Copper Directs ATP7B to the Apical Domain of Hepatic Cells via Basolateral Endosomes

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Abstract

Physiologic Cu levels regulate the intracellular location of the Cu ATPase ATP7B. Here, we determined the routes of Cu-directed trafficking of endogenous ATP7B in the polarized hepatic cell line WiF-B and in the liver in vivo. Copper (10 μM) caused ATP7B to exit the trans-Golgi network (TGN) in vesicles, which trafficked via large basolateral endosomes to the apical domain within 1 h. Although perturbants of luminal acidification had little effect on the TGN localization of ATP7B in low Cu, they blocked delivery to the apical membrane in elevated Cu. If the vesicular proton-pump inhibitor bafilomycin-A1 (Baf) was present with Cu, ATP7B still exited the TGN, but accumulated in large endosomes located near the coverslip, in the basolateral region. Baf washout restored ATP7B trafficking to the apical domain. If ATP7B was staged apically in high Cu, Baf addition promoted the accumulation of ATP7B in subapical endosomes, indicating a blockade of apical recycling, with concomitant loss of ATP7B at the apical membrane. The retrograde pathway to the TGN, induced by Cu removal, was far less affected by Baf than the anterograde (Cu-stimulated) case. Overall, loss of acidification-impaired Cu-regulated trafficking of ATP7B at two main sites: (i) sorting and exit from large basolateral endosomes and (ii) recycling via endosomes near the apical membrane.

Keywords: bafilomycin-A1, bile canaliculus, endosomes, hepatocytes, polarity, proton ATPase, retromer, Wilson’s disease

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Copper (Cu) is essential for life because it is a cofactor of certain oxidative enzymes, including superoxide dismutase, cytochrome oxidase, tyrosinase, lysyl oxidase and ceruloplasmin. However, excessive Cu in cells is toxic (1). Thus, cellular Cu homeostasis is highly regulated and is achieved in part by two intracellular Cu-transporting P-type ATPases, ATP7A and ATP7B (2). When Cu is low, these proteins pump cytosolic Cu into the luminal spaces in the secretory pathway to supply Cu to newly synthesized cuproenzymes. When Cu is high, Cu ATPases exit the trans-Golgi network (TGN) in vesicles and move near the plasma membrane, where they extrude Cu from the cell.

Menkes and Wilson’s diseases result from loss-of-function mutations in ATP7A and ATP7B, respectively (3). In Wilson’s disease, loss of ATP7B function leads to excess Cu accumulation in the brain, kidney and particularly in the liver, owing to defective biliary Cu excretion across the apical surface of hepatocytes. ATP7B in hepatocytes shows Cu-regulated localization in vivo (4). The trafficking mechanisms that operate physiologically during Cu-directed trafficking of ATP7B in hepatocytes remain poorly understood. We recently identified a Wilson’s disease mutation that affects the intracellular trafficking of ATP7B, while having little effect on ATPase activity itself, indicating that a mislocalization of ATP7B is sufficient to cause Wilson’s disease (5).

The physiologic maintenance of luminal pH is critical for intracellular trafficking (6). Luminal pH acidifies from approximately pH 6.0 in the Golgi to 5.5 in exocytotic vesicles nearing the cell surface. In the endocytic pathway, the luminal pH forms a gradient beginning with pH ~6.5 in early endosomes to between pH 6 and 5.5 in late endosomes and lysosomes. Organelle acidification is driven primarily by V-ATPase, a large multisubunit,
ATP-dependent proton pump (7). Bafilomycin-A1 (Baf), a fungal metabolite, specifically inhibits V-ATPase activity at nanomolar concentrations (7,8). Both Baf and weak bases perturb membrane trafficking, as they disperse pH gradients and in the process disrupt both intracellular location and receptor–ligand dissociation (9–12).

Previous studies, using a variety of overexpression systems and cell lines, have reached different and sometimes conflicting conclusions about the localization and trafficking itinerary of ATP7B (13–19). This study focuses on endogenous ATP7B, in a cell model for polarized hepatocytes, which are the chief cells that express ATP7B in vivo. Our main finding is that in high Cu, endogenous ATP7B traverses endosomes near the basolateral domain en route to the apical plasma membrane. Furthermore, luminal acidification was required for the cell to redirect ATP7B to the apical domain via this pathway and maintain it there under conditions of high Cu.

Results

Cu levels regulate the reversible trafficking of endogenous ATP7B in polarized WIF-B cells

Free Cu levels in normal human blood are about 1 μM and are 10- to 50-fold higher in patients with Wilson’s disease (20). In our initial study of ATP7B in WIF-B cells, we treated cells with 200 μM Cu for 4 h and observed Cu-directed trafficking of both endogenous and exogenous ATP7B (19). However, exposing cells cultured to super-physiological levels of Cu for periods of hours is unlikely to model the in vivo situation with fidelity. In hepatocytes in vivo, endogenous ATP7B should respond to Cu concentrations typically seen in the blood, and the redistribution toward the apical domain should occur with sufficiently rapid kinetics to avoid Cu toxicity. Indeed, in primary cultures of hepatocytes, endogenous ATP7B redistributes to vesicles within 15 min of exposure to Cu (4). We thus sought conditions for ATP7B trafficking in WIF-B cells that were more analogous to the physiologic situation. In a previous study, we discovered that exposing WIF-B cells to 10 μM Cu was sufficient to redirect exogenous GFP-ATP7B to the apical region (13). We tested the suitability of this condition for studying the Cu-induced trafficking of endogenous ATP7B during the first hour of Cu exposure (Figure 1).

When cells were depleted of Cu following an overnight incubation in 10 μM of the cell-impermeable Cu chelator bathocuproine sulfonate (BCS), fixed and stained with antibodies against ATP7B, the protein was predominantly in the TGN and overlapped with the post-TGN marker syntaxin 6 (Figure 1A–C). When WIF-B cells were transferred to 10 μM CuCl2 for 60 min, most of the ATP7B was localized to the apical region marked by aminopeptidase N (APN) but also became more prominent in puncta dispersed in the cytoplasm (Figure 1D–F). When Cu-treated cells were subsequently incubated in 10 μM BCS for 3 h, ATP7B returned to the TGN region (Figure 1G–I). Colocalization analysis (Figure 1J) confirmed that the fraction of cellular ATP7B that colocalized with the TGN marker decreased from 72% in BCS to 17% after 60 min in Cu, with a concomitant increase in overlap with the apical marker from 7 to 78%. Returning Cu-treated cells to the chelation condition completely restored the predominantly Golgi localization within 3 h. Thus, WIF-B is a good model with which to study the physiological trafficking of endogenous ATP7B in hepatocytes, the chief cells expressing it in vivo.

Cu induces an increase in the number of ATP7B vesicles, which traverse large basolateral endosomes en route to the apical domain

In addition to the prominent localization of ATP7B in the Golgi when WIF-B cells were grown in BCS, they also exhibited a minor pool of ATP7B in puncta (Figure 2A’). These structures appeared much smaller than the TGN, and they localized predominantly in confocal planes near the coverslip, i.e. the peripheral planes, which correspond to the basal membrane attached to the substrate (Figure 2I). As shown in Figure 2C’, when WIF-B cells were exposed to Cu for various times and then fixed and stained with antibodies to ATP7B, localization in the TGN diminished concomitantly with an increased number of small ATP7B puncta, which arose prior to the appearance of ATP7B in the apical region (Figure 2C’). We used three-dimensional (3D) object analysis to estimate the number of small ATP7B vesicles per WIF-B cell (Figure 2G). Addition of Cu approximately triples the number of ATP7B vesicles/cell at 15 min ~ from 50 to 150. At longer term exposure to Cu, when the majority of the ATP7B had reached the apical domain (Figure 2E), the number of circulating ATP7B vesicles diminished somewhat, yet the number remained double what it was during Cu chelation.
Figure 1: Cu directs the trafficking of endogenous ATP7B in WIF-B cells. A–C) WIF-B cells incubated overnight in 10 μM BCS to stage endogenous ATP7B at the TGN region. D–F) Cells switched to 10 μM CuCl₂ for 60 min. G–I) Cu-treated cells that were rinsed and re-incubated in 10 μM BCS for 180 min. After fixation, cells were triple-stained with antibodies to ATP7B (green), syntaxin 6, a post-TGN marker (red) and APN, an apical surface marker (blue). Single confocal planes are shown. J) The fraction of total ATP7B fluorescence that localized to the TGN or apical region in each condition was quantified as the extent of overlap with syntaxin 6 or APN, respectively. Data shown represent the mean ± SEM from at least three confocal stacks (∼28 WIF-B cells/stack, obtained from a single experiment).
In preliminary experiments, a range of antibodies that demark various intracellular compartments involved in the vesicular trafficking were screened. No marker was found, which showed an obviously high level of overlap with ATP7B in trafficking intermediates during its Cu-induced redistribution. However, a number of proteins involved in intracellular trafficking showed partial overlap, including (i) Vti1b, a Qb-SNARE involved in vesicle fusion, and

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(ii) the endosomal marker EEA1. We focused on EEA1 because it is a well-studied intracellular marker that selectively binds endosomal membranes rich in the lipid phosphatidylinositol 3-phosphate (PI3P), the product of the conserved endosomal protein VPS34. In earlier studies, we demonstrated that the VPS34 lipid product is critical for the function of distinct pools of endosomes located near the basolateral and apical membranes of WIF-B cells (21).

When WIF-B cells were incubated with Cu for various times, then fixed and double-stained for ATP7B (green) and EEA1 (red) (Figure 2B,D,F), some intracellular membranes, especially in confocal planes located near the coverslip (Figure 2′,D′,F′), appeared positive for both markers, as indicated by yellow regions (Figure 2D'). Colocalization analysis (Figure 2H) showed that the fraction of ATP7B that overlapped with EEA1 more than doubled during the first 15 min of exposure to Cu. When all of the EEA1 structures in the cell were digitally binned into either ‘small vesicular’ endosomes (cross-sectional area between 0.1 and 1 μm²) or ‘large sorting’ endosomes (area, >1 μm²), the majority of the total cellular EEA1 (60%) occurred in the ‘small vesicular’ pool, with the remainder in larger structures. However, the flux of ATP7B induced by Cu appeared exclusively in the larger EEA1 compartments, with no increased colocalization in the vesicular pool of EEA1 at any time after Cu exposure. When incubated in Cu for more than 15 min, the fractional overlap of ATP7B with EEA1 gradually returned to its starting (Cu chelation) value of about 20% by 90 min in Cu (Figure 2H). Thus, following the addition of Cu, TGN-derived ATP7B traverses a large EEA1-positive endosomal compartment en route to the apical domain.

Because the confocal images indicated that the preponderance of TGN-derived ATP7B vesicles was located in planes near the coverslip in WIF-B cells (i.e. the peripheral planes, which correspond to basal membrane attached to the substrate, Figure 2C'), we devised an analysis method to plot the normalized location of cellular markers in the axial plane (Figure 2I–K). The total fluorescence intensity attributed to each marker was normalized across all slices in the stack (i.e. the whole cell), which allowed us to plot the peak expression of each marker in z-orientation (Figure 2J). The Golgi region, demarked by TGN38, showed peak fluorescence at approximately 5 μm from the coverslip, while the apical marker APN showed a broader peak, with a maximal fluorescence at about 6 μm above the coverslip. In contrast, endosomes demarked by EEA1 peaked at 2 μm – about six confocal planes closer to the coverslip compared with the Golgi and apical markers.

Axial distribution analysis of the cellular pool of ATP7B under Cu-chelation conditions produced a profile very similar to that of TGN38 (Figure 2K, green). In contrast, after 15 min in Cu, the distribution of ATP7B had shifted much closer to the coverslip/basolateral pole of the cell and became very similar to that of EEA1 (Figure 2K, red). By 90 min in Cu, the ATP7B distribution had assumed a

Figure 2: Cu-directed anterograde (TGN to apical) traffic occurs via TGN-derived vesicles that traverse an EEA1-positive compartment. A and B) WIF-B cells were incubated overnight in 10 μM BCS to stage ATP7B at the TGN region and then (C–D) switched to 10 μM CuCl₂ for 15 min, or (E–F) 10 μM CuCl₂ for 60 min. Cells were fixed and double-stained with antibodies to ATP7B (green) and EEA1 (red), then imaged using confocal microscopy. A–F) Single nuclear confocal planes. A′–F′) Single peripheral planes. Renderings of representative stacks from these time-points are shown in the top row of Movie S2. G) The number of ATP7B vesicles/cell was estimated using 3D objects-based analysis. H) Fraction of ATP7B fluorescence that was coincident with EEA1-positive small and large endosomes. Data shown in (G) and (H) represent the mean ± SEM of at least four confocal stacks from a single experiment. I) Schematic cartoon of a WIF-B cell grown on a glass coverslip and rotated 90°. J) Confocal stacks were analyzed by displaying the normalized fractional distribution per slice of various organelle markers, to show the distribution of each marker along the z-plane of the cells. The Cu status of the cells had no effect on the distribution of these markers; thus, the data shown are pooled with no respect to Cu status, from 7 to 12 confocal stacks, from at least two independent experiments. EEA1-positive endosomes (red) distribute closer to the coverslip (~2 μm above it) compared to the TGN38 (Golgi, green, ~5 μm above coverslip) or APN (apical membrane, blue, ~6 μm above coverslip). K) Cu-induced redistribution of ATP7B into a large basolateral EEA1 endosomes (15 min, red profile).
profile similar to that of the apical marker APN (Figure 2K, blue). Thus, the large endosomal compartments traversed by ATP7B following Cu administration are concentrated near the coverslip in WIF-B cells, which corresponds to the blood sinusoidal/basolateral domain of hepatocytes in vivo (22).

We wondered whether a similar trafficking itinerary (Golgi to basolateral endosome to apical domain) occurred in hepatocytes in vivo after Cu administration. To test this, rats raised on a Cu-deficient diet were given Cu and then killed after 0, 1 or 2 h. Figure 3 shows liver triple-immunostained cryosections for ATP7B (Figure 3A,D,G, green), TGN38 (Golgi, Figure 3B,E,H, red) or dipeptidyl peptidase DPP4 (apical, Figure 3B,E,H, blue). In Figure 3J, the concomitant decrease in Golgi localization and the increase in apical localization that occurs after Cu administration were quantified. The majority of ATP7B colocalized with the apical marker DPP4 2 h after Cu treatment. The arrows (Figure 3D–F) indicate the transient appearance of ATP7B near the basolateral domain following 1 h of Cu administration in vivo.

The transient basolateral pool of ATP7B in hepatocytes was more easily observed in 3D renderings. Movie S1, Supporting Information, depicts rendered confocal stacks at 0, 1 and 2 h after Cu administration. ATP7B fluorescence is in green color, with the areas of ATP7B that overlap with the apical marker DPP4 highlighted in cyan. Consistent with the colocalization analysis (Figure 3J), a small pool (about 18% of the cellular total) of ATP7B that appeared coincident with the apical marker even in the low-Cu condition in vivo were observed. The line of red arrows in the 1-h 3D image demarks a population of non-Golgi non-apical ATP7B, which appears transiently in the basolateral region at 1 h after Cu administration and is the pool presumably en route to the apical domain. By 2 h, this pool is diminished (3D image on right). Thus, the unique Cu-directed trafficking pathways of ATP7B appear similar in polarized WIF-B cells and in hepatocytes in vivo.

Deacidification prevents Cu-directed delivery to apical domain

Intracellular trafficking and sorting via endosomes requires precise physiological control of luminal pH, which depends critically on proton pumping by V-ATPases. Disruption of luminal pH gradients perturbs intracellular trafficking in a range of cells and tissues (23). We tested whether disruption of luminal pH affected the intracellular trafficking of ATP7B. Figure S1 depicts WIF-B cells treated with three different perturbants known to neutralize luminal pH. Cells were labeled by uptake of the cellular dye 3-(2,4-dinitroanilino)-3′-amino-N-methylpropylamine (DAMP), which accumulates physiologically in acidic intracellular compartments (A). Cells treated with Baf, chloroquine (CQ) or NH₄Cl (B–D) showed almost complete loss of acidic compartments.

Figure 4 illustrates how deacidification affects Cu-directed apical delivery of ATP7B. When the pH pertubants were present for 30 min under Cu-chelation conditions, the fraction of ATP7B coincident with TGN38 did not change appreciably (Figure 4M, left set of bars). In contrast, when the perturbants were present during the 1-h Cu exposure, each prevented Cu-directed apical delivery (Figure 4M, right set of bars). In all cases, apical delivery was reduced to less than 20% of control levels, with Baf being the most efficacious (93% reduction in apical delivery). Thus, maneuvers that disrupt luminal pH via different mechanisms show the common property of blocking apical delivery, suggesting that it is low pH itself – rather than a selective, pH-independent effect of Baf on the V-ATPase (24) – that is responsible for the blockade of ATP7B’s Cu-directed apical delivery.

To further explore the apparent lack of effects of Baf when Cu is chelated, WIF-B cells were treated with BCS + 50 nM Baf for 4 h and refreshed Baf once per hour in case the drug was rapidly metabolized by the cells (Figure S2). If cells were kept in chelator, deacidification, even for 4 h, had only a modest effect on ATP7B localization. The degree of overlap with the post-Golgi marker syntaxin 6 was modestly reduced from 47 to 35%, but the number of circulating ATP7B vesicles was not affected by a 4-h treatment in BCS + Baf. Thus, the effect of luminal acidification on ATP7B trafficking was remarkably selective for the Cu-directed pathway, suggesting that the sorting mechanisms that redirect ATP7B to the apical domain when Cu levels rise require luminal pH gradients, while retention and/or recycling of ATP7B at the TGN in low-Cu level do/does not.
Figure 3: Cu-directed TGN-to-apical trafficking occurs via a basolateral compartment in hepatocytes in vivo. A–C) Liver section from a rat raised on a low-Cu diet for 38–45 days since birth. D–F) Liver section from a rat with low Cu level that was given with Cu and then killed after 60 min or (G–I) 120 min. Rat livers were fixed by immersion, and cryosections were immunolabeled for ATP7B (green), TGN38 (red) or apical surface marker DPP4 (blue) and then imaged using confocal microscopy. G–I) Arrows indicate the transient appearance of ATP7B in the basolateral region 60 min after Cu injection. J) Quantification of Cu-induced trafficking in vivo. The graph depicts colocalization analysis on confocal stacks from liver sections stained above. Cu causes a decrease of ATP7B in the Golgi (solid line), with a concomitant increase in ATP7B trafficking to the apical domain (dotted line).
Luminal pH Affects ATP7B Trafficking Through Endosomes

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Baf causes ATP7B to accumulate in large endosomes at the basal end of the cell

Figure 4L′ depicts single-plane images of large, heterogeneous ATP7B-positive compartments that form predominantly in confocal sections near the coverslip in WIF-B cells after a 1-h Cu treatment in the presence of Baf. Analysis of the axial distribution of ATP7B in these cells confirmed that Cu-directed ATP7B accumulates near the coverslip, preventing delivery to the apical membrane (Figure 4N, gray line). In Figure 5, we characterized the large basal EEA1 structures in more detail. Figure 5A–C depicts a typical group of five ATP7B clusters co-labeled for EEA1 (each cluster approximately of 5 μm in diameter). The clusters were already apparent as early as 15 min after Cu + Baf exposure. After 60 min of treatment with Baf and Cu, ATP7B had accumulated in two distinct pools of large EEA1-positive endosomes, apical and basolateral (Figure 5E,E′).

To characterize the Baf-induced ATP7B clusters, masks were created to isolate the large EEA1-positive structures, which formed quickly upon exposure to Baf and were more homogeneously labeled across the cluster when labeled by antibodies to EEA1 compared with those to ATP7B (compare Figure 5A and B). Focusing on large EEA1 structures allowed for a more robust particle analysis. Following exposure to Baf, nearly 100% of these large clusters were triple-positive for ATP7B, EEA1 and Vti1b. When we size-segregated small and large EEA1 compartments as performed previously in our analysis of Cu-directed intermediates, a remarkable fraction (∼70%) of the ATP7B in the cell was localized to the large compartments by 60-min Cu + Baf treatment (Figure 5F). Conversely, the amount of ATP7B localized to the small EEA1 (vesicular) compartment decreased to <5% (Figure 5F, dotted line), compared with ∼10% in controls (Figure 2H, dotted line).

Baf treatment also affected the size of large EEA1 structures themselves (Figure 5G). The mean cross-sectional area of the large structures (those with an area >1 μm²) approximately doubled, from 2 to 4 μm², while the fraction of the total EEA1 pool that accounted for them also doubled, from about 40 to 80% (Figure 5H). Baf also caused an obvious reduction in the abundance of small, vesicular-like EEA1 structures; this was not quantified. The overall effect of Baf on EEA1 was to promote pooling and accumulation of the protein into large endosomal compartments.

Within the large clusters shown in Figure 5A–C, it was clear that ATP7B and EEA1 were distributed heterogeneously, suggesting micro-organization of the membrane. Specifically, it was unclear whether the ATP7B punctae occurred in continuity with the main EEA1 compartment or rather consisted of closely associated, but distinct, vesicles. It was also unclear whether the EEA1 was organized as one big structure or consisted of aggregates of smaller vesicles. To address these questions, we applied a confocal/deconvolution technique that used a higher pixel density and a smaller pinhole to increase our ability to image these structures. We examined three time-points (15, 30 and 60 min) after Cu + Baf treatment. Altogether, we analyzed 22 examples. Figure 6 shows an example of one of these structures. The overall dimensions among the structures showed considerable heterogeneity, especially at the 60-min time-point when the cross-sectional area...
Figure 5: Legend on Next page.
ranged between 3 and 28 μm², and their thicknesses varied between 1.5 and 3 μm. EEA1 and ATP7B typically exhibited a complementary relationship within the structures, and sometimes we observed what appeared to be distinct ATP7B vesicles (which were negative for EEA1) in the local vicinity of the main EEA1 structure, which appeared as a more contiguous tubular network. In Figure 6C, we show that the size of the structures increased with Cu + Baf treatment duration, confirming the prior analysis. Colocalization analysis (Figure 6D) indicated that the amount of ATP7B fluorescence that was coincident with EEA1 varied between 42 and 49%, and did not change significantly with time of exposure to Cu + Baf. These data are consistent with a model in which Baf creates a blockade in the budding, pinching off or tubulation processes that operate in high Cu to sort ATP7B from large, tubular EEA1+ endosomes, prior to delivery to the apical membrane.

Movie S2 provides a visual summary of the effects of Cu in the absence and presence of Baf. The top row of renderings shows ATP7B fluorescence merged with the red mask for the large EEA1 compartment. When Cu was added in the absence of Baf, a transient increase in overlap with large EEA1 compartment (middle panel) was seen prior to apical delivery (right panel). The bottom row shows the accumulation of ATP7B in these large compartments as early as 15 min after Cu + Baf treatment, and more ATP7B accumulated there after 60 min. By manually counting these structures in 3D renderings, the number of large Baf-induced EEA1 structures per cell is found to be less – fewer than 10 per cell.

**Baf washout restores Cu-directed apical localization**

As deacidification inhibits degradative processes in lysosomes and can both inhibit (25) and enhance (26) autophagy, we wondered whether the large EEA1 structures were created by changes in trafficking to these compartments. In Figure S3, we labeled vehicle or Baf-treated cells with markers against lysosomes (LAMP1, A–B), Cathepsin D (C–D) or autophagosomes (LC3B, E–F) and observed no overlap between these organelles and the ATP7B clusters. This result indicates that it is unlikely that ATP7B had accumulated in a degradative compartment.

Furthermore, if ATP7B had accumulated in terminal degradative compartments, then removal of the drug would not be expected to restore apical delivery. Figure 7 shows that the blockade of ATP7B apical delivery produced by Baf is reversible following Baf washout. Baf exposure, even for 3 h, did not affect ATP7B protein levels (Figure 7J). This indicates that changes in the rate of synthesis and degradation do not account for the effect of Baf on ATP7B trafficking. Moreover, the half-life of ATP7B in hepatic cells is ~8 h (27), which is considerably longer than the timeline of our experiments. Following washout, apically localized ATP7B became apparent by 3 h (compare Figure 7C and E, with concurrent disappearance of the large ATP7B clusters in the periphery (Figure 7D′,F′). The effects of Baf washout were quantified in Figure 7G,H,I using the methods we developed to characterize Cu-directed trafficking. The accumulation in large structures near the coverslip was almost completely

**Figure 5: Baf plus Cu causes ATP7B to accumulate in large EEA1-positive endosomes located predominantly at the peripheral region of WIF-B cells.** Cells were treated same as Figure 4 but fixed at 15-min intervals during the 60 min of Cu treatment plus DMSO or 50 nm Baf and then double-stained for ATP7B (green) and EEA1 (red). Substantial overlap between (A) ATP7B and (B) EEA1 was seen in large clusters at the peripheral region of cells as early as 15 min after Cu plus Baf. C) Magnification of a group of typical Baf-induced clusters (each ~5 μm across), with ATP7B concentrated in subdomains (arrows) of the large and more homogeneous EEA1-positive endosomes. D) 60 min after Cu plus DMSO or (E) Cu plus Baf. Single confocal planes from the (D–E) nuclear or (D′–E′) peripheral region show overlap between ATP7B and EEA1 persisted in Baf-treated cells. 3D renderings of confocal image stacks representing 0-, 15- and 60-min time-points are shown in Movie S2. F) The extent of overlap between ATP7B and EEA1 during 60 min of Cu plus Baf was quantified from at least four confocal stacks from a single experiment. The 0- and 60-min time-points represent the mean of 10 confocal stacks, obtained from three independent experiments. G) To characterize the effect of Baf on the EEA1-positive endosomes, the mean cross-sectional area of the large EEA1 structures was measured across all experimental conditions and plotted against the time that the cells were incubated in Baf or DMSO. H) The fraction of total EEA1 that was present in ‘large’ EEA1 organelles, defined as having >1 μm² cross-sectional area in the 2D analysis.
reversed (Figure 7I), and the number of circulating ATP7B vesicles was restored to control levels (Figure 7H). The restoration of apical delivery following Baf washout was also observed, but it was partial (Figure 7G). This is not unexpected, owing to the high binding affinity of Baf for the V-ATPase, which has a dissociation constant ($K_D$) of approximately $10^{-8}$ M (28). Overall, our results showed that, upon Baf washout in the presence of Cu, ATP7B could be rescued from the large peripheral structures and redirected toward the apical domain.
Baf blocks apical recycling, which maintains ATP7B at the apical surface

We focused next on the pathways involved in the maintenance of ATP7B at the apical membrane in Cu-treated cells (Figure 8). In these experiments, cells were treated for 1 h with Cu to stage ATP7B at the apical domain, and then Baf was added in the continued presence of Cu. Baf caused a progressive loss of ATP7B colocalization with APN, in conjunction with increased colocalization with EEA1 (Figure 8H). The effect could be measured already after 30-min exposure to Baf (left panels), which suggests that significant amounts of apical ATP7B are recycling through subapical EEA1 endosomes in high Cu. The effect of Baf was more pronounced after 3 h (right panels), when approximately 70% of the apical colocalization was lost (Figure 8M), and ATP7B had accumulated in...
Figure 8: Legend on Next page.
large endosomal structures throughout the cell. During quantitative western blot, ATP7B (Figure 8N) showed no change in ATP7B protein levels, ruling out perturbed rates of degradation and/or new synthesis. Thus, our results suggest that ATP7B is actively maintained at the apical membrane via recycling, and that Baf causes a selective blockade in exocytosis from endosomes located near the apical plasma membrane.

**Discussion**

A major finding of this study is that, in WIF-B cells, the Cu-directed trafficking of endogenous ATP7B occurs via large basolateral endosomes prior to appearance at the apical domain. The physiological relevance of this finding was validated in hepatocytes *in vivo*, where ATP7B exhibited a similar trafficking itinerary: TGN to the basolateral region to the bile canaliculus. A second major finding is that Cu-directed trafficking depends on luminal acidification at two main intracellular sites: exit from a basolateral sorting endosome and exocytosis to the apical plasma membrane.

In low-Cu conditions, the localization of ATP7B was barely affected by loss of acidification. Yet, when Cu levels were raised, delivery to the apical domain was almost completely blocked. The exquisite sensitivity of ATP7B localization to luminal pH when Cu levels are high implies that pathological processes or mutations that disrupt luminal pH in hepatocytes would reduce the capacity of ATP7B-dependent Cu sequestration and extrusion into the bile, leading to Cu toxicity. Further study will be required to test this idea directly.

A summary of our findings in WIF-B cells is depicted in Figure 10. In low-Cu levels, the majority of ATP7B (∼85%) is associated with the Golgi, with the remaining 15% in vesicles concentrated near the basolateral membrane – a localization that is analogous to the sinusoid, the site of Cu uptake from the blood in hepatocytes. If deacidification is induced under this condition, only minor effects on ATP7B localization occur. The ATP7B vesicles are positioned to function as Cu sentinels in the cytosol, near sites of Cu uptake from the blood and equipped with ATP7B for rapid Cu sequestration. In low Cu, we presume that these vesicles (∼50/WIF-B cell) recycle to the TGN. In hepatocytes with low-Cu levels, any Cu sequestered by such vesicles near

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**Figure 8: Cu-dependent localization of ATP7B at the apical domain is maintained by local recycling through apical endosomes and is perturbed by Baf.** A) WIF-B cells were incubated overnight in 10 μM BCS, switched to 10 μM CuCl₂ for 60 min to stage ATP7B at the apical region, and then fixed. B–G) Parallel sets of cells were maintained in Cu for an additional 30 min or (I–L) 180 min in the presence of (B–D), (I–J) DMSO, or (E–G), (K–L) 50 nM Baf. Cells were stained with antibodies to ATP7B (green), EEA1 (red) and APN (blue), and imaged using confocal microscopy. A–G and I–L) Single planes from the nuclear region. D′, G′, J′ and L′) Single planes from the peripheral region. H) Quantification of the extent of overlap between ATP7B and EEA1 or APN after 30 min or (M) 180 min in Cu ± Baf. Data shown in (H) are the mean of seven confocal stacks from two independent experiments (∼196 cells); data in (M) represent three stacks from a single experiment (∼84 cells). N) Western blots of ATP7B from duplicate coverslips treated as in (A), (I) and (K) (in three separate experiments) indicate no significant changes in ATP7B protein levels.
Figure 9: Cu washout initiates retrograde traffic (apical to TGN) of ATP7B, which is largely unaffected by Baf. ATP7B was staged at the apical region of WIF-B cells with Cu (Figure 7A). Cells were then pre-incubated for 30 min in DMSO or Baf to deacidify intracellular compartments [see Figure 7B (+DMSO) and Figure 7E (+Baf)]. Cells were thereafter rinsed and incubated for an additional 180 min in 10 μM BCS to initiate retrograde traffic of ATP7B. A–C) Kinetics of ATP7B retrograde trafficking in the presence of DMSO or (D–F), 50 nM Baf. Cells were fixed at various times after initiating retrograde trafficking, triple-stained with antibodies to ATP7B (green), EEA1 (red) and APN (blue), analyzed, and imaged using confocal microscopy. A–F) Single planes from the nuclear region. C′ and F′) Single planes from the peripheral region. G) Image analysis was used to quantify the fraction of ATP7B that overlapped with EEA1 or (H) TGN38. Data for each time-point represent four to seven confocal stacks from one or two independent experiments, depending on the time-point.

the sinusoid would therefore be returned to the secretory pathway for use in metallation reactions.

When intracellular Cu levels rise, the number of ATP7B vesicles triples and the predominant Golgi-like localization of ATP7B disappears, as the protein is redirected apically; this would presumably increase the capacity of rapid Cu sequestration in the cytosol followed by extrusion at the apical membrane when Cu is in excess. Notably, even at long times of Cu exposure, the fractional overlap of
Figure 10: Summary of results. A) When Cu levels are low, about 80% of ATP7B resides in the TGN, with the remainder in small vesicles that localize near substrate side of the cell, close to the coverslip. Treatment of WIF-B cells with Baf in low Cu does not perturb localization in the TGN. B) When Cu levels are increased, this induces a rapid doubling of ATP7B vesicles, presumably derived from the TGN. These vesicles transiently traverse a pool of large EEA1-positive endosomes located near the substrate. Segregation and exit from these ‘basal’ endosomes is inhibited by loss of luminal acidification. A second site of Baf action was revealed to be rapid recycling through an endosomal pool that lies in close association with the apical plasma membrane. Treatment with Baf promotes accumulation in these endosomes. C) When Cu is removed to induce ATP7B trafficking back to the TGN, the process proceeds similarly in the absence or presence of Baf. Thus, the role of acidification is selective for ATP7B trafficking routes induced by Cu.

ATP7B and TGN38 remained at 15%, which implies that ATP7B-dependent delivery of Cu to the TGN lumen persists in high Cu. Thus, when intracellular Cu levels are high, some ATP7B continues to return to the TGN but perhaps does not reside there long before exiting again as vesicles. A similar continual flux through the TGN in high Cu has been shown for ATP7A (29).

We find that endogenous ATP7B traverses large basal endosomes en route to the apical domain. A similar itinerary to the plasma membrane (via EEA1-positive endosomes) was suggested in another recent study, which used exogenous ATP7B in COS-7 cells (16). Once ATP7B exits the TGN, it appears to be committed to a trafficking route toward the basolateral domain prior to segregation and retrieval from large endosomes for apical delivery. The microtubules in polarized WIF-B cells are organized such that the plus ends localize near the basal membrane and the minus-end rich microtubule-organizing centers are located in close vicinity to the apical plasma membrane and Golgi (22). This strongly suggests that long-range transport of ATP7B is mediated by microtubule motors, both minus-end and plus-end directed, as suggested previously by a requirement for dynactin in ATP7B trafficking (30). We estimate that the distance an ATP7B molecule originating at the TGN must traverse to reach the apical membrane in a WIF-B cell is ~7 μm; that is, 3 μm to go from TGN to basal endosomes, plus 3 μm to go from basal endosomes to apical region, plus 1 μm for apical delivery.

In Cu-treated cells exposed to different pH neutralizers, ATP7B vesicles still exited the TGN but they failed to reach the apical region. Instead, the protein accumulated in large EEA1 endosomes, which themselves grew larger with increasing times in Baf. Within these structures, ATP7B and the endosomal marker EEA1 exhibited a complementary relationship and approximately 50% overlap (Figure 6). Our experiments indicate that Cu-directed ATP7B trafficking involves a Baf-sensitive mechanism, which functions to segregate ATP7B out of large endosomes prior to retrieval and delivery to the apical region. The presence of Baf blocks one or more of the processes involved in segregation, budding or tubulation from the main EEA1 structure, prior to the creation of cargo destined for the apical domain.

A number of previous studies on ATP7B have employed overexpressed protein (31) using nonpolarized cells (32) and/or treated cells with Cu for as much as 10× longer and/or at a 10× higher concentration (18) than in this study. Under conditions of pathological Cu overload, increased amounts of both Cu and lysosomal hydrolases appear in the bile, owing to Cu toxicity (33,34). These studies have led to the suggestion that ATP7B resides physiologically in hepatic lysosomes, which fuse directly
with the apical membrane. One recent report, which employed exogenous GFP-ATP7B in nonpolarized cells, concluded that secretory lysosomes are a major pathway for the delivery of ATP7B to the plasma membrane (17). In this study, endogenous ATP7B in polarized WIF-B cells was not found in lysosomes. Our finding that ATP7B traverses sorting endosomes prior to retrieval to the apical domain suggests that the different results of previous studies are explained by saturation of the physiologic segregation and redirection systems in endosomes during super-physiological Cu conditions and/or if ATP7B is overexpressed. In such a scenario, ATP7B cargo would be expected to escape rescue from endosomes and accumulate non-physiologically in late endosomes or lysosomes.

In endosomes near the apical plasma membrane, we uncovered a second site of ATP7B sensitivity to Baf. Loss of luminal acidification blocked ATP7B recycling through apical endosomes. In the presence of Cu, Baf blocked exocytosis of ATP7B, preventing apical delivery. Thus, physiologic luminal acidification is required not only for the apical targeting of ATP7B but also for its maintenance at the apical membrane in continued high Cu. A number of previous studies have shown a requirement for luminal acidification for exocytosis at the plasma membrane (35).

On the basis of the results of this study, one might hypothesize that all apical trafficking is blocked by deacidification. However, the apical resident protein APN, which takes a transcytotic route (36), was not affected by Baf. In experiments where we compared ATP7B and APN (not shown), the amount of ATP7B found in the APN ‘small’ compartment, which consists mainly of vesicles en route to the apical domain, was never more than 3% – nor did it change with Cu levels or following loss of luminal acidification. Additional experiments using continuous uptake of anti-APN from the basolateral surface of WIF-B cells showed that vesicles in the transcytotic pathway, which also target the apical surface (22), were successfully delivered in the presence of Baf and did not form large clusters as the apically targeted ATP7B did. Thus, the Cu-directed route of ATP7B is different from the route taken by newly synthesized resident apical proteins in hepatocytes.

What mediates the Baf-sensitive retrieval mechanism of ATP7B from large endosomes in high Cu? One attractive candidate mechanism involves retromer (37), which was recently shown to interact with ATP7A, the Menkes disease protein (38). Retromer mediates minus-end-directed cargo retrieval from EEA1-positive endosomes via the transient formation of tubules. We did not observe such tubules, but they are labile and may not be preserved in fixed cells. Our images of these structures are consistent with the idea that luminal acidification did not prevent the micro-segregation of ATP7B cargo within the endosomal membrane, but rather blocked the separation of this cargo prior to long-range trafficking to the apical domain. Interestingly, a genetic link between retromer-mediated trafficking and luminal pH has been shown in yeast (39,40).

Does the post-endosomal itinerary of ATP7B proceed via vesicles, tubules or both? In contrast to the apparently long distances traveled by ATP7B en route to the apical domain, the return trip (apical to Golgi) appears to be much shorter (<1 μm), owing to the close juxtaposition of the apical membrane and the Golgi. Live cell imaging, in conjunction with direct knockdown of retromer machinery, will be required to address these questions.

Materials and Methods

Materials
Bathocuproine disulfonic acid (BCS), cupric chloride (CuCl2), Baf, CQ, dimethyl sulfoxide (DMSO), imidazole, AEBSF and Pipes were purchased from Sigma, while ammonium chloride (NH4Cl) was from J.T. Baker. NuPAGE Novex 4–12% gradient Bis–Tris gels and MOPS SDS Running buffer were from Invitrogen, while PVDF Immobilon-P membrane was from Millipore. Halt protease inhibitors and West Pico super signal substrate were from Thermo Scientific. All other chemicals were of the highest purity and obtained from sources identified in previous publications.

Antibodies
Primary antibodies used for indirect immunofluorescence were as follows: Figures 1, 4, 6 and S3 show rat anti-ATP7B (S. Lutsenko, Johns Hopkins School of Medicine); Figures 2, 5, 7, 8, S2 and Movie S2 show rabbit anti-ATP7B (#ab124973; Abcam); Figures 3 and Movie S1 show rabbit anti-ATP7B (Dr J. Gitlin, Brown Alpert Medical School); rabbit anti-APN [#1637, (22) guinea pig anti-DPP4, (41)] goat anti-EEA1 (#sc-6415, Santa Cruz Biotechnology); mouse anti-TGN38, and syntaxin 6 (BD Biosciences); mouse anti-LAMP1 (#H4A3-s, Developmental Studies Hybridoma Bank); rabbit anti-Cathepsin D (Dr A. Hasilik); rabbit anti-LC3B (#2775, Cell Signaling Technology). Secondary antibodies conjugated to Cy3 or Cy5 were from Jackson ImmunoResearch Laboratories, while those conjugated to Alexa 488, 568, or 647 were from...
Molecular Probes. Primary antibodies used for immunoblotting included rabbit anti-ATP7B (#3985) (13), mouse anti-alpha tubulin, and DM1A (Sigma). Secondary antibodies conjugated to horseradish peroxidase (HRP) were from G.E. Healthcare.

Cell culture
WIF-B cells were seeded at 2 \times 10^4 cells/cm^2 on glass coverslips (22 \times 22 mm), cultured as described (22,42) and used at 11–13 days later when maximal polarity had been achieved.

Anterograde and retrograde trafficking assays
For ATP7B anterograde trafficking, cells were incubated overnight in 10 \mu M BCS to stage ATP7B at the TGN and then fixed, while a parallel set was rinsed and switched to 10 \mu M CuCl_2 for 60 min to direct ATP7B to the apical region and then fixed. In some cases, cells were fixed every 15 min during the 1-h Cu treatment (Figure 2). For ATP7B retrograde (apical region-to-TGN) trafficking, cells were treated as mentioned above, rinsed, and then incubated in 10 \mu M BCS for 180 min to promote ATP7B return to the TGN.

Baf, CQ and NH_4Cl treatments
To BCS or Cu-media, 50 \mu M Baf, 300 \mu M CQ, or 30 \mu M NH_4Cl was added 30 min prior to and during anterograde or retrograde trafficking assays. Controls were treated with DMSO (up to 0.05% of final concentration). For ATP7B retrograde trafficking in the presence of Baf (Figure 9), cells exposed to 10 \mu M CuCl_2 for 60 min were treated for an additional 30 min in Cu plus Baf or vehicle and then switched to BCS for 180 min, in the continued presence of Baf or vehicle. Prolonged treatment with Baf was used in some experiments to examine steady-state localization of ATP7B at the TGN (BCS-treated cells; Figure S2) or apical region (Cu-treated cells; Figure 8). For assays in chelator, cells were treated overnight in 10 \mu M BCS to stage ATP7B at the TGN and incubated an additional 240 min in BCS plus Baf and then fixed. For assays in Cu, cells were treated in Cu for 60 min to stage ATP7B at the apical region and incubated up to an additional 180 min in Cu plus Baf and then fixed (Figure 8K). Parallel sets of cells, in duplicate, were used as samples in western blots to assess ATP7B protein levels. In experiments where Baf was washed out to test reversibility, WIF-B cells were washed thrice with medium containing 10 \mu M CuCl_2 plus vehicle and then incubated in Cu for an additional 3 h. A parallel set of cells, in duplicate, were used as samples in western blots to assess ATP7B protein levels.

Copper deficiency and copper administration in vivo
Animal handling and experimental procedures were approved by the Johns Hopkins Animal Care and Use Committee. Copper-deficient Sprague-Dawley rat pups born to pregnant dams (Charles River Breeding Labs) were fed with a Cu-deficient chow (Harlan Tekland) beginning at 5–7 days prenatal and during the weaning period. The weaned pups were maintained on the same chow until they were 38–45 days old. Before the exposure to Cu, the Cu-deficient rats were starved overnight and then Cu was administered via intraperitoneal injection of CuSO_4 in saline (3 mg Cu/kg body weight). At 0, 1, 2 or 3 h later, rats were lightly anesthetized, decapitated, and their livers excised. Livers were rinsed briefly in cold 0.9% saline and then cut into 5 \times 20 mm blocks prior to immersion in fixative and cryosectioned as previously described (43). ATP7B was visualized with a rabbit polyclonal antibody (a gift of Dr J. Gitlin).

Indirect immunofluorescence
For experiments that used the rat anti-ATP7B antibody, WIF-B cells were processed at room temperature as follows. Cells were briefly rinsed in PBS, fixed 30 min in 4% paraformaldehyde/PBS and then permeabilized for 5 min in 0.2% Triton-X 100 in PHEM buffer (60 mM pipes, 25 mM hepes, 10 mM EGTA and 2 mM MgCl_2; pH 6.8). Fixed and permeabilized cells were incubated for 30 min in blocking buffer (PBS containing 1% BSA and 0.1% Triton-X 100) and then in primary antibodies (1 h) and secondary antibodies (30 min) diluted in blocking buffer. For experiments that employed the rabbit polyclonal ATP7B (ab124973; Abcam), cells were fixed on ice and permeabilized as previously described (22).

Confocal microscopy
Images were observed using a confocal microscope (Zeiss LSM 510 Meta), with a 100X oil objective (NA = 1.4) and a pinhole size of one airy unit. We collected twelve-bit confocal image stacks (512 \times 512 lateral dimensions; 0.176 \times 0.176 \mu m pixels) of 18–25 slices at 0.4 \mu m Z-step sizes from dual- or triple-labeled cells. Single-plane images in the figures were collated with Adobe Photoshop and Illustrator CS5 software.

Two-dimensional image analysis
Single channels from the confocal stacks were opened in Fiji, and the middle slice in the stack was auto-thresholded using the Triangle method (44); this threshold was applied to all images in the stack. ImageJ particle counting was used to identify and classify particles in each slice with a cross-sectional diameter of 0.1 \mu m^2 or larger, and create a binarized mask. Thus, the size cutoff considers structures in the slice 2 \times 2 pixels or larger (0.124 \mu m^3), while particles with a cross-sectional area smaller than 0.1 \mu m^2 were assumed to be a mixture of sub-resolution cellular structures and noise and were not analyzed. In analyses where we segregated organelle size, particles having a cross-sectional area between 0.1 and 1 \mu m^2 were designated ‘small’ and those with an area over 1 \mu m^2 area were ‘large’. The total fluorescence value for a given marker was calculated to be the sum of pixel intensity values in that channel’s masked region. For colocalization analysis, pixels common to two masks were found by combining the masks for each channel using the Fiji image calculator ‘AND’ function. Percent colocalization was expressed as the ratio of summed pixel values present in common mask divided by the summed pixel values in the ATP7B channel. Analyses were performed on between 3 and 12 confocal stacks obtained from between one and five independent experiments. In cases where analysis from a single experiment is shown, qualitatively similar results were observed in at least two independent replications.

Deconvolution confocal microscopy and analysis
WIF-B cells treated with 10 \mu M Cu + 50 \mu M Baf for 15, 30 and 60 min were fixed and labeled for ATP7B (green), EEA1 (red) and APN (blue).
We used a Zeiss LSM 710 confocal microscope with the pinhole set to 0.5 airy units (green channel) to collect stacks in 0.25–μm Z-steps. Using a 5x digital zoom to focus on the large EEA1/ATP7B-positive structures located approximately 1–3 μm above the coverslip, we collected 16-bit images of dimensions 1024 × 1024 pixels, using a 100× NA 1.4 oil objective. Thus, in this analysis, the lateral dimensions of the pixels were 0.021 × 0.021 μm, which is nearly 10× smaller than what was used in the prior analyses. All stacks were collected from fully polarized WIF-B cells, as judged by the presence of an apical surface labeled by APN. The stacks were further subjected to 10 rounds of blind, reiterative 3D deconvolution using the default settings (median noise) of the Autodeblur algorithm in the Autoquant package (version X. 1.4.1). Deconvoluted images were exported as single-channel .tif images and imported into Fiji, where contrast was adjusted by use of a stack histogram, in which 0.1% of the pixels were saturated. To obtain dimensions of the structures and perform colocalization analysis, individual structures were cropped from the original images and also cropped in the axial plane such that only structures located near the coverslip remained in the stack, while more nuclear planes were excluded. Dimensions of the structures in XY were measured from maximal projections of the EEA1 channel. Dimensions in Z were estimated by multiplying the number of Z-sections encompassing the structure by the step size (0.25 μm). Colocalization analysis for ATP7B and EEA1 was performed similarly to the other analyses, except that the masks were not size-selected and the Otsu thresholding routine from Fiji was used. Images in Figure 6A are maximal Z-projections. Images in Figure 6B are single confocal planes. Altogether, we analyzed a total of 22 distinct EEA1/ATP7B structures over the three time-points.

3D analysis
To estimate the number of ATP7B vesicles/cell, the binarized masks used in the two-dimensional (2D) analysis were opened in Fiji and processed using in the 3D Particle Analyzer from the BoneJ plugin (45). Trial and error testing established that an object volume cutoff of 0.176 μm3, which corresponds structures containing between 4 and 164 voxels (0.176 × 0.176 × 0.4 μm dimensions), reliably segregated the TGN pool from the vesicular pool of ATP7B. Thus, objects called ‘vesicles’ in this analysis had an estimated volume between 0.004 and 3 μm3. Images were rendered in 3D using the 3D viewer in Fiji.

To estimate the number of cells in a microscopic field, the ATP7B fluorescence channel was used to identify and count the (unstained) nuclear profiles, including those from cells on the edges of the stack. The number of cells in the stack was estimated to be the number of full-cell nuclei in the stack plus half the number of those nuclei of cells at the edge of the stack. Among the stacks collected over multiple experiments, the estimated number of WIF-B cells per stack ranged from 19 to 33, with a mean of 27.5 ± 6.2 (SD); N = 32 confocal stacks.

Digital analysis of distribution in the axial plane
To determine the z-distribution of a fluorescent label, the distribution of fluorescence units for each fluorescence channel after thresholding and masking (describe above) was expressed as the percent of total per slice in the stack. The closest slice to the coverslip showing detectable fluorescence was defined as being 0.4 μm above the coverslip (i.e. slice 1), and subsequent slices were spaced at 0.4-μm steps above. Thus, in these analyses, the area under each curve equals 1 (the total fluorescence of that marker) defined by the summed pixel intensities contained within the mask.

Detection of acidic organelles using DAMP stain
The extent of deacidification by 50 nM Baf, 300 μM CQ or 30 mM NH4Cl was confirmed using DAMP labeling as described in (46), with some modifications. Briefly, WIF-B cells were incubated in basal media containing Baf, CQ or NH4Cl for 30 min. DAMP (30 μM) was added for an additional 60 min in the continued presence of the drug in basal media. Cells were fixed for 30 min in 4% paraformaldehyde in PBS, permeabilized at room temperature for 5 min in 0.2% Triton-X 100 in PHEM buffer, then blocked in 1% BSA/PBS for 30 min. Incubations with primary (Goat anti-DNP, Oxford Biomed Research) and secondary (Donkey anti-goat Cy3) antibodies were carried out at 37 °C for 1 h each in 1% BSA/PBS. Confocal images were acquired at the same laser, detector, and pinhole settings to allow direct comparison of the fluorescent signal after each treatment.

Immunoblotting
Quantitative immunoblotting was used to assess ATP7B protein levels in WIF-B cells at various stages of Cu-responsive assays ± Baf. Briefly, WIF-B cells on coverslips were rinsed twice in PBS, scraped into 200 μL of imidazole buffer per coverslip (25 mM imidazole, pH 7.5, 250 mM sucrose, 1 mM AEBSE, Halt protease inhibitors), and sonicated. Whole-cell homogenates were diluted sixfold using imidazole buffer to a final volume of 1200 μL, of which 5 or 6 μL was added to 2x gel sample buffer (final: 20 mM Tris HCl, pH 8.8, 5% SDS, 0.1 M DTT, 15% sucrose, 2 mM EDTA and 5 μL urea) and heated at 50–55 °C for 10 min. Samples were analyzed using SDS–PAGE and western blotting (19). Membranes were probed with rabbit anti-ATP7B #3985-3 or mouse anti-alpha tubulin at 1:5000 or 1:6000, respectively. For each assay, three to four experiments were carried out with duplicate coverslips for each condition.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Figure S1: WIF-B cells treated with Baf or weak bases show rapid luminal deacidification. WIF-B cells in basal media were pretreated for 30 min with (A) DMSO; (B) 50 μM Baf; (C) 300 μM chloroquine or (D) 30 mM NH4Cl. DAMP was added in the continued presence of...
the various drugs for an additional 60 min before fixation. Antibodies against DAMP were used to visualize acidic compartments. The absence of intense DAMP labeling in (B–D) indicates lumenal deacidification. To compare the relative levels of lumenal deacidification by the each drug, confocal images were acquired using the same laser power and detector gain for all conditions.

**Figure S2: Prolonged treatment with Baf in the presence of BCS does not grossly perturb ATP7B localization at the TGN, or the number of circulating vesicles.** A) WIF-B cells were incubated overnight in 10 μM BCS to stage ATP7B at the TGN region. (B–D) Cells incubated an additional 240 min in BCS plus DMSO, or (E–G) BCS plus 50 nM Baf. Cells were fixed, double-stained with antibodies to ATP7B (green) and syntaxin 6, a post-TGN marker (red), then analyzed by confocal microscopy. (A–G) Single planes from the nuclear region. D' and G' single planes from the peripheral region. (H) The fraction of total ATP7B fluorescence that localized to the TGN was quantified as the extent of overlap with syntaxin 6. (I) The number of ATP7B vesicles per cell after 240 min in BCS ± Baf was quantified. The overlap with syntaxin 6 in the Golgi shown in (H) (∼50%) is lower than that in Figure 1 (∼72%) because of variation observed among different passages of WIF-B cells grown in BCS. Likewise, the estimated number of circulating ATP7B vesicles per cell in BCS shown in (I) (∼100) differs from the estimate shown in Figure 2G (∼50) for the same reason. Data shown for each condition represent three confocal image stacks from a single experiment, which corresponds to approximately 84 WIF-B cells.

**Figure S3: Baf does not redirect ATP7B to lysosomes or autophagosomes.** A, C, and E) WIF-B cells treated similarly as in Figure 4D,J with DMSO or (B, D, F) with 50 nM Baf, then fixed and double-stained with antibodies to ATP7B (green) and lysosomal markers (LAMP1 or Cathepsin D, red in (A–D) or autophagy marker (LC3B, red in (E, F)). Cells were analyzed and imaged by confocal microscopy. (A–F) Single planes from the nuclear region. (A′–F′) Single planes from the peripheral region.

**Movie S1:** 3D rendering of Cu-dependent localization of ATP7B in vivo: appearance of a transient basolateral pool. The movie shows 3D-rendered confocal stacks of liver sections, as described in Figure 3. The ATP7B is labeled green. Light blue regions indicate areas of ATP7B that colocalize with the apical marker DPP4. Arrows indicate ATP7B transiently localized in the basolateral region. Note: DPP4 is also observed in endothelial cells lining the sinusoids, but it is not depicted here because ATP7B is specific to hepatocytes.

**Movie S2:** Baf plus Cu induces the accumulation of ATP7B in large EE1-positive endosomes. The top row of 3D renderings shows the Cu-directed transient appearance of ATP7B in large endosomes when Baf is not present. The bottom row of renderings shows the accumulation of ATP7B in the presence of Baf, in large EE1-positive endosomes located predominantly at the cell periphery.

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