Biometals (2010) 23:681–694
DOI 10.1007/s10534-010-9332-2

Conservation of copper-transporting P(IB)-type ATPase function

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Received: 24 January 2010 / Accepted: 25 March 2010 / Published online: 7 April 2010

Abstract Copper-transporting P(IB)-type ATPases are highly conserved, and while unicellular eukaryotes and invertebrates have only one, a gene duplication has occurred during vertebrate evolution. Copper-induced trafficking of mammalian ATP7A and ATP7B from the trans-Golgi Network towards the plasma membrane is critical for their role in copper homeostasis. In polarized epithelial cells ATP7A and ATP7B traffic towards the basolateral and apical membranes respectively. We examined the localization and function of DmATP7, the single *Drosophila melanogaster* orthologue, in cultured *D. melanogaster* and mammalian cells to explore the conservation of P(IB)-type ATPase function. Comparative genomic analysis demonstrated motifs involved in basolateral targeting and retention of ATP7A were conserved in DmATP7, whereas ATP7B targeting motifs were not. DmATP7 expression was able to correct the copper hyper-accumulation phenotype of cultured fibroblasts from a Menkes disease patient expressing a null *ATP7A* allele. DmATP7 was able to transport copper to the cupro-enzyme tyrosinase and under elevated copper conditions DmATP7 was able to traffic towards the plasma membrane and efflux copper, essentially phenocopying ATP7A. When expressed in polarized Madin-Darby Canine Kidney cells, DmATP7 translocated towards the basolateral membrane when exposed to elevated copper, similar to ATP7A. These results demonstrate DmATP7 is able to functionally compensate for the absence of ATP7A, with important trafficking motifs conserved in these distantly related orthologues.

Keywords Menkes · Wilson · DmATP7 · Copper · *Drosophila*

Abbreviations

MDCK	Madin-Darby canine kidney
PM	Plasma membrane
TGN	trans-Golgi network

Introduction

Copper-transporting P(IB)-type ATPases are highly conserved in aerobic organisms and function to deliver copper to cupro-enzymes in the secretory pathway, transport copper across epithelial and

Electronic supplementary material The online version of this article (doi:10.1007/s10534-010-9332-2) contains supplementary material, which is available to authorized users.

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endothelial cell membranes and efflux excess copper (reviewed by (Lutsenko et al. 2007; Veldhuis et al. 2009a). Interestingly, while unicellular eukaryotes and invertebrates utilize one copper-transporting P(IB)-type ATPase, a gene duplication has occurred during vertebrate evolution resulting in *ATP7A* (*MNK*) and *ATP7B* (*WND*). Orthologues of both of these genes are present in chicken and fish species suggesting the duplication occurred early in chordate evolution. The human paralogues share approximately 54 percent sequence identity but differ in their tissue expression profile. ATP7A is expressed almost ubiquitously with the exception of the liver, whereas ATP7B is predominantly expressed in the liver as well as the brain, kidney, placenta and mammary glands (La Fontaine and Mercer 2007; Lutsenko et al. 2007). Mutations in *ATP7A* are responsible for the systemic copper deficiency in Menkes disease due to copper accumulation in the gut and kidney, whereas mutations in *ATP7B* cause Wilson disease with copper toxicity due to accumulation in the liver (Menkes 1999a; Menkes 1999b).

The P(IB)-type ATPases are critical for intracellular copper distribution, as ATP7A transports copper into the lumen of organelles in the secretory pathway for use by cupro-enzymes, such as tyrosinase (Petris et al. 2000; Setty et al. 2008), while ATP7B delivers copper to apo-ceruloplasmin in hepatic cells (Terada et al. 1998). Under elevated copper conditions, ATP7A traffics from the trans-Golgi Network (TGN) towards the plasma membrane (PM) facilitating copper efflux to restore copper homeostasis (La Fontaine et al. 1998b; Petris et al. 1996). ATP7B functions similarly, translocating from the TGN to vesicles at or near the PM (Cater et al. 2006; Hung et al. 1997). In polarized epithelial cells ATP7A traffics towards the basolateral membrane (Greenough et al. 2004; Nyasae et al. 2007), consistent with systemic copper absorption. In contrast, ATP7B traffics towards the apical membrane (Braiterman et al. 2009; Guo et al. 2005), consistent with copper transport from hepatic cells into the bile for excretion.

Several motifs are required for copper-responsive trafficking of mammalian P(IB)-type ATPases. The C-terminal di-leucine motif of ATP7A (L1487L1488) is involved in endocytic retrieval and basolateral targeting, as mutating these residues to di-alanine results in constitutive plasma membrane localization of ATP7A in non-polarized cells (Petris et al. 1998) and mislocalization near the apical membrane in polarized Madin-Darby Canine Kidney (MDCK) cells (Greenough et al. 2004). In hepatic cells, the comparable tri-leucine motif of ATP7B functions similarly in endocytic retrieval (Cater et al. 2006). A Class I PDZ domain (DTAL), conserved in the C-terminus of mammalian ATP7A proteins, but absent in ATP7B, is necessary for the correct targeting and retention of ATP7A at the basolateral membrane of MDCK cells (Greenough et al. 2004). Conversely, the FAFDNVGYE motif in the N-terminus of ATP7B, absent in ATP7A, is necessary for apical targeting and TGN retention in hepatic cells (Braiterman et al. 2009; Guo et al. 2005). Kinase phosphorylation is also important for the regulation of ATP7A (Cobbold et al. 2002; Voskoboinik et al. 2003) and ATP7B (Bartee et al. 2009; Vanderwerf et al. 2001). We recently identified twenty phosphorylation sites in ATP7A, including several that are copper-responsive and some of these regulate copper-responsive trafficking of ATP7A in MDCK cells (Veldhuis et al. 2009b). Many of these residues are also conserved in mammalian ATP7B.

Drosophila melanogaster is an important model organism for the study of copper homeostasis (Balamurugan et al. 2007; Zhou et al. 2003). We identified *DmATP7* as the single orthologue of both *ATP7A* and *ATP7B* in *D. melanogaster* (Norgate et al. 2006; Southon et al. 2004). *D. melanogaster* therefore appears to represent the most complex model organism utilizing only one P(IB)-type ATPase for copper transport. DmATP7 is localized at the basolateral membrane of both the midgut and the Malpighian tubules (analogous to the mammalian kidneys) of *D. melanogaster* larvae, consistent with ATP7A-like copper transport into the circulatory system (Burke et al. 2008). Furthermore, the absence of functional DmATP7 causes reduced copper transport from the gut, decreased cupro-enzyme activity and early lethality similar to that seen in patients with classical Menkes disease (Norgate et al. 2006). Despite this phenotypic similarity between DmATP7 and ATP7A, copper-responsive trafficking of DmATP7 has not been demonstrated.

We sought to determine whether copper-responsive translocation of endogenous DmATP7 occurs in vitro in *D. melanogaster* using cultured embryonic S2 cells and larval neuronal Bm3-c2 cells. We used comparative genomics to demonstrate that membrane targeting, retention and retrieval motifs are conserved

in orthologous proteins from twelve Drosophila species. In addition, we utilized non-polarized and polarized mammalian cell lines to investigate whether copper-induced trafficking of DmATP7 occurs in these cells. We found remarkable conservation of P(IB)-type ATPase function despite hundreds of millions of years since mammals and insects shared a common ancestor, with DmATP7 able to compensate for the absence of ATP7A.

Materials and methods

Cell lines and DNA constructs

D. melanogaster S2 cells were propagated at 27°C in Schneider's Complete Media (Invitrogen, Mount Waverly, Vic, Australia) with 10% fetal calf serum (Trace Scientific, Melbourne, Vic, Australia) as previously reported (Southon et al. 2004). Full-length *DmATP7* cDNA lacking the C-terminal STOP codon (according to FlyBase annotation; The FlyBase Consortium, 2003; http://flybase.org/) was amplified from *D. melanogaster* S2 cell-derived cDNA. This was cloned into pAc3.1V5-HisA (Invitrogen) and then subcloned into pcDNA3.1 (Invitrogen) containing a Geneticin selectable marker and modified to contain an in-frame N-terminal Myc epitope tag (EQKLISEEDL) for expression in mammalian cells (pcDNA-DmATP7/Myc).

Wild-type (GM2069) and ATP7A null (Me32a) human fibroblast cells have been described previously (La Fontaine et al. 1998b). Cells were maintained at 37°C in BME (Thermo Scientific, Melbourne, Vic, Australia) supplemented with 10% fetal calf serum (Trace Scientific). Me32a cells maintaining stable overexpression of DmATP7 (Me32a-DmATP7) were generated by transfection of pcDNA-DmATP7/Myc using Fugene6 according to the manufacturer's instructions (Roche, Mannheim, Germany) and propagated in media containing 50 μg/ml Geneticin (Invitrogen) to select for cells maintaining stable expression. All fibroblasts were seeded in growth medium without additional copper one day prior to analysis.

MDCK cells were maintained at 37°C with 5% CO_2 in DMEM (Thermo Scientific) with 10% fetal calf serum (Trace Scientific) as previously reported (Greenough et al. 2004). MDCK cells maintaining stable overexpression of DmATP7 (MDCK-DmATP7) were generated by transfection of pcDNA-DmATP7/Myc using Lipofectamine2000 according to the manufacturer's instructions (Invitrogen) and propagated in media containing 50 μg/ml Geneticin (Invitrogen). MDCK cells were seeded 6 days prior to analysis to ensure polarization. All cell culture experiments were conducted in growth media with fetal calf serum and copper was added as $CuCl_2$ at the concentration specified. The copper content of basal media was 0.8 μM.

Immunocytochemistry

Cells were grown on glass coverslips and prepared as previously described (Veldhuis et al. 2009b). DmATP7 was detected in *D. melanogaster* cells with a polyclonal rabbit antibody targeted to the N-terminus of mouse ATP7B (1:400, Michalczyk et al. 2000). Rabbit pre-immune serum was used to confirm antibody specificity (data not shown). Anti-Myc (1:100, 9B10, Cell Signaling Technology, Danvers, MA, USA) was used for detection of DmATP7-Myc in mammalian cells. Mouse anti-Golgin 97 (1:200, Invitrogen) and rabbit anti-GCC88 (1:200, Luke et al. 2003) were used as TGN markers and rabbit anti-Zo1 (1:100, Invitrogen) was used as a tight junction marker in polarized MDCK cells. Anti-Tyrosinase (1:300, T311, Santa Cruz Biotechnology) was used to detect tyrosinase. Secondary antibodies were, Alexa 488 anti-mouse and Alexa 594 anti-rabbit (1:400, Invitrogen). Rhodamine conjugated phalloidin (1:100, Invitrogen) was used to detect F-actin at the cell periphery and DAPI (300 nM, Invitrogen) was used to detect the nucleus. Images were recorded at 100× magnification using an Olympus FluoView 1000 confocal microscope with Olympus FluoView ver1.6a software (Olympus, Center Valley, PA, USA). Images at each wavelength were captured sequentially and multi-color XY and XZ images were prepared using Image J (NIH). XY images are shown as maximum brightness stacked images unless otherwise specified in the figure legend. Representative images are shown.

Cell surface biotinylation and western blotting

Cell surface proteins were labeled with 0.5 mg/ml sulpho-NHS-SS-biotin (Thermo Scientific) and

precipitated with streptavidin-agarose beads (Thermo Scientific) as previously described (Pase et al. 2003). Protein samples were analyzed on NuPAGE 4–12% Bis–Tris gels (Invitrogen) and transferred to nitrocellulose membranes for western blotting. DmATP7 was detected with a polyclonal rabbit antibody targeted to the N-Terminus of human ATP7A (1:2000, Camakaris et al. 1995). Rabbit pre-immune serum was used to confirm antibody specificity (data not shown). Horseradish peroxidase coupled secondary antibodies were: rabbit anti-mouse and goat anti-rabbit (1:7000, Dako). Chemiluminescence was detected using ECL (GE Healthcare, Buckinghamshire, UK) and images were captured with a Fujifilm LAS-3000 (Fujifilm LifeScience, Stamford, Connecticut, USA).

Copper accumulation and retention assays

Copper accumulation and retention experiments were conducted as previously reported (Southon et al. 2004). Briefly, cells were seeded in a 12 well plate and incubated with approximately 0.4 MBq ^{64}Cu (Australian Radioisotopes, Lucas Heights, NSW, Australia) and 2 μM non-radioactive copper for 24 h. Cu accumulation was stopped by washing cells four times with 1 ml cold HBSS containing 1 mM histidine (Sigma-Aldrich-Aldrich, Castle Hill, NSW, Australia). For copper retention experiments cells were incubated with copper for 24 h, washed as described above, and incubated for an additional 24 h in basal media. Cells were lysed in 100 μl of 0.1% SDS, containing 2 mM EDTA. Radioactivity was measured with a γ-counter (1282 CompuGamma, LKB Wallac, Turku, Finland) and intracellular copper levels were standardized to total cellular protein, which was determined using BioRad protein reagent according to the manufacturer's instructions (BioRad, Gladesville, NSW, Australia). A one-way ANOVA with Bonferroni's post-hoc test was used for statistical analysis (Prism 4, GraphPad Software Inc, La Jolla, CA, USA).

Cell viability assays

Cell viability following copper exposure was determined colorimetrically using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) as previously described (La Fontaine et al. 1998a). The absorbance was read at 600 nM using a PowerWave XS microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA) and percentage viability determined by expressing the average absorbance from a given copper treatment relative to the average absorbance from a reference sample treated with basal media. A two-way ANOVA was used for statistical analysis (Prism 4, GraphPad Software Inc).

Tyrosinase assay

A human tyrosinase expression construct (pcDNA-hTyr, provided by Dr. Bi-Xia Ke, Deakin University, Victoria, Australia) was transfected into human fibroblasts using Fugene6 according to the manufacturer's instructions (Roche). Tyrosinase expression was confirmed by immunocytochemistry and in situ tyrosinase activity was detected by the conversion of L-DOPA to Dopachrome as described previously (Petris et al. 2000). Bright field images were recorded at 100x magnification using an Olympus FluoView 1000 confocal microscope with Olympus FluoView ver1.6a software (Olympus). Representative images are shown.

Results

Sequence conservation of mammalian and insect copper-transporting P(IB)-type ATPases

DmATP7 is the sole *D. melanogaster* P(IB)-type ATPase and shares 59 percent sequence similarity to both human ATP7A and ATP7B proteins. It has a putative di-leucine motif (L1225L1226) and ATP7A specific Class I PDZ motif (STEL) three residues upstream of the C-Terminus (Norgate et al. 2006). As human and mouse ATP7A and ATP7B are the best characterized mammalian P(IB)-type ATPases we conducted a protein sequence alignment with DmATP7 to determine whether additional trafficking motifs are conserved in this orthologue (Fig. S1). The FAFDNVGYE apical targeting motif conserved in mammalian ATB7B orthologues (Braiterman et al. 2009) is not present in the N-terminus of DmATP7 or elsewhere in the protein. The N-terminal region between metal binding domains three and four of ATP7A contains six residues recently shown to be phosphorylatable and there are an additional eight

phosphorylatable serines in the C-terminus that are likely to be functionally important (Veldhuis et al. 2009b). Serine 346 is conserved in the N-terminus of DmATP7 and C-terminal serines 1432 and 1469 are also conserved (Fig. S1).

If these residues are important for DmATP7 function we hypothesized that they would be conserved in P(IB)-type ATPase orthologues from other Drosophila species and we used comparative genomics to determine whether this was the case. Genome sequences are available for twelve Drosophila species that diverged from a common ancestor approximately 60 million years ago (Clark et al. 2007). We performed tBLASTn searches to identify an additional eleven P(IB)-type sequences from these genomes (Fig. S2A). As with *D. melanogaster* there was no evidence for duplication of these genes in these insect species. Phylogenetic analysis revealed the relationship between the Drosophila ATP7 protein sequences closely resembles that of the relationship between these species (Fig. S2B), suggesting the genes encoding these proteins were under similar selective constraints as these species evolved. The N-terminal serine corresponding to ATP7A serine 346 (Veldhuis et al. 2009b) was conserved in all twelve orthologues (Fig. S2A). Within the C-terminus the serine corresponding to ATP7A residue 1432 was completely conserved, whereas serine 1469 was not. Both the putative di-leucine and PDZ domain were conserved in all twelve orthologues. The PDZ target motif involved in basolateral targeting of ATP7A is a Class 1 PDZ target motif defined by a serine or threonine at position two and a hydrophobic residue at position four. Although usually encoded by the last four amino acids, PDZ target motifs can be more proximal (Hung and Sheng 2002) as seen with these Drosophila proteins where an additional three residues were present after the putative domain. Five serine residues were conserved in the C-terminal region and could potentially be phosphorylated similarly to those in ATP7A. The absence of apical targeting motifs in DmATP7, together with the presence of a putative Class I PDZ target motif and conservation of two residues phosphorylated in ATP7A suggests this protein more closely resembles ATP7A than ATP7B. To test this hypothesis we investigated DmATP7 localization in Drosophila and mammalian cells under basal and elevated copper conditions.

DmATP7 localization in Drosophila cells

We previously demonstrated DmATP7 expression in embryonic *D. melanogaster* S2 cells where it functions to efflux excess copper (Southon et al. 2004). In order to determine whether DmATP7 undergoes copper-responsive trafficking, S2 cells were cultured in basal media or media supplemented with 0.8 mM copper for 2.5 h. This is the highest concentration of copper tolerated by these cells over 48 h (Southon et al. 2004). DmATP7 localization was examined using immunocytochemistry (Fig. 1a–f) and cell surface biotinylation (Fig. 1g). Under basal conditions, DmATP7 was localized to the TGN and partially co-localized with Golgin 97 (Fig. 1a–c). The Golgi apparatus of S2 cells is more dispersed than that of mammalian cells and the distribution of Golgin 97 is consistent with that previously reported for this TGN protein in these cells (Sinka et al. 2008). The localization of DmATP7 was unaffected by elevated copper, remaining at the TGN (Fig. 1d–f). To more accurately determine whether any DmATP7 was present at the PM we used biotinylation to detect proteins at the cell surface (Fig. 1g). DmATP7 was predominantly present in the non-biotinylated intracellular fraction, but was also detected in the biotinylated cell surface fraction. The amount of DmATP7 at the PM was not increased in response to elevated copper. Taken together, these studies demonstrate that there does not appear to be a detectable shift in the localization of DmATP7 from the TGN to the PM under elevated copper conditions in S2 cells. To determine whether this result was specific to these cells, we repeated the above experiments in *D. melanogaster* Bm3-c2 cells, a larval neuronal cell line (Ui et al. 1994). As with S2 cells, immunocytochemistry demonstrated DmATP7 was localized to the TGN under both basal and excess copper conditions (Fig. S3A–F), with a small proportion detected at the PM by cell surface biotinylation (Fig. S3G). There was no evidence for copper dependent translocation of DmATP7 in cultured Drosophila cells. The presence of DmATP7 at both the TGN and the PM could presumably be due to constitutive recycling between these compartments or the result of two discrete pools of this protein. In either case, the conserved motifs and residues important for mammalian P(IB)-type ATPases trafficking may be important for distribution of DmATP7 in specific cell

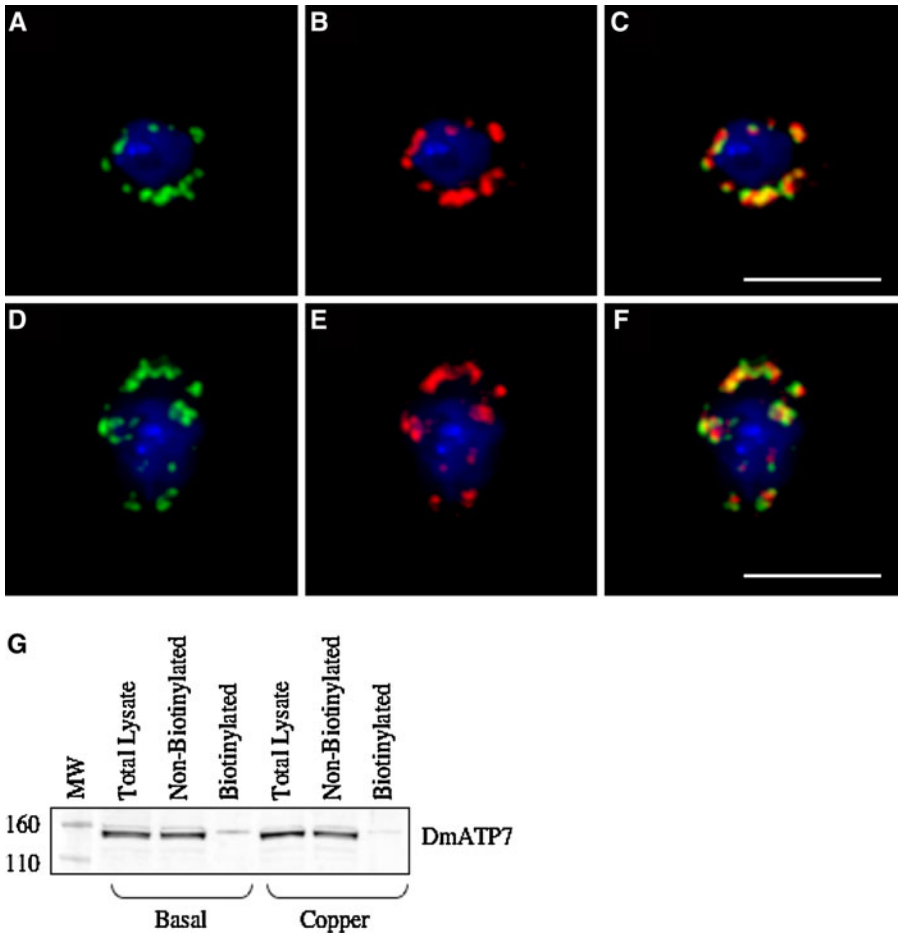


Fig. 1 DmATP7 does not undergo copper-responsive trafficking in S2 cells. **a**–**e** Immunocytochemical detection of DmATP7 in S2 cells exposed to basal media (**a**–**c**) or media supplemented with 0.8 mM copper for 2.5 h (**d**–**f**). DmATP7 was detected with anti-ATP7B (*Green*: **a**, **d**). Anti-Golgin 97 was used to detect the TGN (*Red*: **b**, **e**) and DAPI was used to detect the nucleus (*Blue*). Merged images are also shown (**c**, **f**). Scale bar = 10 μm. DmATP7 partially co-localized with Golgin 97 at the TGN under both basal and elevated copper conditions. **g** Western blot detection of DmATP7 in S2 cells exposed to basal media or media supplemented with 0.8 mM copper for 2.5 h. Biotin was used to label cell surface proteins as described in the "Materials and methods" section. Biotinylated samples represent protein at the cell surface whereas non-biotinylated samples represent intracellular protein, with total lysate also shown. DmATP7 was detected with anti-ATP7A in the biotinylated sample demonstrating that this protein is present at the PM, under both basal and elevated copper conditions. Similar results were seen with immunocytochemical and biotinylation experiments in *D. melanogaster* Bm3-c2 cells (Supplementary Fig. 3)

types or developmental stages. We therefore sought to determine whether DmATP7 contains sufficient similarity to ATP7A to translocate and function in mammalian cells.

DmATP7 localization, trafficking and function in mammalian cells

Me32a human fibroblast cells derived from a patient with classical Menkes disease hyper-accumulate copper due to a null *ATP7A* allele. We have previously demonstrated that expression of ATP7A corrects this phenotype (La Fontaine et al. 1998b). These cells provide a unique system to examine the localization and function of orthologous P(IB)-type ATPases. DmATP7 containing an N-terminal Myc epitope tag was cloned and expressed in these ATP7A null cells.

We first used immunocytochemistry to determine whether DmATP7 can undergo copper-responsive

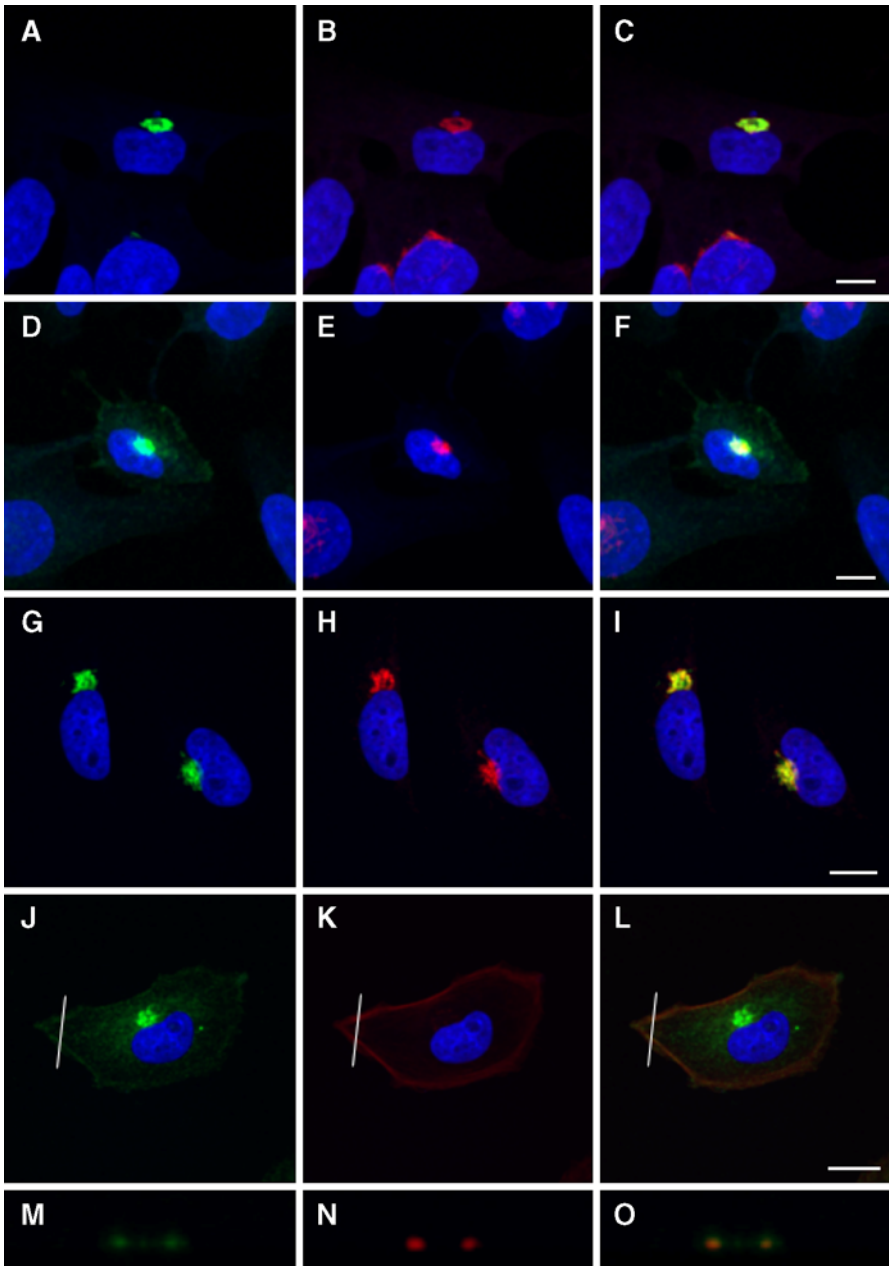


Fig. 2 DmATP7 undergoes copper-responsive trafficking when expressed in human fibroblast cells. Me32A-DmATP7 cells were exposed to basal media (**a**–**c**), media supplemented with 189 μM copper for 2.5 h (**d**–**f**) or media with 189 μM copper for 2.5 h followed by basal media for 2.5 h (**g**–**i**). Anti-Myc was used to detect DmATP7 (*Green*: **a**, **d**, **g**). Anti-GCC88 was used to detect the TGN (*Red*: **b**, **e**, **h**) and DAPI was used to detect the nucleus (*Blue*). Merged images are also shown (**c**, **f**, **i**). Scale bar = 10 μm. Under basal conditions DmATP7 was localized to the TGN (**a**, **c**), whereas copper exposure induced translocation of a discernable proportion of DmATP7 towards the PM (**d**, **f**). When returned to basal media following copper exposure DmATP7 returned to the TGN (**g**, **i**). To more clearly demonstrate PM localization additional cells were exposed to media supplemented with 189 μM copper for 2.5 h (**j**–**o**) and phalloidin was used to detect the cell periphery (*Red*: **k**, **n**). X–Z cross sections are shown (**m**–**o**) with the corresponding X–Y position indicated with white lines. Partial co-localization of DmATP7 with phalloidin can be seen in the merged images (**l**, **o**), demonstrating copper induces translocation of DmATP7 to the cell surface

translocation. Me32a-DmATP7 cells were cultured in basal media (Fig. 2a–c), media supplemented with 189 μM copper for 2.5 h (Fig. 2d–f), or media with189 μM copper for 2.5 h followed by basal media for an additional 2.5 h (Fig. 2g–i). Under basal conditions DmATP7 co-localized with GCC88 at the TGN, whereas addition of excess copper caused a discernable proportion of DmATP7 to traffic towards the PM. This translocation was reversible, as returning copper treated cells to basal media caused DmATP7 to recycle to the TGN. To better define the localization of DmATP7 under elevated copper conditions we used the peripheral cytoskeletal marker phalloidin (Fig. 2j–o). DmATP7 partially co-localized with phalloidin at the cell periphery as shown in the X–Y (Fig. 2j–l), and more clearly in the X–Z (Fig. 2m–o), dimensions of these cells. These results demonstrate DmATP7 is sufficiently similar to human ATP7A to be recognized by the trafficking machinery of these mammalian cells and translocate to the PM in response to elevated copper.

We next sought to determine whether DmATP7 could rescue the copper hyper-accumulation phenotype of Me32a cells by assaying copper accumulation and retention, copper tolerance, and cupro-enzyme activity. Wild-type human fibroblast cells (GM2069) and ATP7A null cells (Me32a) were used as positive and negative controls respectively.

Copper accumulation was measured in cells cultured in media supplemented with 2 μM copper for 24 h (Fig. 3a). Absence of functional ATP7A caused Me32a cells to accumulate significantly more copper than wild-type GM2069 cells, as previously reported (La Fontaine et al. 1998b). Me32a-DmATP7 cells accumulated significantly less copper than Me32a. This was not statistically different to GM2069, demonstrating expression of DmATP7 was able to rescue the copper hyper-accumulation phenotype of ATP7A null cells. We investigated copper turnover with cells exposed to 2 μM copper for 24 h and copper retention was measured following an additional 24 h in basal media. As previously reported (La Fontaine et al. 1998b), Me32a cells retained most of the copper they had accumulated, consistent with the Menkes disease phenotype. In contrast both GM2069 and Me32a-DmATP7 cells retained significantly less copper than Me32a cells. To assess copper tolerance, cells were exposed to excess copper

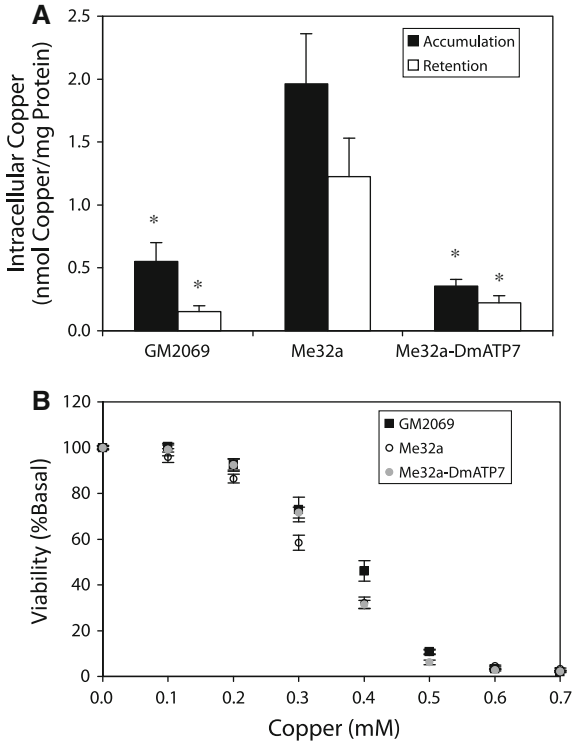


Fig. 3 DmATP7 expression restores copper homeostasis to ATP7A null human fibroblast cells. Copper accumulation and retention (**a**) and copper tolerance (**b**) was examined in wild-type fibroblast cells (GM2069), ATP7A null fibroblasts (Me32a) and Me32a cells expressing DmATP7 (Me32a-DmATP7). **a** Intracellular copper accumulation was measured in cells exposed to 2 μM copper for 24 h. Retention was measured following 24 h copper exposure with an additional 24 h exposure to basal media. Values are expressed as mean ± S.E.M. of twelve replicates from four independent experiments. A one-way ANOVA with Bonferroni's post hoc test was used to determine statistically significant differences, * $P < 0.05$ compared to Me32a. Absence of ATP7A caused Me32a cells to accumulate significantly more copper than GM2069 cells. Me32a-DmATP7 cells accumulated significantly less copper than Me32a cells and were not different to GM2069. Copper retention remained significantly lower in GM2069 and Me32a-DmATP7 cells compared to Me32a. DmATP7 expression therefore rescues the copper hyper-accumulation phenotype of ATP7A null Me32a cells. **b** Cells were exposed to 0.1–0.8 mM excess copper for 48 h to determine copper tolerance. Data is expressed as a percentage of the viability of cells exposed to basal media and values are mean ± S.E.M. of nine replicates from three independent experiments. A two-way ANOVA found Me32a cells were significantly less tolerant to copper than GM2069 cells ($P < 0.001$). Me32a-DmATP7 were significantly more copper tolerant than Me32a cells ($P < 0.001$), but less tolerant than GM2069 ($P < 0.001$). DmATP7 expression therefore partially rescued the copper sensitivity phenotype of ATP7A null Me32a cells

for 48 h and cell viability was determined (Fig. 3b). As expected, Me32a cells were significantly more sensitive to copper than GM2069 cells. In contrast, Me32a-DmATP7 cells were significantly more copper tolerant than Me32a but less tolerant than GM2069 cells. Thus expression of DmATP7 partially rescued the copper sensitivity of ATP7A null Me32a cells.

The cupro-enzyme tyrosinase has previously been used to demonstrate that ATP7A transports copper into the lumen of the secretory pathway of human fibroblast cells (Petris et al. 1998). A human tyrosinase construct was transiently transfected into GM2069, Me32a and Me32a-DmATP7 cells (Fig. 4). Tyrosinase expression was confirmed with immunocytochemistry showing strong staining in each cell line (Fig. 4a–c), consistent with the secretory pathway localization previously reported in human fibroblasts (Petris et al. 1998). In situ tyrosinase activity was determined from the conversion of L-DOPA to the brown Dopachrome. Tyrosinase activity was not detected in Me32a cells (Fig. 4e), whereas strong activity was observed in GM2069 (Fig. 4d) and Me32a-DmATP7 cells (Fig. 4f), demonstrating ATP7A and DmATP7 were both able to transport copper into the lumen of organelles in the secretory pathway for incorporation into this cupro-enzyme.

Taken together these results show that DmATP7 essentially phenocopies ATP7A when expressed in cultured human fibroblasts. Polarized MDCK cells have previously been used to demonstrate that ATP7A translocates towards the basolateral membrane when stimulated by copper (Greenough et al. 2004). We examined DmATP7 localization in these cells to determine whether DmATP7 traffics in response to elevated copper in polarized epithelial cells, and if so towards which membrane domain.

DmATP7 was expressed in wild-type cells (MDCK-DmATP7) and localization was examined using immunocytochemistry. Cells were cultured in basal media (Fig. 2a–c), media supplemented with 189 μM copper for 2.5 h (Fig. 2d–f), or media with 189 μM copper for 2.5 h followed by basal media for an additional 2.5 h (Fig. 2g–i). Under basal conditions DmATP7 co-localized with GCC88 at the TGN, whereas exposure to elevated copper caused a discernable proportion of DmATP7 to traffic towards

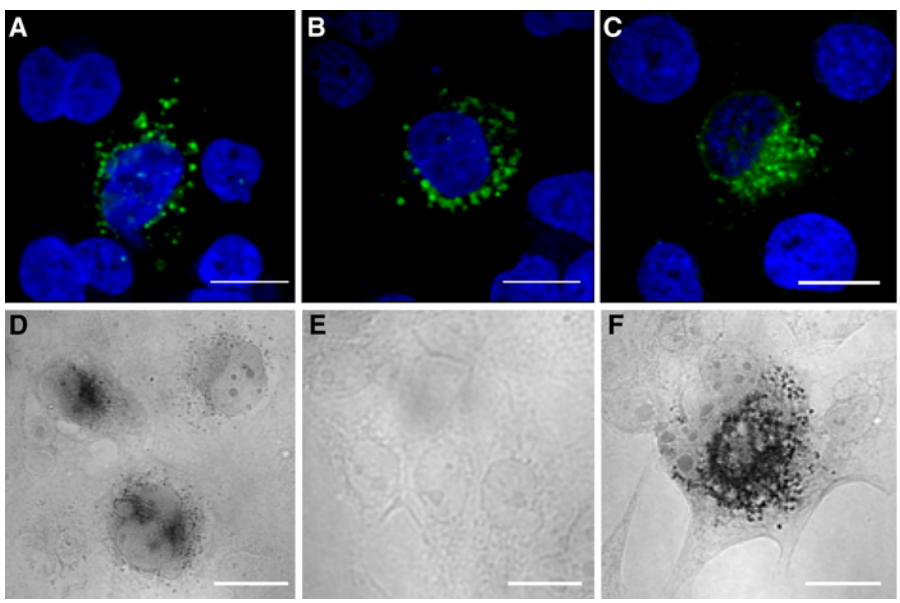


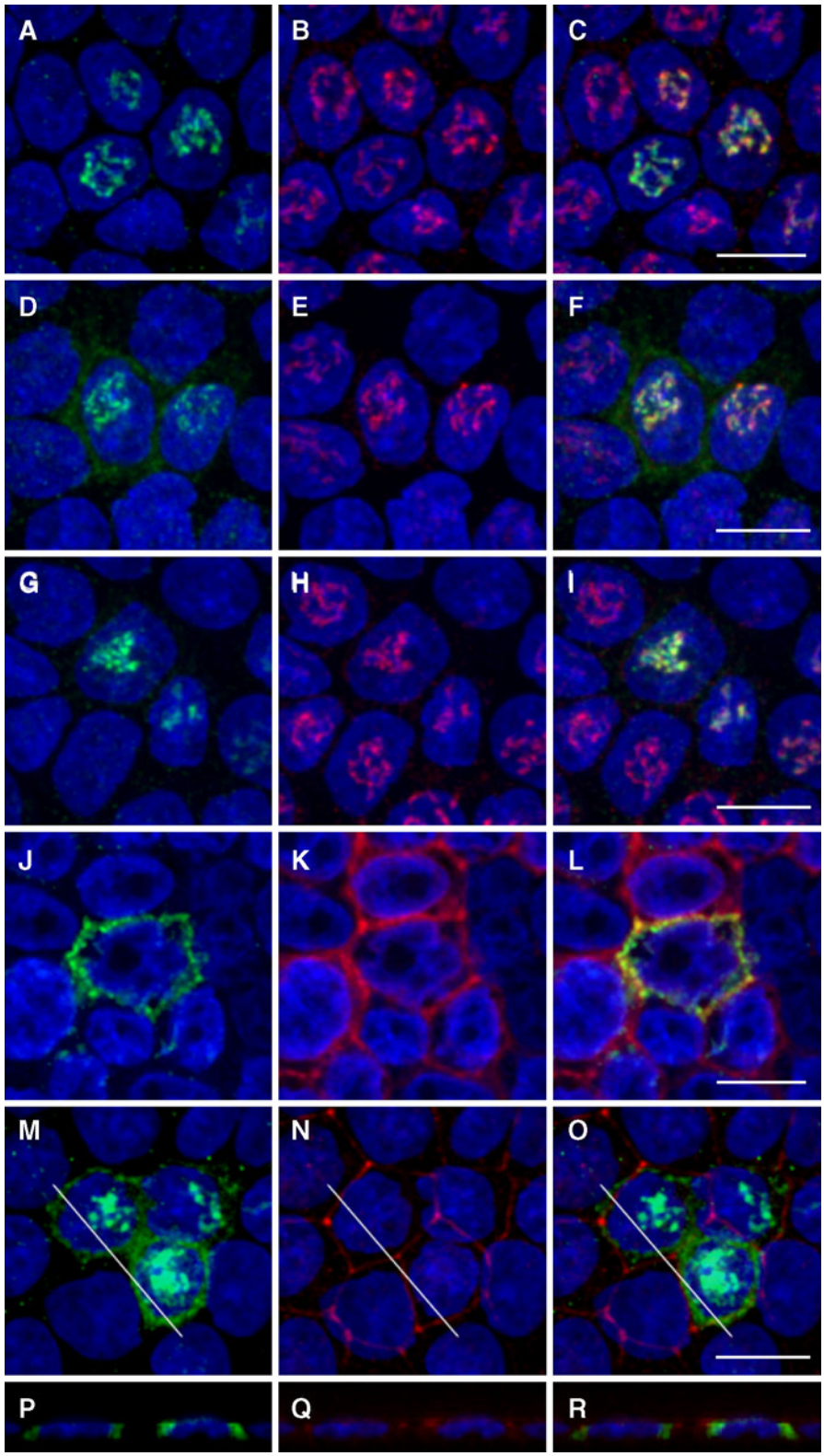
Fig. 4 DmATP7 transports copper to tyrosinase when expressed in human fibroblast cells. Tyrosinase was transfected into GM2069 (**a**, **d**), Me32a (**b**, **e**) and Me32a-DmATP7 (**c**, **f**) cells. Tyrosinase expression (**a**–**c**) was detected with anti-tyrosinase (*Green*) and DAPI was used to detect the nucleus (*Blue*). Tyrosinase activity (**d**–**f**) was measured in situ by the conversion of L-DOPA to Dopachrome. Scale bar = 10 μm. Tyrosinase activity was detected in GM2069 (**d**) and Me32a-DmATP7 cells (**f**), but not Me32a (**e**) cells, demonstrating DmATP7 expression restores tyrosinase activity in ATP7A null Me32a cells

the basolateral membrane. This translocation was reversible, as returning copper exposed cells to basal media caused DmATP7 to recycle to the TGN. To better define the localization of DmATP7 under copper excess conditions we again used the peripheral cytoskeletal marker phalloidin, and partial co-localization can be seen at the cell periphery (Fig. 5j–l). To determine more specifically where copper-stimulated DmATP7 is localized we used the tight junction marker Zo-1 to identify the boundary between the apical and basolateral membranes (Fig. 5m–r). Zo-1 can clearly be seen in the X–Y dimension (Fig. 5m–o) indicating that these cells are polarized. The images in the X–Z dimension (Fig. 5p–r) show DmATP7 to be localized to the lateral membrane of these cells, below the tight junctions. The absence of apical DmATP7 above Zo-1 suggests copper-induced translocation of DmATP7 is restricted to the basolateral membrane of MDCK cells. As with human fibroblasts, these results demonstrate that DmATP7 is sufficiently similar to ATP7A to be recognized by the trafficking machinery of MDCK cells, suggesting that the putative C-terminal di-leucine and PDZ domains are likely to be functional in this system and one or more of the conserved phosphorylatable serines involved in trafficking of ATP7A (Veldhuis et al. 2009b) may function similarly.

Fig. 5 DmATP7 undergoes copper-responsive trafficking when expressed in polarized MDCK cells. MDCK-DmATP7 cells were exposed to basal media (**a**–**c**), media supplemented with 189 μM copper for 2.5 h (**d**–**f**) or media with 189 μM copper for 2.5 h followed by basal media for 2.5 h (**g**–**i**). Anti-Myc was used to detect DmATP7 (*Green*: **a**, **d**, **g**). Anti-GCC88 was used to detect the TGN (*Red*: **b**, **e**, **h**) and DAPI was used to detect the nucleus (*Blue*). Merged images are also shown (**c**, **f**, **i**). Scale bar = 10 μm. Under basal conditions DmATP7 was localized to the TGN (**a**, **c**), whereas copper exposure induced translocation of a proportion of DmATP7 towards the basolateral membrane (**d**, **f**). When returned to basal media following copper exposure DmATP7 returned to the TGN (**g**, **i**). To more clearly demonstrate basolateral membrane localization additional cells were exposed to media supplemented with 189 μM copper for 2.5 h (**j**–**l**) with single confocal slices shown. Phalloidin was used to detect the cell periphery (*Red*: **k**) and partial co-localization with DmATP7 can be seen in the merged images (**l**), demonstrating copper induces translocation of DmATP7 to the lateral cell surface. To demonstrate DmATP7 does not translocate to the apical membrane additional cells were exposed to media supplemented with 189 μM copper for 2.5 h (**m**–**r**) and Zo-1 was used to detect the tight junctions between the apical and basolateral membranes (*Red*: **n**, **q**). X–Z cross sections are shown (**p**–**r**) with the corresponding X–Y position indicated with white lines. Merged images are also shown (**o**, **r**). DmATP7 was localized to the lateral membrane and could not be detected above Zo-1 demonstrating copper does not induce translocation of DmATP7 to the apical cell surface

Discussion

The emergence of *D. melanogaster* as a model organism for the study of copper homeostasis has revealed remarkable conservation of copper homeostasis mechanisms in this species when compared with mammals (Balamurugan et al. 2007; Norgate et al. 2006; Zhou et al. 2003). DmATP7 is expressed in all *D. melanogaster* tissues tested to date (Chintapalli et al. 2007), transports copper to cuproenzymes in the secretory pathway, and is responsible for copper transport across the basolateral membrane of the midgut, thereby delivering dietary copper into the circulation. DmATP7 therefore fulfils a similar role to that of ATP7A in mammals, yet appears to do so without the need to undergo copper-induced trafficking. Nevertheless, the localization of DmATP7 does change at different developmental stages (Norgate et al. 2006). During embryogenesis endogenous DmATP7 is localized to the PM, and this localization becomes progressively more cytoplasmic as the embryo develops. Expression of FLAG-tagged DmATP7 could be seen at either the PM or dispersed throughout the cytoplasm of the larval midgut (Norgate et al. 2006). Localization motifs may be active in different cells or during different developmental stages. The results presented here provide additional evidence that the localization of endogenous DmATP7 is not copper dependent in cultured *D. melanogaster* cells, with this protein predominantly restricted to the TGN of embryonic S2 cells and larval Bm3-c2 cells with very little detected at the PM. In these cells a subset of DmATP7 may constitutively cycle between these compartments. This is consistent with the in vivo expression patterns described above and constitutive trafficking has been reported for ATP7A under basal conditions in a variety of mammalian cells (Cobbold et al. 2002; Greenough et al. 2004; Petris and Mercer 1999). Alternatively, DmATP7 may load copper into vesicles destined for the PM, rather than completely translocating to the PM similarly to that proposed for ATP7A (Nyasae et al. 2007) and ATP7B (Lutsenko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et al. 2007). Although copper dependent translocation of DmATP7 has not been seen in *D. melanogaster* or the two cultured cell lines tested in the present study, we cannot rule out the possibility that this occurs in other cell types or under different conditions.

Both cell-specific trafficking mechanisms and DmATP7-specific sequences are likely to regulate the localization of this protein. The C-terminal di-leucine, conserved in all twelve Drosophila orthologues, is an obvious candidate for regulating the TGN localization of DmATP7 given the C-terminal di-leucine of ATP7A and corresponding tri-leucine of ATP7B are necessary for endocytosis and retention of these proteins at the TGN. Basolateral targeting of DmAPT7 was seen in larval midgut and Malpighian tubules (Burke et al. 2008) indicating the conserved di-leucine and Class I PDZ target motif may regulate this membrane-specific targeting of DmATP7. Phosphorylation of the C-terminal serine 1432 was also required for basolateral trafficking of ATP7A in MDCK cells (Veldhuis et al. 2009b), suggesting this conserved residue may function similarly in DmATP7. Future studies involving mutating these residues will be required to determine whether they regulate localization of this protein in *D. melanogaster*.

The FAFDNVGYE apical targeting motif is conserved in diverse ATP7B orthologues (Braiterman et al. 2009), but is not present in DmATP7. It is possible that insects such as *D. melanogaster* do not need a P(IB)-type ATPase to localize to an apical membrane. In mammals the apical targeting of ATP7B is critical in placental and mammary tissues as well as the liver for copper transport into the bile for excretion (reviewed by (Lutsenko et al. 2007; Veldhuis et al. 2009a). Insects do not have these mammalian reproductive tissues, while the fat body (Sondergaard 1993) and oenocytes (Gutierrez et al. 2007) are the closest equivalent to the liver However, there is no evidence that either of these tissues remove excess copper from *D. melanogaster*. Both larvae and adult flies accumulate excess copper yet are highly copper tolerant (Balamurugan et al. 2007; Norgate et al. 2006), suggesting copper sequestration is an important detoxification mechanism in this organism.

Although copper-induced translocation of DmATP7 was not observed in cultured *D. melanogaster* cells, the localization of DmATP7 was copper responsive when expressed in mammalian cells and DmATP7 was able to rescue the copper hyper-accumulation of ATP7A null human fibroblasts. To the best of our knowledge this is the first report of a non-mammalian P(IB)-type ATPases trafficking in mammalian cells and demonstrates a remarkable level of conservation between these proteins, given mammals and insects shared a common ancestor over 600 million years ago (Doolittle et al. 1996). The zebrafish ATP7A orthologue is also able to induce tyrosinase activity in ATP7A null fibroblasts, however copper induced trafficking was not examined (Madsen and Gitlin 2008). Our results demonstrate functionally important regulatory motifs of ATP7A are sufficiently conserved in DmATP7 to be recognized by these cultured fibroblasts, allowing DmATP7 to transport copper to the secretory pathway and across the PM.

The copper-induced translocation of DmATP7 was very similar to that reported for ATP7A in cultured human fibroblasts (La Fontaine et al. 1998b) and human ATP7A expressed in polarized MDCK cells (Greenough et al. 2004; Veldhuis et al. 2009b). In both cell types copper-induced DmATP7 was able to recycle from the PM to the TGN when cells were returned to basal media, suggesting membrane specific targeting, retention and retrieval motifs are conserved. In contrast, human ATP7B underwent copper-induced trafficking towards the apical membrane when expressed in MDCK cells (Barnes et al. 2009). We found that a pool of DmAPT7 remained at the TGN when cells were incubated in elevated copper, similar to previous studies expressing human ATP7A in these cells (La Fontaine et al. 1998b; Veldhuis et al. 2009b). Biotinylation studies with endogenous ATP7A in CHO cells demonstrated that only a subset of this protein is able to translocate to the PM with the remainder retained at the TGN (Pase et al. 2003), consistent with our DmATP7 trafficking results in mammalian cells.

Although, the overall sequence similarity shared between mammalian and Drosophila P(IB)-type ATPases is comparable, functionally important trafficking motifs are shared between ATP7A and DmATP7, whereas ATP7B specific motifs are not. The localization studies presented here also demonstrate greater functional similarity between DmATP7 and ATP7A, suggesting the single ancestral P(IB)-type ATPase may have been more like these proteins than ATP7B. The emergence of genome sequences from more distantly related chordates should

facilitate a more accurate prediction of when in evolutionary time the ancestral *ATP7A* and *ATP7B* gene duplication occurred. Although speculative, the gene duplication may have coincided with the evolution of a liver-like organ in an early chordate, leading to the evolution of this ATP7B-like orthologue developing a restricted distribution and copper excretion function.

In summary, DmATP7 is localized to the TGN and PM in cultured *D. melanogaster* cells under both basal and elevated copper conditions, yet is able to undergo copper-responsive translocation when expressed in mammalian cells and also functionally compensates for the absence of ATP7A. Putative membrane specific targeting, retention and retrieval motifs are conserved in these distantly related Drosophila orthologues, suggesting ATP7A may more closely resemble ancestral P(IB)-type ATPases than the paralogue ATP7B.

Acknowledgements We gratefully acknowledge Dr. Sharon La Fontaine for providing the GM2069 and Me32a cell lines, Dr. Bi-Xia Ke for the pcDNA-hTyr construct and Prof. Paul Gleeson for the GCC88 antibody. We thank Mark Greenough for constructive comments. The Australian Research Council and the Australian Institute of Nuclear Science and Engineering provided grants. The J.C Rowden White Trust provided a grant for partial funding of the Olympus FluoView 1000 confocal microscope. Adam Southon is the recipient of a PhD scholarship from the National Health and Medical Research Council of Australia and the 2009 Dawson Bursary.

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