Title. Basolateral sorting and transcytosis define the Cu⁺-regulated translocation of ATP7B to the bile canaliculus

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Summary Statement. Excess Cu⁺ induces the transport of ATP7B from the TGN to the membrane of the bile canaliculus via basolateral sorting and transcytosis for biliary Cu⁺ excretion without lysosomal involvement.
ABSTRACT

The Cu⁺ pump ATP7B plays an irreplaceable role in the elimination of excess Cu⁺ by the hepatocyte into the bile. The traffic and site of ATP7B action is a subject of controversy. One current proposal is that an increase in intracellular Cu⁺ results in the translocation of ATP7B to the lysosomes and excretion of excess Cu⁺ by lysosomal mediated exocytosis at the bile canaliculus. Here we show that ATP7B is transported from the trans-Golgi network to the bile canaliculus by basolateral sorting and endocytosis, and microtubule mediated transcytosis through the subapical compartment. Trafficking ATP7B is not incorporated into lysosomes and addition of Cu⁺ does not cause relocalization of lysosomes and the appearance of lysosome markers in the bile canaliculus. Our data describes the pathway of the Cu⁺-mediated transport of ATP7B from the TGN to the bile canaliculus and indicates that the bile canaliculus is the prime site of ATP7B action in the elimination of excess Cu⁺.
INTRODUCTION

The redox properties of Cu\(^+\) endow the metal with the simultaneous ability to be both essential and potentially damaging to the cell. Thus, as the cell only needs Cu\(^+\) in trace amounts, to counter the relative high levels of Cu\(^+\) in the environment and food, animals have developed sophisticated mechanisms to chaperone, store and eliminate excess Cu\(^+\). In the liver, the hepatocyte acts as the major captor of the Cu\(^+\) absorbed in the digestive tract, the main reservoir and distributor of Cu\(^+\) to other tissues and organs as well as the key mediator of Cu\(^+\) elimination by excretion in the bile (Harris, 2000; Lalioti et al., 2009). Because of these three functionalities the liver plays an essential role in Cu\(^+\) homeostasis in mammals, birds and reptiles.

In the hepatocyte, the elimination of Cu\(^+\) is mediated by ATP7B, a membrane pump specialized for the vectorial transport of Cu\(^+\) (Bull et al., 1993; Tanzi et al., 1993). At physiological concentrations of Cu\(^+\), ATP7B is localized to the trans-part of the Golgi apparatus where it functions in the transfer of Cu\(^+\) to secretory proteins such as ceruloplasmin. Increased Cu\(^+\) levels in the hepatocyte results in the release of the ATP7B sequestered in the trans-Golgi network (TGN) and its relocation to other membrane compartments. However, though the direct relocalization of ATP7B to the bile canaliculus (BC) would appear to be the most likely trafficking route (Guo et al., 2005; Hernandez et al., 2008; Roelofsen et al., 2000b), studies on the Cu\(^+\)-induced relocation of ATP7B have resulted in identification of other membrane targets, including late endosomes and lysosomes (Harada et al., 2003a; Polishchuk et al., 2014; Weiss et al., 2008). In addition a stable pool of ATP7B has been localized to the tight junctions (Hernandez et al., 2008). The detection of Cu\(^+\) within lysosomes carrying ATP7B in their membranes and the increase traffic of ATP7B to the BC upon activation of lysosomal exocytosis, together with the recent report that brief exposure to Cu\(^+\) activates lysosomal exocytosis, has led to the proposal that ATP7B pumps excess Cu\(^+\) into lysosomes and the lysosomes loaded with Cu\(^+\) then fuse with the membrane of the bile canaliculus (Polishchuk et
al., 2014). These observations are in contrast with studies indicating that ATP7B is transported from the Golgi to the BC and recent evidence indicating that transport to the BC occurs through a trans-endosomal pathway (Lalioti et al., 2014; Nyasae et al., 2014).

While more than 300 mutations in the ATP7B gene affecting the function and cellular distribution of ATP7B have been associated with the Wilson disease, a rare autosomal Cu⁺ toxicosis (Kenney and Cox, 2007), the tight association between the traffic and function of ATP7B in Cu⁺ donation and elimination makes it likely that some Cu⁺ metabolism abnormalities could be caused by the dysfunction of the mechanisms of membrane protein traffic and their regulation, dysfunction that may also affect the traffic of other proteins. With regard to this, it is interesting that mutation of the AP1S1 gene, which codes for the σ₁A subunit of the clathrin adaptor AP-1 involved in the intracellular trafficking of Cu⁺ transporters ATP7B and ATP7A (Holloway et al., 2013; Jain et al., 2015; Lalioti et al., 2014), causes the MEDNIK syndrome described as a new disorder of copper metabolism (Martinelli et al., 2013). Furthermore, deficiency of the COMMD1, a protein component of the CCC complex that plays an important role in the trafficking of retromer cargo, results in defective Cu⁺ homeostasis (Phillips-Krawczak et al., 2015; van De Sluis et al., 2002).

It is thus important to characterize the traffic pathway of ATP7B in the hepatocyte and the site of ATP7B action, not only to enable the identification of the genetic defects associated with Cu⁺ toxicosis, but also to understand the presentation and course of those disorders, and to design specific strategies to prevent and treat them.

We describe here the characterization of the Cu⁺-dependent pathway of ATP7B translocation from the TGN to the BC in the polarized rat hepatoma Can 10 cell. Our results indicate that on Cu⁺-induced release from the trans-Golgi network, ATP7B is initially sorted to the basolateral membrane and then transported by transcytosis to the BC via a route that includes the subapical compartment and excludes the lysosomes.
RESULTS

Polarized hepatoma Can 10 cells use the inwards-positioned apical surface to assemble BC of different sizes and, when growing in small islets, arrange their bodies perpendicular to the BC and project their large basolateral surface outwards (Fig. S1A-D) (Cassio et al., 2007; Hernandez et al., 2008), this arrangement greatly facilitates to monitoring the changes in the distribution of ATP7B in response to increased Cu⁺ levels in the cell.

ATP7B IS BASOLATERAL SORTED at the TGN by ACIDIC VESICLES.

Careful titration of the Cu⁺ dose/ATP7B response in Can 10 cells showed that additions of Cu⁺ >1 µM to the cell medium caused the mobilization of the ATP7B retained in the TGN at low Cu⁺ concentrations. To study the pathway of ATP7B translocation from the TGN to the BC we used 40 µM Cu⁺, concentration that caused the fast release of ATP7B from the TGN and a traffic rate suitable to monitor the redistribution of ATP7B and to characterize the pathway of transport.

ATP7B began to leave the TGN within 5 min after addition of Cu⁺ and the release was completed in 15-20 min (Fig. 1A-C). The acidic pH of the TGN led us to study if increase in the pH affected the ATP7B release. To this end, we studied the release of ATP7B from the TGN in Can 10 cells preincubated for 3h with 50 µM chloroquine (CQ), an acidotropic agent that accumulates in acidic organelles, producing the loss of their acidity and their osmotic swelling. The study revealed that 15 min after the addition of Cu⁺ many of the vesicles transporting ATP7B out of the Golgi remained attached to the TGN and were swollen, hence indicating that the acidity of the TGN was critical for the release of the vesicles loaded with ATP7B (Fig. 1D).

Since V-type H⁺ ATPases inhibitor are essential for organelle acidification and deacidification by specific inhibitors blocks TGN to plasma membrane delivery (Yilla et al., 1993), we next studied the effect of bafilomycin A₁ (BF) on the release of ATP7B from the TGN. We found in cells treated for 3h with 100 nM BF, and then challenged for 15 and 25 min with Cu⁺, that the
drug caused the segregation of a grape-looking cisterna loaded with ATP7B from the cisterna hosting the TGN marker TGN38 (Figs. 1E-G) before its breaking into clusters of EEA1 positive-endosomes (Fig. 1H) (see Discussion). While the rapid incorporation of the marker of early endosomes EEA1 into the vesicle clusters originated from the Golgi has been already observed in cells transfected with ATP7B mutants (Lalioti et al., 2014) it might explain the localization of ATP7B in clusters of basolateral endosomes in cells treated with BF (Nyasae et al., 2014) (see Discussion).

Remarkably, much of the ATP7B released from the TGN in cells incubated for 5-10 min with Cu⁺ was found in vesicle-like structures hoarded at the far end of the basolateral domain, opposite to the apical BC (Figs. 2A, B₁, B₂). Besides, many of those vesicles retained the EEA1 marker (Fig. S1E-G). Next, to study the presence of ATP7B in basolateral endosomes we incubated the cells for 12 min with Cu⁺ and the last 8 min of the incubation with the marker of basolateral endocytosis Alexa 647-transferrin (Tf). The detection of Tf in vesicles loaded with ATP7B at the far end of the basolateral domain, opposite to the BC, was consistent with the step of ATP7B through basolateral endosomes after its release from the TGN (Fig. 2C₁-C₃; Table S1).

**BASOLATERAL SORTED ATP7B is not INCORPORATED into LYSOSOMES.**

The acidity of the vesicles loaded with ATP7B that pinch-off from the TGN and the reported ability of Cu⁺ to induce the direct and fast incorporation of ATP7B into late endosomes/lysosomes (Polishchuk et al., 2014), prompted us to examine those vesicles and the incorporation of ATP7B into lysosomes during the ATP7B relocation to basolateral endosomes. To this end and to minimize the chances that ATP7B would be incorporated into a discrete/small population of lysosomes, causing the event to pass unnoticed, we separately compared the distributions of ATP7B and four lysosomal integral membrane proteins (LIMPs), CD63, LIMPII, LAMP1 and LAMP2, which are unevenly distributed among late endosomes,
plasma membrane, specialized phagosomes and secretory vesicles (Figs. 3A) (Barriocanal et al., 1986; Metzelaar et al., 1991). Two of these proteins, CD63 and LAMP1, have been used in the study that described the Cu⁺-induced incorporation of ATP7B into lysosomes of HepG2 cells (Polishchuk et al., 2014).

The possible incorporation of ATP7B into lysosomes and LIMPs into ATP7B vesicular carriers was next studied by comparing separately the distributions of ATP7B and the LIMPs CD63, LIMP II and LAMP2 in cells that were treated for 15 min with Cu⁺ to have the bulk of ATP7B moving through the basolateral domain (Figs. 3B, C1, D1, E1). Under these conditions the average ATP7B/LIMPs coincidence ratio in ATP7B vesicles and lysosomes was never higher than 0.0206/1 (Table S2). Furthermore, subjection of pairs of the CD63, LIMP II and LAMP1 populations with distributions that were not normal (Fig. S3A, Table S3) to the non-parametric Kolmogorov and Wilcoxon tests (Statistics, Suppl. Exp. Proc. Tables S4, S5), indicated that the association of ATP7B and LIMPs in ATP7B vesicular carriers and lysosomes was described by two separate populations of CD63 and LIMPII/LAMPI, with intervals of confidence of [4.156 x 10⁻³, 8.861 x 10⁻³]/[1.315 x 10⁻², 2.082 x 10⁻²] and [3.247 x 10⁻³, 6.93 x 10⁻³]/[1.153 x 10⁻², 1.886 x 10⁻²], respectively (Table S6), intervals that reassured the calculated ATP7B/LIMPs coincidence ratio lower than 0.0206/1, value that fell dramatically short of the 0.40/1 ratio reported in HepG2 cells (Polishchuk et al., 2014). Altogether these data indicated that the vesicles that transported ATP7B through the basolateral domain were structures distinct from lysosomes.

**ATP7B IS INSERTED INTO the BASOLATERAL MEMBRANE and ENDOCYTOSED.**

To know if the presence of ATP7B in basolateral endosomes resulted from the fusion of endosomes with the vesicles that transported ATP7B to the basolateral domain, or from the ATP7B insertion into the basolateral membrane and subsequent endocytosis, we studied the effect of dynasore (DYN), the inhibitor of dynamin and clathrin-dependent endocytosis, and
repeated the Tf loading experiment in cells transfected with exofacially HA-tagged ATP7B that were simultaneously incubated with Alexa647-Tf and anti-HA antibody. Treatment of cells for 60 min with DYN before challenge with Cu⁺ showed a marked retention of ATP7B in the basolateral surface and a complete inhibition of its incorporation into the BCs, effect which was strongly reversed after washing out the drug (compare Figs. 4A₁-A₃ and 4B₁-B₃). Moreover, in cells transfected with exofacially HA-tagged ATP7B we localized the endocytosed HA antibody and Alexa647-Tf in endosomes transporting ATP7B (Fig. 4C₁-C₃). These two studies strongly indicated that after its release from the TGN, ATP7B was targeted to the basolateral domain and inserted into the basolateral membrane, and was then endocytosed.

To investigate if ATP7B interacted with AP-2, the clathrin adaptor that works in cargo internalization by clathrin-mediated endocytosis. To this end we examined the ability of ATP7B and the α subunit of the AP-2 adaptor to pull each other down (Fig. 4D). Confirmation of this ability suggested that the surface localized AP-2 worked in the clathrin-mediated endocytosis of ATP7B inhibited by DYN.

ATP7B is VECTORIALLY TRANSPORTED from the BASOLATERAL to the APICAL DOMAIN by a MECHANISM that REQUIRES MICROTUBULES.

To examine the redistribution of the basolateral endosomes loaded with ATP7B and Tf, we extended the treatment of cells with Cu⁺ to 25 min and postponed the addition of Tf to the last 10 min of incubation with the metal (Fig. 2D₁-D₃). Under these conditions ATP7B began to appear in the apical pole of the cells and to disappear from basolateral endosomes loaded with Tf (compare Figs. 2C and 2D; Table S1). It is well established that vesicle-mediated transcytosis from the basolateral to the apical domain requires microtubules(Hunziker et al., 1990). To explore if transport of ATP7B to the apical pole required microtubules, we incubated Can 10 cells for 15 min with Cu⁺, to target the ATP7B retained in the TGN to the basolateral domain, and then for 60 min at 4ºC with the microtubule inhibitor nocodazol (NOC) before incubation
for 40 min at 37°C with or without NOC and with Alexa 647–Tf, in the continuous presence of 

\(\text{Cu}^+\) (Fig. 5). The results showed that in cells warmed in NOC, and therefore without 

microtubules, the translocation of ATP7B to the apical domain was strongly inhibited and the 

protein was retained in numerous vesicles dispersed throughout the cytoplasm, many of them 

loaded with Tf (Fig. 5B1-B3). In contrast, the washing of NOC immediately before warming of 

the cells, resulted in complete reconstruction of the microtubule network and in translocation 

of ATP7B to the apical BC (Fig. 5C1-C3). 

**ATP7B ENTERS THE SUBAPICAL COMPARTMENT and BFA BLOCKS TARGET of ATP7B from the 

SAC to the BC.**

Accumulation of ATP7B in the cell apical pole, where the subapical compartment (SAC) 

operates as a protein sorting hub (Hoekstra et al., 2004), occurred within the 15-30 min 

window after the addition of \(\text{Cu}^+\) (Fig. 6A1-A3). This movement was preceded by the 

segregation of the ATP7B and Tf loads (Fig. S2A1-A3), with Tf being targeted to the area 

adjacent to the nucleus where clusters of common recycling endosomes recover it back to the 

basolateral surface.

Exam of the ATP7B distribution in the apical pole showed a high degree of coincidence 

between ATP7B and Rab11a, the small GTPase associated with the SAC that regulates the 

apical transport/recycling of proteins (Welz et al., 2014) (Figs. 6A1-A3, 6B). Furthermore, 

microscopy studies provided evidence of the transient codistribution between the two 

proteins, consistent with the passage of ATP7B through the Rab11a-positive SAC before its 

incorporation into the BC (Fig. 6C1-6F3). Separate studies of the CD63, LIMPII and LAMP1 

association with the ATP7B transiting through the SAC in cells incubated for 25 min with 40 \(\mu\)M 

\(\text{Cu}^+\) showed no evidence of association (Figs. 3C2, D2, E2; Statistics Suppl. Exp. Proc., Table S7), 

and analysis of the experimental data using a geometric distribution that allows the estimation 

of the probability to detect one LIMP signal in the SAC, produced a \(p_{\text{SAC}} < 0.049\) (\(\overline{p}_{\text{SAC}} =\)
0.033) at a 95% confidence level that strongly endorsed the experimental observations (Fig. S3B, Table S7).

**TRANSPORT OF ATP7B FROM THE APICAL POLE to the BILE CANALICULUS is INHIBITED by the ARF-GEF INHIBITOR, BREFELDIN A**

The fungal toxin brefeldin A (BFA) specifically targets the large ARFs, GTPases which regulate trafficking pathways by recruiting the vesicle coat components AP-clathrin adaptors, COPI and GGA proteins (Cox et al., 2004). Consistent with the localization of large GEFs in the TGN (BIG1/BIG2) and in endosomes (BIG2), BFA caused the translocation of ATP7B from the TGN to the apical pole of HepG2 cells (Roelofsen et al., 2000b), and in Can 10 cells created a characteristic patchy distribution of ATP7B that flanked the BCs (Fig. S2B1-C3) To study if the inhibition affected the translocation of ATP7B from the apical SAC area to the BC, we treated Can 10 cells for 15 min with Cu\(^{+}\) to release the protein from the TGN and then for 1h at 4ºC with BFA/ NOC in the presence of the metal, before raising the temperature to 37ºC and continuing the incubation with the two drugs, with BFA alone and without drugs (Fig. 6). The results showed that whereas in cells incubated with NOC and BFA the ATP7B protein was retained in vesicles distributed throughout the cytoplasm (Fig. 6G1-G4); the removal of NOC allowed the advance of ATP7B to the apical area and its retention in SAC (Figs. 6H1-H4). Moreover, removal of NOC and BFA resulted in incorporation of ATP7B into the BC (Fig. 6I1-I4). We therefore concluded that BFA inhibited the transport of ATP7B from the SAC to the BC, probably by inhibiting apical BIG2. Our results were similar to those described for the transcytosis of the polymeric immunoglobulin receptor in polarized neurons, where BFA inhibited the redistribution of the receptor from the basal-dendritic domain to the apical-axonal domain (de Hoop et al., 1995). The relocation of the ATP7B released from the TGN was culminated after its arrival and incorporation into the BC as shown by its overlap with the marker of the BC membrane, HA4 (Figs.7A1-B3). The whole translocation process required
approximately 60 min (Fig. 7C). It was important that we never observed any apically oriented movement of lysosomes during the Cu⁺-dependent ATP7B translocation to the BC. Moreover, in three separate studies of the association of CD63, LIMPII and LAMP1 with the BC of cells incubated for 90 min with 40 µM Cu⁺ we did not detect any presence of LIMPs in the BC (Fig. 3C, D, E). Analysis of the experimental data using a geometric distribution, produced a $p_{BC} < 0.019$ ($p_{BC} = 0.013$) at a 95% confidence level, values that strongly validated the experimental observations (Fig. S3B and Table S7). With regard to this it is significant that we never found a significant incorporation of ATP7B into the lysosomes and of lysosomal markers into the BC of Can 10 or HepG2 cells incubated under stringent Cu⁺ conditions (200 µM Cu⁺ for 24 h) (Fig. S4).

**APICAL ENDOCYTOSIS OF ATP7B IS INHIBITED by DYN and RECYCLING by BFA.**

Treatment of cells with the non-permeable Cu⁺ chelating agent, bathocuproine disulphonate (BCS), induced only a slow return of ATP7B from the BC to the TGN, probably due to the requirement of functional ATP7B in the BC to feed extracellular BCS with excreted Cu⁺ (Fig. 7D). In contrast, the use of the cell permeable Cu⁺ chelating agent neocuproine (NEC), to directly reduce intracellular Cu⁺, produced a more rapid transit and after a 2h incubation with the drug the bulk of ATP7B was returned to the TGN (Fig. 7D; compare Figs. 7E-E and 7F-F). In the experiments using NEC, we observed that DYN strongly inhibited the exit of ATP7B from the BC suggesting that the ATP7B was endocytosed by clathrin coated vesicles (compare Figs.7F-F and 7H-H). In addition, we also observed that while organelle de-acidification, induced by BF, did not inhibit the apical endocytosis of ATP7B, it did inhibit its apical recycling and return to the TGN, retaining it in large vesicular bodies abundant in the subapical area adjacent to the BC (Fig. 8A-C). Since the treatment with BFA inhibited the target of ATP7B to the BC (Fig. 6H-H), we studied the possibility that the SAC was involved in the apical recycling of ATP7B by examining the distribution of ATP7B in cells that were first treated with Cu⁺ for 90
min and then incubated for a further 30 min after the addition of BFA. The incubation with BFA induced the partial re-localization of the ATP7B from the BC to the Rab11-positive SAC (compare Figs 7A₁-B₃ and 7I₁-J₃), resulting in the same patchy pericanalicular distribution of the apically bound ATP7B observed in the SAC of cells treated with BFA (compare Figs. 7I₁-J₃ with Figs. 6H₁-H₄ and Figs S2B₁-C₃). These results demonstrate that in cells with elevated levels of Cu⁺, the ATP7B transported from the TGN to the BC membrane was continuously endocytosed and cycled between the SAC and the BC.
DISCUSSION

In this study we have characterized the pathway of ATP7B transport from the TGN to the BC in response to the elevation of intracellular Cu\(^{+}\) levels in the rat hepatoma cell line Can 10.

The Cu\(^{+}\) induced release of ATP7B from the TGN was found to be a rapid process that begins within 5 min of the addition of 40 µM Cu\(^{+}\) and is completed within 20-25 min. Treatment with BF delayed the detachment of the cisterna carrying ATP7B from the TGN, demonstrating that cisterna detachment is a prerequisite to the generation of transport vesicles as has previously been described in plants (Uemura et al., 2014). This process is pH dependent and the rapidity of its occurrence in mammalian cells is probably why it has not been previously observed in others than plants. Our studies also show that the vesicles loaded with ATP7B budding from the TGN do not contain lysosomal membrane proteins, suggesting that these are instead segregated into vesicles distinct from those transporting ATP7B. The ATP7B carriers are then rapidly transported to a subcellular location in the large basolateral domains of Can 10 cells, directly opposite the apical domains where the BC are located. The basolateral sorting of ATP7B at the TGN can be explained by the Cu\(^{+}\)-mediated interaction of the C-terminal sequence motif DKWSLLL with the clathrin adaptor AP-1A resident in the TGN (Lalioti et al., 2014) since this adaptor operates in the basolateral sorting of membrane proteins in the hepatocyte lacking AP-1B (Gravotta et al., 2012). The role of AP-1A in the basolateral sorting and recovery of ATP7B from endosomes to the TGN (Braiterman et al., 2011) explicate that mutations in σ1A, the small subunit of the AP-1 adaptors, produce severe perturbations of copper metabolism in humans (Martinelli et al., 2013).

Our detection of basolaterally endocytosed transferrin in the ATP7B vesicular carriers localized at the distal end of the basolateral domain suggests that ATP7B is transported by basolateral endosomes/endocytic vesicles. This suggestion is supported by recent studies demonstrating that de-acidification of the luminal pH results in the trapping of ATP7B in
aggregates of EEA1-positive endosomes localized to the basolateral domain of polarized WIF-B cells (Nyasae et al., 2014). Furthermore, the co-localization of both endocytosed anti-HA antibody and Alexa 647-Tf in the basolateral endosomes of cells transfected with HA-tagged ATP7B and incubated for 30 min with Cu⁺, indicates that basolateral sorted HA-ATP7B is first inserted into the basolateral membrane and is then subsequently endocytosed. This is supported by the observed accumulation of ATP7B in the basolateral membrane of cells treated with DYN, an inhibitor of both dynamin and clathrin-mediated endocytosis, and the concomitant reduction in the incorporation of the ATP7B into the BC, and is consistent with the mutual pulldown of ATP7B and the α-subunit of the clathrin adaptor AP-2 implicated in endocytosis. With respect to the movement of ATP7B from the basolateral to the apical domain is interesting that ATP7B contains a FAFDNVGYE signal motif, a variant of the [FY]XNPX[YF] signal recognized by the basolateral retromer and absent in ATP7A (Donoso et al., 2009), which is essential for its apical targeting in elevated Cu⁺ and mediates the retention of the transporter in the TGN at low-Cu⁺ concentrations (Braiterman et al., 2009).

The accumulation of ATP7B in the SAC of cells incubated with BFA following its endocytosis from the basolateral membrane, together with its incorporation into the BC after removal of the drug, both implicate a large ARF-GEF localized in recycling endosomes, such as BIG2, in the targeting of ATP7B from the SAC to the BC. Our finding that BFA inhibits the recycling of apically endocytosed ATP7B to the BC by retaining the protein in the SAC suggests that the same mechanisms of transport that operate in the SAC may function in both the transcytosis of ATP7B and in its apical recycling.

The inhibition of apical ATP7B endocytosis by DYN is consistent with the BC being the site of ATP7B functioning in Cu⁺ excretion. It should be noted that this observation is confirmatory of the originally observed Cu⁺-induced translocation of ATP7B from the TGN to the BC in HepG2 cells and the localization of ATP7B in the BC of liver (Guo et al., 2005; Roelofsen et al., 2000a;
Schaefer et al., 1999b), and that these studies were subsequently confirmed in other cells by other laboratories (Guo et al., 2005; Hernandez et al., 2008).

Is intriguing to note that while most of the membrane proteins, resident in the BC, that contain multiple transmembrane spanning sequences are directly targeted from the Golgi (Kipp and Arias, 2002), ATP7B in contrast uses a distinct transcytosis route to reach the BC, a route that was previously thought to be restricted to apical proteins with a single transmembrane domain (Bastaki et al., 2002).

The conclusions of the studies reported here are in contrast with those of a recent study in HepG2 cells that proposes that ATP7B is rapidly and directly targeted from the TGN to lysosomes in response to Cu\(^{+}\) and that fusion of the Cu\(^{+}\) loaded lysosomes with the BC membrane results in the excretion of excess Cu\(^{+}\) into the bile (Polishchuk et al., 2014). The latter studies extended previous reports that interpreted the presence and functioning of transfected GFP-ATP7B and ATP7B-DsRed constructs in late endosomes as part of the lysosome-mediated excretion of Cu\(^{+}\) into the bile. In these studies the ATP7B constructs were not detected in the TGN and were not relocated in response to excess Cu\(^{+}\) (Harada et al., 2005; Harada et al., 2003b; Harada et al., 2000a; Harada et al., 2000b). In contrast, in our studies in highly polarized Can 10 cells we observe that excess Cu\(^{+}\) induces the rapid translocation of endogenous ATP7B from the TGN to the BC by basolateral sorting and transcytosis. Moreover, during this rapid trafficking process we did not find any association of ATP7B with lysosomes and, repeating these studies using HepG2 cells we could not find any ATP7B in the lysosomes in response to Cu\(^{+}\) treatment in these cells. Furthermore, we did not observe any Cu\(^{+}\)-induced movement of lysosomes towards the BC, nor did we detect any incorporation of four distinct LIMPs into the BC as it would be expected if the excretion of Cu\(^{+}\) was primarily mediated by lysosomal exocytosis.
The picture emerging from studies in human and rat frozen sections of liver is that endogenous ATP7B is localized in the TGN and in the bile canaliculus (Schaefer et al., 1999a; Schaefer et al., 1999b). However more studies are needed to establish the response of the ATP7B retained in the TGN to excess Cu⁺ in liver.

It is important that abnormal intracellular trafficking of the Cu⁺ transporters has been detected in multisystemic diseases that include Menkes and Wilson symptoms. Consequently with the transport of ATP7B from the TGN to the BC by basolateral sorting and transcytosis two of those diseases are produced by mutations that target AP-1A (Martinelli et al., 2013), the clathrin adaptor resident in the TGN implicated in the basolateral sorting of protein in the hepatocyte (Gravotta et al., 2012), and COMMD1 (van De Sluis et al., 2002), the component of the CCC complex that in cooperation with the WASH complex and the retromer regulates the traffic of cargo through basolateral endosomes in polarized cells and is required for transcytosis (Phillips-Krawczak et al., 2015; Verges et al., 2004). These observations emphasize the importance of elucidating the mechanisms regulating the traffic of Cu⁺ transporters to facilitate the diagnoses and understand the development and prognosis of Cu⁺ disorders.

In closing, this study describes that the Cu⁺-dependent relocation of ATP7B from the TGN to the bile canaliculus includes the basolateral sorting at the TGN, transport to and endocytic retrieval at the basolateral membrane, followed by microtubule-dependent transcytosis through the SAC and incorporation into the BC. These results indicate that the site of ATP7B action in vectorial Cu⁺ excretion is the membrane of the BC.
MATERIALS AND METHODS

CELL CULTURE

Rat hepatoma Can 10 and human hepatoma HepG2 cells were grown in Coon’s modification medium supplemented with 1.5 g/L NaCO\textsubscript{3}, 10% fetal calf serum, 1% glutamax penicillin and streptomycin (CS). Can 10 cells were cultured for 5-days for complete surface polarization and media was changed daily. Counting of basolateral vesicles was facilitated by growing the Can 10 cells in polylysine-coated coverglasses and incubating the cells for 10 h in Coon’s medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine.

cDNA CLONING, CONSTRUCTS AND TRANSFECTIONS

Mouse ATP7B cDNA was cloned by PCR amplification of a mouse liver Marathon Ready cDNA library (Lalioti et al., 2014). Exofacial HA-tagging of ATP7B was performed by introducing the tag into the third exofacial loop of ATP7B (Lalioti et al., 2014). Cells were transfected for 11 h using Lipofectamine 2000 to minimize protein overexpression, and the last 3h were incubated with 50 µg/mL cycloheximide to inhibit new ATP7B synthesis.

ANTIBODIES

Polyclonal anti-ATP7B antibodies (Hernandez et al., 2008), antibodies to TGN38 (Yuan et al., 1987) and the lysosomal membrane proteins CD63, LIMPII, LAMP1 and LAMP2 (Barriocanal et al., 1986) were developed in the laboratory. Further details on these antibodies and on other primary and secondary antibodies used in this study are provided in Additional Methods.

CHEMICALS

Neocuproine (NEC), bathocuprine disulfonate (BCS) and brefeldin A (BFA) were from Sigma Aldrich (MO); nocodazol (NOC) and dynasore (DYN) were from Santa Cruz Biotech.
**IMMUNOFLUORESCENCE MICROSCOPY**

Cell fixation was adapted to antibody reactivity; fixation/permeabilization was performed by treatment with cold (-20°C) methanol for 4 min (anti-ATP7B, ZO-1, YOL 1/34, TGN38, β catenin CD63, LIMPII, LAMP1 and LAMP2 antibodies); for 20 min with 2% paraformaldehyde and 5 min with 0.2% Triton X100 (anti- ATP7B, Zo-1, Rab11a, YOL 1/34, HA tag and EEA1 antibodies) and for 1 min with 3% paraformaldehyde and 10 min with methanol at 5°C (ATP7B, ZO-1 and HA4 antibodies). For co-localization studies, cells were grown on 0.1-0.16 mm glass coverslips and mounted in gelvatol (refractive index 1.376). Stable Alexa488 and Alexa647 dyes were used to avoid spectral bleed and the stained cells were examined using a Zeiss Plan-apochromat x63/1.4 oil DIC objective and oil with a refractive index of 1.51. Capture Images were de-convoluted and studied using Coloc 2 and JACoP modules of Image J software. Data was subjected to robust statistics (see Statistics Suppl. Exp. Proc.).

**ATP7B TRAFFICKING ASSAYS.**

The de-acidification agent BF, the dynamin inhibitor DYN, the microtubule inhibitor NOC, the ARF-GEF inhibitor BFA and incubation at low temperature were used to treat cells and help dissect the pathway of transport. Translocation of ATP7B from the TGN to the BC was studied by challenging Can 10 cells with 40 µM Cu²⁺ for periods between 5 min and 2h; the translocation process was best monitored in small islets organized by highly polarized cells. The effective retention of ATP7B in the TGN at 0.3 µM Cu²⁺ made unnecessary the use of Cu²⁺ chelating agents to reset the start of the protein traffic at the TGN. In a set of traffic experiments we established as landmarks of the ATP7B anterograde traffic, the beginning (5-7 min) and completion (10-15 min) of its release from the Golgi; its appearance in basolateral endosomes loaded with endocytosed Tf (8-20 min); its approach and entrance into the SAC (15-35 min), and its incorporation into the BC (30 min-1h) (see Fig. 7C). These landmarks
helped to set a series of traps to dissect the pathway of ATP7B transport: bafilomycin-A1 (BF) and chloroquine (CQ) were employed to slowdown the release of ATP7B by acidic vesicles from the TGN; dynasor (DYN), the inhibitor of dynamin and clathrin-dependent endocytosis, was used at 80 µM in serum free media to exam the traffic of ATP7B through the basolateral membrane; nocodazol (NOC) the microtubule depolymerizing agent was a powerful tool to study the second leg of traffic between the basolateral membrane and the SAC and the incorporation of ATP7B into the BC; we incubated cells for 15 min with Cu⁺ to target ATP7B from the TGN to basolateral endosomes, and then for 1h with Cu⁺, NOC and BFA, at 4ºC, to disrupt the microtubules and to set the stage to study the redistribution of basolateral ATP7B after warming the cells in the absence of NOC, and the presence or absence of BFA; the trapping of ATP7B in the SAC of cells warmed up in the absence of NOC and in the presence of BFA, helped to characterized the passage of ATP7B through the SAC and indicated the involvement of a BFA-sensitive ARF-GEF in the regulation of the ATP7B target from the SAC to the BC, probably the apical BIG2; for studies of apical ATP7B endocytosis and retrograde transport the cell permeable neocupreine (NEC) was the Cu⁺ chelating agent of choice for its quick effects in eliciting the backwards ATP7B traffic in response to decrease Cu⁺ levels, the cell impermeable bathocuproine disulfonate (BCS) was less effective; DYN was a strong tool to study the apical endocytosis of ATP7B in cells deprived of Cu⁺ by treatment with NEC, whereas BFA was effective in trapping the apically endocytosed ATP7B in cells continuously incubated with Cu⁺. The experiments were repeated an average of three times.

IMMUNOPRECIPITATION and WESTERN BLOT STUDIES.

All the procedures were performed at 4º C. Can 10 cells were washed twice with TBS, scraped, suspended in 1mL of cold buffer A (20mM HEPES pH 7.5, 130 mM NaCl, 1mM EGTA, and one tablet of the cocktail of protease inhibitors Complete Mini, Roche) and incubated in 1% NP40 for 10 min, and nuclei removed by centrifugation at 100x g for 10 min. The resulting
postnuclear supernatant was incubated at 4ºC for 1h, aggregates removed by centrifugation at 15000 x g for 10 min, and dilution in buffer A to 0.6% NP40 by. Equal extract aliquots were separately incubated 4h with the rabbit anti- ATP7B antibody or the goat anti AP-2α antibody bound to Protein G- Sepharose, prepared by mixing 5 μL of each antibody with 15 μL of Protein G-Sepharose (GE Healthcare) and then washing off excess antibody. Immunoprecipitation complexes were collected by low-speed centrifugation and, after three washes in buffer A and one in water, were resuspended and heated (85ºC, 3min) in Laemli buffer and resolved by 10% SDS–PAGE. The resolved proteins were blotted onto nitrocellulose and studied using bioluminescence, donkey anti-rabbit-HRP (GE Healthcare) and donkey anti-goat-HRP (Merck Millipore) antibodies and the Clean Blot IP detection kit (Thermo Scientific). The ATP7B/AP-2α experiment was repeated two times.
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COMPETING INTERESTS.

No competing interests declared.

AUTHORS CONTRIBUTIONS.

V.L., I.V.S. Planning, performance cell biology and biochemical experiments, antibody development, manuscript writing; R.P. Statistics, manuscript editing; M.P.B. ATP7B apical endocytosis and retrograde transport assays, manuscript editing. Y.T. ATP7B anterograde transport assay, antibody development; A. M., T.V. Confocal microscopy, deconvolution and image analysis. C.S. Conventional and confocal microscopy, manuscript writing.

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**Fig. 1** ATP7B is quickly released from the TGN in response to Cu*. Can 10 cells were treated with 40 µM Cu* for the indicated times and immunostained for ATP7B and the TGN marker TGN38 (A1-C2); untreated controls (w/o). ATP7B is transported out of the TGN by acidic vesicles: chloroquine (CQ) induces the swelling and retards the release of ATP7B-loaded vesicles emerging from the TGN upon Cu* stimulation: Can 10 cells treated for 3h with 50 µM CQ and then for 15 min with 40 µM Cu* and the drug, were stained for ATP7B and the lysosomal
integral membrane protein LAMP1 and studied by confocal microscopy in a 0.04 µm optical section (D1, D2): the intense fluorescence produced by the ATP7B is retained in the vesiculated TGN and in swollen vesicles attached to the TGN that are distinct from the lysosomes.

Inhibition of the V-H⁺ pump slows down the packing of ATP7B at the TGN into transport vesicles. Cells treated for 3h with 100 nM BF and then with 40 µM Cu⁺ and the drug for the times indicated, were stained for ATP7B, TGN38 and EEA1 and studied by microscopy (E-H). Note the grape-surface of the cisternae loaded with ATP7B and its physical segregation as a distinct replica of the TGN38-positive cisterna (F, G), before its budding off into vesicles accompanied by the acquisition of the early endosome marker EEA1 (H). Arrowed areas are enlarged in the inserts in separate color channels. Bars 10 µm.
**Fig. 2** ATP7B-loaded vesicles are rapidly relocated to the cell basolateral domain after its release from the TGN. Can 10 cells treated with 40 µM Cu⁺ for the times indicated were stained for ATP7B (A, B₁, B₂), the TJ marker ZO-1 (A) or the marker of the BC membrane, HA4 (B₁, B₂); nuclei were stained with DAPI.

*Localization of ATP7B in basolateral endosomes loaded with endocytosed Tf.* Cells treated for 12 min with Cu⁺ and with Alexa647-Tf for the last 8 min (C₁-C₃) or for 25 min with Cu⁺ and with Alexa647-Tf for the last 10 min (D₁–D₃); arrowed areas are enlarged in the inserts. Treated cells showed the loading of basolateral endosomes with ATP7B and endocytosed Tf. Bars 10 µm.
Fig. 3 ATP7B is not incorporated into lysosomes during trafficking through the somatobasolateral domain of Can 10 cells. Reactivity of mouse monoclonal antibodies with CD63, LIMP II and LAMP1 in CAN10 cells extracts and of rabbit polyclonal antibody with LAMP2 (A). Optical sections from CAN 10 cells incubated for 15 min with 40 µM Cu⁺ and stained for ATP7B and LIMP II are shown in panels B1-B6: upper-case numerals, ratio of ATP7B in lysosomes; bottom-case numerals, optical section number and distance to the plane of cell attachment; nuclei were stained with DAPI. Can 10 cells incubated for 15, 25 and 90 min with 40 µM Cu⁺ to compare the cellular distributions of ATP7B and the lysosomal membrane markers CD63, LIMP II and LAMP1 during ATP7B trafficking through the basolateral domain (C₁, D₁, E₁) and the SAC (C₂, D₂, E₂), and after its incorporation into the BC (C₃, D₃, E₃). (J). Bars 10 µm.
Fig. 4 Basolateral sorted ATP7B is inserted into the basolateral membrane. DYN blocks transport of ATP7B to the BC at the basolateral membrane. Cells preincubated for 120 min with 100 µM NEC, to confine ATP7B to the TGN, were washed and treated for 60 min with 80 µM DYN and then for 90 min with 40 µM Cu, in the continuous presence of DYN (A1-A3) or in the absence of DYN for the last 40 min of incubation with the metal (B1- B3); after fixation-permeabilization the cells were stained for ATP7B, Zo-1 and the basolateral membrane marker β catenin. Note the accumulation of ATP7B in the β catenin-positive basolateral membrane of the cells continuously treated with DYN and its partial release and arrival to the BC after DYN removal. Transfected HA-ATP7B is inserted into the basolateral membrane and endocytosed. Cells transfected for 11 h with exofacially-HA tagged ATP7B were incubated for 3 h in complete Coon’s medium with 50 µg/ml cycloheximide to inhibit new ATP7B synthesis, and the last 2 h with 100 µM NEC to retain the bulk of ATP7B in the TGN; following the cells were washed and then incubated for 30 min with 100 µM Cu⁺ and with 2.5 µg/ml Alexa 647-Tf and 5 µg/ml
monoclonal anti-HA antibody. The fixed-permeabilized cells were studied by IFM using specific antibodies to the HA tag and ATP7B; magnifications of ATP7B, endocytosed Alexa647-Tf (false blue) and HA-antibody are shown in the inserts. Bars 10 µM. **Pulldown of ATP7B and the clathrin adaptor AP-2α subunit.** Detergent-solubilized postnuclear supernatants from CAN 10 cells incubated for 15 min with or without 40 µM Cu⁺, were incubated with specific antibodies to either ATP7B or the α-subunit of the AP-2 clathrin adaptor directly bound to protein G-Sepharose (Prot. G). The immunoprecipitates were subjected to Western blot analyses (W) using antibodies against the pulldown proteins. Controls included analysis of the total cell extracts (INPUT) and proteins immunoprecipitated by protein G-Sepharose alone (Prot. G). The results show the reciprocal immunoprecipitation of ATP7B (*) and the AP-2 α-subunit (**) and the similar amounts of the AP-2 α subunit pulled down by the ATP7B antibody from extracts of cells incubated in the absence or presence of Cu⁺.
**Fig. 5 ATP7B transcytosis requires microtubules.** Can 10 cells were incubated for 60 min at 4°C with 20 µM NOC and then for 40 min at 37°C with both NOC and Alexa647-Tf (A1-A3), or incubated for 15 min with 40 µM Cu⁺, to release the ATP7B retained in the TGN, followed by incubation with NOC and Alexa647-Tf performed in the presence of the metal (B1-B3). Microtubules were reconstituted by washing the NOC and performing the last incubation with Cu⁺ and Alexa647-Tf in the absence of the drug (C1-C3). All cells were stained for ATP7B, TGN38 and microtubules. Panels on the right side (A4-A5, B4-B5, C4-C5) show magnifications of the arrowed areas. Bars 10 µm. Notice the retention of ATP7B in vesicles after its release from the Golgi and the disruption of microtubules and the resumption of the ATP7B transport to the apical BC after reassembly of microtubules.
**Fig. 6** Apically bound ATP7B transits through the SAC compartment. Can 10 cells were treated for 25 min with 40 µM Cu⁺ and stained for ATP7B and for the SAC marker Rab11a (A₁-A₃); notice the characteristic two-fold and trefoil arrangement of the SACs and the different rates of ATP7B entrance in the SAC; arrowed SACs are magnified in the inserts. Bars 10 µm. Pearson’s and Manders co-distribution coefficients (co-distribution tends to 1; Median +/- S_w0.2) were computed using 17 confocal SAC images and the Fiji Coloc 2 and JACoP software (B); observe the high degree of ATP7B and Rab11a co-distribution. Passage of ATP7B through the SAC was studied in cells incubated 25 min with 40 µM Cu⁺ to record the ATP7B approach (C₁-C₃) and entrance into the SAC (D₁-D₃), and its subsequent incorporation into the inner circular BC (E₁-F₃) Bars 5 µm (C₁-F₃).
Transport of ATP7B from the SAC to the BC is inhibited by BFA. Can 10 cells were incubated for 15 min with 40 µM Cu⁺ and then for 60 min at 4°C after the addition of 10 µg/ml BFA and 20 µg/ml NOC, before removal of BFA (G₁-G₄), NOC (H₁-H₄) or BFA and NOC (I₁-I₄), followed by incubation for 40 min with the metal at 37°C. Cells were stained for ATP7B, ZO-1 and TGN38 as indicated. Panels on the right side G₃-G₄, H₃-H₄ and I₃-I₄ show magnifications of the arrowed areas. Bars 10 µm. Notice the resumption of the apically bound ATP7B transport after removal of NOC and the retention of ATP7B in the SAC of cells incubated with BFA (see also Fig. 4S) as well as the translocation of ATP7B from the SAC to the BC after removal of BFA.
Fig. 7 ATP7B incorporation into the BC, DYN-sensitive apical endocytosis and BFA-sensitive apical recycling. ATP7B incorporation into the BC was studied in cells with long and short BCs treated for 90 min with 40 µM Cu⁺ and stained for ATP7B and ZO-1 (A₁-A₃) and the BC membrane marker HA4 (B-B₃). Notice the retention of ATP7B in the BC boxed and sealed by the ZO-1-positive tight junctions and its co-localization with the apical membrane marker HA4; ATP7B loaded apical endosomes are marked with an asterisk.
Time course of the Cu\(^{+}\)-dependent ATP7B relocation from the TGN to the BC. Cells fixed and permeabilized after incubation with 40 µM Cu\(^{+}\) for the indicated times were stained for ATP7B, TGN38, Rab11aA and HA4 (C); single organelles (TGN, SAC, BC) and basolateral vesicles (Median +/- S_{W,0.2}) were computed in 50 cells per time point. The experiment shown was representative of three experiments.

ATP7B apical endocytosis and retrograde transport to the TGN. Cells preincubated for 90 min with Cu\(^{+}\) were incubated after washing out the metal with 100 µM of the cell-permeable and impermeable Cu\(^{+}\) chelating agents, NEC and BCS, respectively, for the times indicated (D); notice the strongest action of NEC; incubation with NEC induced the complete translocation of ATP7B to the TGN after 120 min incubation (D; compare E and F); p<0.05 (*), p<0.01 (**), p<0.001 (***)

ATP7B apical recycling and retrograde transport. Apical ATP7B endocytosis was studied in Can10 cells incubated for 90 min with 40 µM Cu\(^{+}\) and then for 60 min with 80 µM DYN and Cu\(^{+}\) (G\(_1\)-G\(_4\)), before removal of the metal and incubation for 2h with DYN and 100 µM NEC (H\(_1\)-H\(_4\)); treatment showed the strong inhibition of ATP7B apical endocytosis by the dynamin inhibitor DYN.

Apical recycling of endocytosed ATP7B. Recycling was studied in cells incubated for 90 min with 40 µM Cu\(^{+}\) and then for a further 45 min with 10 µg/ml BFA in the presence of the metal; compare panels I\(_1\)-J\(_3\) with panels A\(_1\)-B\(_3\) and notice the retention of ATP7B in the RAB11a-positive SAC, flanking the TJ, of the BFA treated cells. Bars 10 µm.
**Fig. 8** Bafilomycin A₁ blocks ATP7B apical recycling and retrograde transport to the TGN. Can 10 cells first incubated for 90 min with 40 µM Cu⁺ to induce the translocation of ATP7B to the BC (A) were then incubated for 3h with 60 nM BF and the metal (B) and, after washing out the metal, were incubated for 2h with BF and 100 µM NEC (C). The cells treated with BF in the presence of Cu⁺ showed the accumulation of ATP7B in vesicular bodies distinct from the BC and TGN (compare A and B, arrows), and BF blocked the retrograde transport of ATP7B to the TGN after removal of the metal by NEC treatment (C₁, arrows). Intensity fluorescence of ATP7B in the BC (D) and TGN (E) and size of ATP7B-positive vesicular bodies (F). Data collected from 20 BC and 50 TGN contained in three randomly selected fields (M +/- SD; p<0.01 (**), p<0.001 (***)). Bars 5 µm.