with 200  $\mu\text{M}$   $\text{CuCl}_2$  (+ Copper). Detection of ATP7B was performed as described in Figure 2.

variants to traffic [17,31] could be due to either (i) the mutation preventing formation of the acyl-phosphorylated intermediate or (ii) slowing of the rate of catalysis to such an extent that the rate of anterograde trafficking is less than the rate of retrieval back to the TGN. In the latter case, ATP7B would not accumulate at the cytosolic vesicles. To distinguish between these two possibilities, the

six N-terminal copper-binding site mutations were combined with the phosphatase domain mutation (MBS1-6c/s + TGE-AAA). The mutant protein localized to vesicular compartments in both basal medium (Figure 4E) and medium supplemented with 200  $\mu\text{M}$  copper (Figure 4F), with a pattern of labelling indistinguishable from that of the TGE-AAA protein (Figures 2E and

2F). These results suggested that mutation of the six N-terminal copper-binding sites alone perturbs, but does not completely abrogate the trafficking of ATP7B.

If copper binding to ATP7B is responsible for initiating catalytic activation and trafficking, then mutation of the copper-binding site(s) involved would be expected to abolish these activities. The CPC motif in the copper-ATPases (ATP7A and ATP7B) is 43 amino acids upstream of the phosphorylation domain [DKTGT(L,L)T] (Figure 1) and is critical for the function of both copper-ATPases [6,17,35,36]. It has been proposed that the CPC motif may facilitate the translocation of copper through the transmembrane region [7]. It is conceivable that binding of copper to the CPC motif leads directly to acyl-phosphate formation. Mutation of both cysteine residues in the CPC motif to serine residues (CPC-SPS) prevented trafficking of ATP7B in CHO-K1 cells (Figures 4G and 4H). The CPC-SPS protein localized to the perinuclear region under both basal and elevated copper conditions (Figures 4G and 4H), consistent with previous observations [6]. Surprisingly, when the TGE-AAA mutation was introduced into the CPC-SPS protein, the double mutant localized to vesicles in the absence and presence of added  $\text{CuCl}_2$  (Figures 4I and 4J). This observation suggested that mutation of the CPC motif retards rather than completely blocks the trafficking process, as observed with the six N-terminal MBS mutations.

Possibly copper binding to either the N-terminal MBSs or the CPC would be sufficient for acyl-phosphate formation and hence trafficking. To investigate this hypothesis, a mutant protein in which the six N-terminal MBSs, the CPC motif and the phosphatase domain were mutated (MBS1-6c/s + CPC-SPS + TGE-AAA) was expressed in CHO-K1 cells. This mutant protein localized to vesicular compartments in both basal and 200  $\mu\text{M}$   $\text{CuCl}_2$ -supplemented medium (Figures 4M and 4N). When the six N-terminal MBSs and the CPC motif were mutated but the phosphatase domain was left unchanged (MBS1-6c/s + CPC-SPS), the protein localized to the perinuclear region (Figures 4K and 4L). This result indicated that the combination of the MBS1-6c/s and the CPC-SPS mutations was not responsible for the constitutive vesicular localization of the MBS1-6c/s + CPC-SPS + TGE-AAA protein. To demonstrate that the vesicular localization of this protein was not due to folding irregularities caused by the multiple mutations, CHO-K1 cells that expressed this mutant protein were incubated at 30 °C for 3 days before being evaluated by immunofluorescence microscopy [Figure 3A (k and l)]. Reducing the temperature had no effect on the subcellular localization of the MBS1-6c/s + CPC-SPS + TGE-AAA protein [Figure 3A (k and l)]. These results suggested that while the six N-terminal MBSs and the CPC motif are required for the efficient trafficking of ATP7B, surprisingly they are not essential for trafficking to occur and, when mutated together with the phosphatase domain (TGE-AAA), lead to accumulation of ATP7B at vesicular compartments.

### Copper chelation restores TGN localization of TGE-AAA mutants

If ATP7B is required to bind copper to initiate catalytic activation and trafficking, then depleting intracellular copper should prevent, or slow, the redistribution of the TGE-AAA mutants. To test this hypothesis, cell lines that expressed the TGE-AAA mutants were cultured in a medium containing the copper chelators BCS (200  $\mu\text{M}$ ) and D-penicillamine (200  $\mu\text{M}$ ). With the exception of the D-E + TGE-AAA protein (Figure 5F), all TGE-AAA mutants localized to the perinuclear region when cells were incubated for 3 days with the copper chelators (Figure 5). Three days of incubation were necessary to shift the steady-state localization of the TGE-AAA mutants from cytosolic vesicles to the perinuclear region (Figure 5); after only one or two days the proteins were still

observed in vesicles (results not shown). The D-E + TGE-AAA protein retained the hazy punctate pattern under copper-depleted conditions (Figure 5F). The overall number and appearance of the cells was unchanged by the copper chelation treatment, suggesting that the cells were still viable. Taken together, these results demonstrate that copper availability was necessary for the constitutive vesicular localization of the TGE-AAA mutants. Since the two known copper-binding regions were mutated in the MBS1-6c/s + CPC-SPS + TGE-AAA protein, these results raise the possibility of an additional unrecognized copper-binding site(s) in ATP7B.

### The role of the key domains in the copper-translocation activity of ATP7B

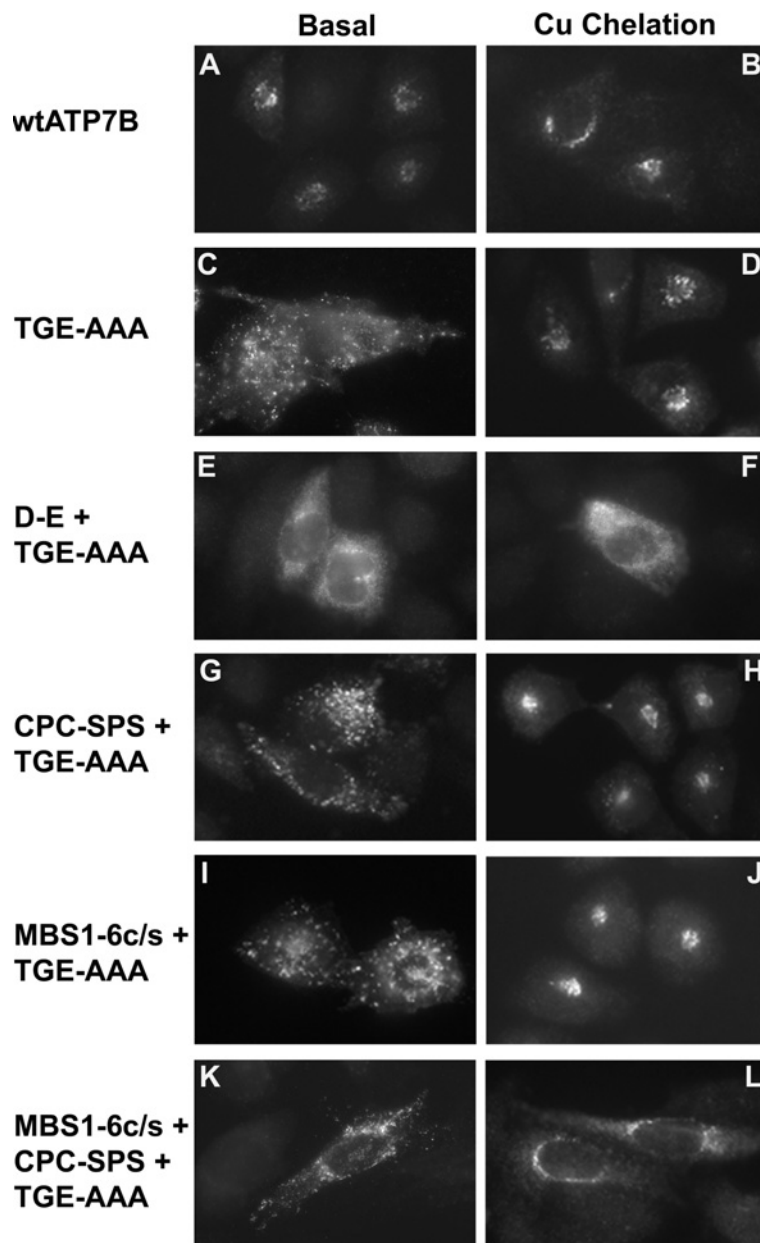
Any mutation that blocks a step in the catalytic cycle of ATP7B will inhibit the translocation of copper by the protein. Thus mutation of the phosphorylation (D-E) or the phosphatase domain (TGE-AAA), both of which are required for ATP7B to complete the copper-translocation cycle, should result in a non-functional protein. The aspartate residue in the phosphorylation domain of ATP7B is essential for catalytic activation [37]; however, the importance of the phosphatase domain has not been evaluated. The ATP7B key domain mutants were expressed in *S. cerevisiae* *ccc2* $\Delta$  and evaluated for their copper-transport ability by assessing the growth of the transformed yeast on iron-limited medium (Figure 6A, -Fe). The *ccc2* $\Delta$  strain that expressed wtATP7B (or wtATP7A) grew uniformly on iron-limited agar plates, while the *ccc2* $\Delta$  strains that expressed the mutant proteins did not grow on the same medium (Figure 6A, -Fe). The viability of the yeast strains was confirmed on iron-sufficient medium (Figure 6A, DOB-Ura). The failure of the ATP7B mutant proteins to complement the copper-transport defect of *ccc2* $\Delta$  was not due to low expression levels, which were equivalent to or greater than wtATP7B (Figure 6B).

## DISCUSSION

In cells exposed to high concentrations of copper, both ATP7A and ATP7B shift their steady-state localization from the TGN to either the plasma membrane (ATP7A) or to cytosolic vesicles (ATP7B) [17,19,23–25]. The copper-induced trafficking of ATP7A previously was shown to be regulated with the catalytic cycle of the ATPase [11]. Consistent with this report, we show here that acyl-phosphorylation was required for the trafficking of ATP7B and dephosphorylation via the phosphatase domain was essential for TGN retrieval of trafficked protein.

The combination of the D-E and TGE-AAA mutations led to instability of the protein at 37 °C. Temperature-sensitive mutations have been identified in ATP7B and include the patient mutations H1069Q and M769V and several C- and N-terminal truncations [6,29,38,39]. Analysis at reduced temperatures previously was used to determine the functional effect of the H1069Q and M769V mutations [6,39]. In the present study, the subcellular localization of D-E + TGE-AAA was corrected at the permissive temperature (30 °C) and we demonstrated that the constitutive vesicular accumulation of the TGE-AAA protein required the aspartate residue (Asp<sup>1027</sup>), consistent with the requirement for acyl-phosphorylation for trafficking of ATP7B to occur.

Copper binding by the copper-ATPases is believed to be responsible for initiating catalytic activation and regulating their subcellular distribution [29,37,40]. The six N-terminal MBSs (GMXCXXC) and the CPC motif are currently the only recognized copper-binding motifs in both copper-ATPases. The



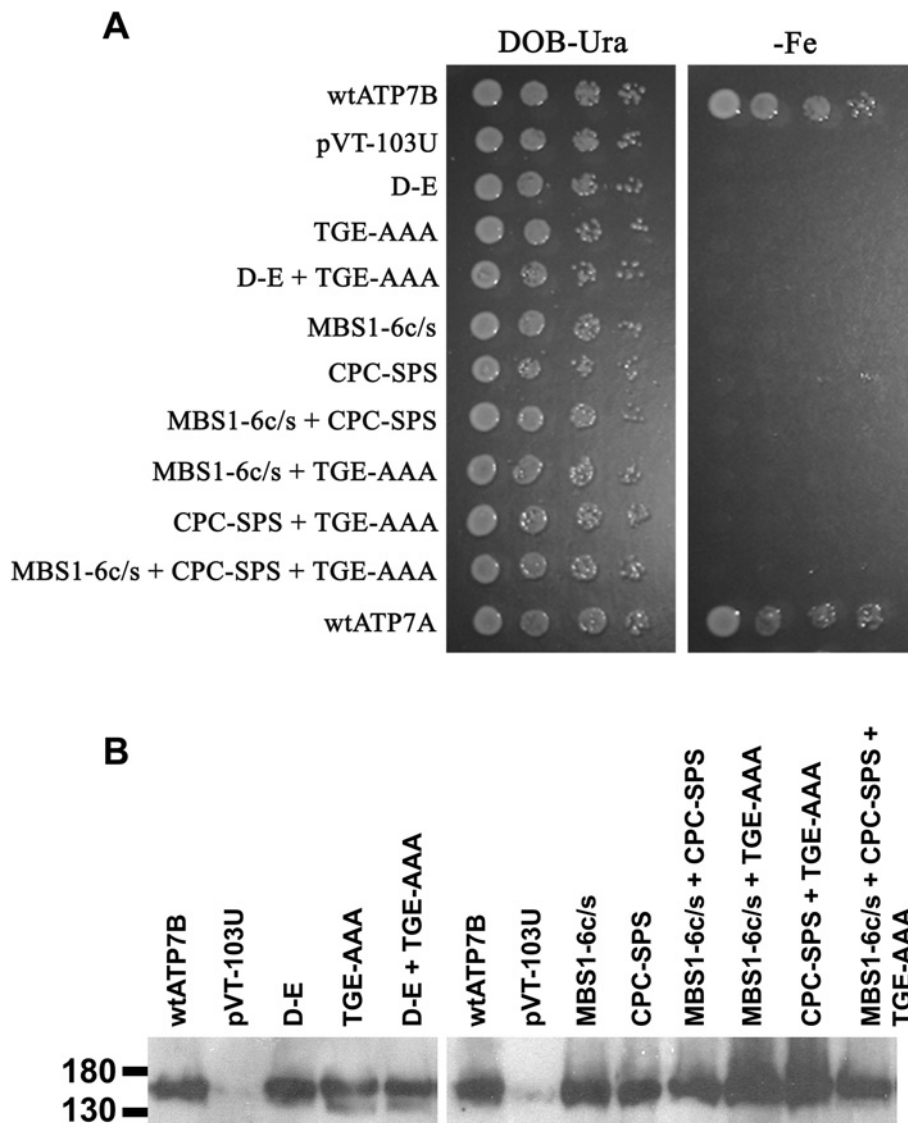
**Figure 5** Effect of copper chelation on the subcellular localization of the TGE-AAA mutants

CHO-K1 cells expressing the indicated wt or TGE-AAA mutant ATP7B proteins were cultured in basal medium or a medium containing the copper chelators BCS (200  $\mu$ M) and D-penicillamine (200  $\mu$ M). Detection of ATP7B was performed as described in Figure 2.

TGE-AAA (phosphatase) mutation provided a valuable tool to determine whether the N-terminal GMXCXXC sites or the CPC are essential for trafficking, because it results in trafficked molecules accumulating at cytosolic vesicles. Although the MBS1-6c/s and CPC-SPS mutant proteins predominantly had perinuclear localization in basal and elevated copper, when each was combined with the TGE-AAA mutation to produce double mutants or together to produce a triple mutant, the resultant proteins were found at cytosolic vesicular compartments. These results show that mutation of the N-terminal MBSs (MBS1-6c/s) and/or the CPC motif (CPC-SPS) did not suppress the effect of the TGE-AAA mutation, suggesting that copper co-ordination by these sites is not essential for the formation of the acyl-phosphate intermediate required for trafficking. Nevertheless, copper was required

for the vesicular localization of the TGE-AAA mutants since copper chelation confined the proteins to the perinuclear region (Figure 5). These observations suggest that copper may bind to another region of ATP7B leading to acyl-phosphate formation and trafficking. Alternatively, the trafficking of these mutants may be stimulated by another copper-binding protein(s) (such as a copper-dependent kinase) that might interact with ATP7B to provide a signal for acyl-phosphorylation to occur. Although the presence of copper was previously shown to be critical for acyl-phosphate formation [9], our results demonstrate that acyl-phosphorylation and trafficking can occur in the absence of N-terminal or CPC copper binding, albeit this process seems less efficient. This result was unexpected, as most models of ATP7B (and ATP7A) function envisage that copper is delivered to the N-terminal MBSs and is





**Figure 6** The ability of the ATP7B key domain mutants to complement *S. cerevisiae ccc2Δ*

(A) *S. cerevisiae ccc2Δ* transformed with wt or mutant *ATP7B* constructs [amino acid substitution(s) indicated on the left] were analysed for their ability to grow on iron-depleted medium (-Fe). Each yeast strain was inoculated in 5 ml of DOB-Ura and grown at 30 °C overnight to reach saturation. Serial 10-fold dilutions in sterile distilled water were made starting from 1:10 and 5  $\mu$ l of each dilution was applied on to the appropriate medium. Yeast strain viability was demonstrated by growth on iron-sufficient plates (DOB-Ura). (B) The *ccc2Δ* strains used in the copper-transport assay described above were analysed for ATP7B expression by Western-blot analysis. Total cellular protein (30  $\mu$ g) was fractionated by SDS/PAGE (7.5% polyacrylamide) and transferred to nitrocellulose. Protein was immunolabelled with ammonium sulfate-precipitated NC36 (diluted 1:1000), followed by horseradish peroxidase-conjugated donkey anti-sheep IgG (diluted 1:4000) and analysed by chemiluminescence.

then transferred to the CPC residues. This latter step is thought to be critical for initiating copper-dependent acyl-phosphorylation [4,6,7]. However, the results obtained in the present study clearly suggest an additional mechanism, namely that there is another copper-binding site(s) to induce acyl-phosphate formation in addition to the N-terminal MBS and the CPC motif. Several methionine and cysteine residues located within transmembrane regions are candidates for additional copper-binding sites, in particular the highly conserved Met<sup>1393</sup> in transmembrane domain 7, since mutation of this residue is responsible for the Wilson disease phenotype in the toxic milk mouse [41] and is important for copper translocation and acyl-phosphate formation in ATP7A [10]. However, there is currently no direct evidence that any of these residues co-ordinate copper.

Further studies are required to distinguish if the anterograde movement of the copper-ATPases is directly linked to formation of the acyl-phosphorylated intermediate or whether further downstream protein modifications are required. Possibly, a specific conformation associated with the acyl-phosphate intermediate exposes a trafficking signal or occludes a TGN retention sequence (not mutually exclusive). Vanderwerf et al. [42] have reported a correlation between kinase-dependent phosphorylation (distinct from acyl-phosphorylation) of ATP7B and its trafficking to cytosolic vesicles. These authors demonstrated that there are at least two different sites in ATP7B that are phosphorylated by unknown kinases and that elevated copper concentrations result in hyper-phosphorylation of ATP7B coinciding with vesicular redistribution [42]. Previously, we discovered that a putative

targeting motif exists within the sixth metal-binding domain of ATP7B, which directs the protein to vesicular compartments [43]. Possibly the exposure or modification (e.g. kinase-dependent phosphorylation) of this region subsequent to acyl-phosphorylation directs both copper-ATPases into the trafficking pathway and the difference in this region between both proteins is responsible for their respective post-Golgi destinations.

Previously, we used complementation of *S. cerevisiae ccc2Δ* to show that mutation of the six N-terminal MBSs inhibits the copper-translocation ability of both ATP7A and ATP7B [29,43]. Using a different assay based on copper translocation into isolated vesicles, Voskoboinik et al. [44] demonstrated that mutation of all MBSs in ATP7A reduced, but did not abolish, copper-translocation activity. This observation is consistent with the constitutive vesicular localization of the ATP7B MBS1-6c/s + TGE-AAA protein, which suggested that the MBS1-6c/s protein possessed reduced activity and this activity was detected by the introduction of the TGE-AAA mutation that trapped the acyl-phosphate intermediate at cytosolic vesicles. Thus we propose that the predominant perinuclear localization of the MBS1-6c/s and CPC-SPS mutant proteins resulted from a reduction in the rate of catalytic activation rather than complete abolition of activity. By slowing down acyl-phosphate formation, the rate of anterograde trafficking becomes less than the rate of perinuclear retrieval of trafficked protein and therefore the mutant proteins display a steady-state perinuclear localization.

Intracellular trafficking represents a switch in function for the copper-ATPases from a role in copper delivery to cuproenzymes at the TGN, to a protective role involving the transport of excess copper into vesicles (ATP7B) and across the plasma membrane (ATP7A) for excretion from the cell. The shift in the steady-state localization of the copper-ATPases from the TGN can be explained by an increase in the number of molecules that are catalytically activated (acyl-phosphorylated). By coupling trafficking with catalytic activity, this provides both autoregulation and an appropriate distribution of the copper-ATPase between the TGN and post-Golgi compartments following copper uptake [11]. When intracellular copper is raised to a certain level, the number of catalytically activated molecules is sufficient to maintain a steady-state post-Golgi subcellular localization. Trafficking allows the removal of excess copper, which results in the shift in the distribution of the copper-ATPases back to the TGN.

The findings presented in this report substantiate the fact that the intracellular trafficking of ATP7B requires acyl-phosphate formation. Importantly, formation of the acyl-phosphate intermediate was not entirely dependent on copper co-ordination by the six N-terminal MBSs or the CPC motif, although these sites clearly enhance the efficiency of the trafficking process. We have demonstrated that copper was indeed still essential for the trafficking of ATP7B and thus our data allude to the presence of an as yet unrecognized copper-binding site(s) that contributes to the efficiency of ATP7B trafficking.

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