ATP7B expression and trafficking in the intestine and ovary: insights into specialized function

by

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A THESIS DISSERTATION

Presented to the Department of Biochemistry and Molecular Biology

School of Medicine

Oregon Health & Science University

In partial fulfillment of the requirements for the degree of

Master of Science

July 2010

School of Medicine

Oregon Health & Science University

CERTIFICATE OF APPROVAL

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Table of Contents

List of figures iv-v

Abbreviations vi-viii

Acknowledgements ix

Abstract x-ix

Chapter I: Introduction 1-8

I: Copper is an essential component of metabolism

II: Cuproenzyme catalysis is vital to many biochemical pathways

III: An array of proteins orchestrate copper homeostasis

IV: Copper-ATPases catalyze the translocation of copper across biological membranes

V: Mutations in ATP7A cause Menkes disease, and mutations in ATP7B cause Wilson's disease

Chapter II: Expression and trafficking of ATP7B in the intestine 9-45

- Introduction
- Materials& Methods
- Results
- Discussion

Appendix I: ATP7B expression and trafficking in the ovary and cisplatin resistance 46-61

- Summary
- Introduction
- Materials& Methods
- Results
- Discussion

Appendix II: Intracellular trafficking of ATP7A and ATP7B **62-68**

- Summary
- Introduction
- A review of trafficking
-

References **69-74**

Figures

1a. A simplified illustration of the pathways involved in copper homeostasis

1b. Representation of colon histology

2a. Detection of ATP7B expression in Caco-2 cells

2b. Caco-2 cells grown in basal media show partial co-localization of ATP7B and a TGN marker

3. ATP7B protein expression in Caco-2 cells visualized by Western blotting. Caco-2, Hek293, and HepG2 cells appear to express comparable amounts of ATP7B

4. Loss of co-localization (white arrows) between ATP7B and Syntaxin-6 in copper-treated Caco-2 cells. ATPB is largely observed in cytosolic vesicular structures (red arrows)

5. Caco-2 cells treated with 50 μM BCS for increasing lengths of time show a sequential increase in the proportion of ATP7B located in the peri-nuclear region of Caco-2 cells

6. There is partial co-localization between ATP7A and ATP7B in BCS-treated Caco-2 cells (Panel A) and this is lost when cells are treated with $CuCl_2$ (Panel B) (50 μM, 1 hr).

7. ATP7B appears to be expressed in human colon mucosa, and tissue morphology is identifiable

8. Higher magnification (1000x) enables visualization of sub cellular regions of epithelial cells lining a colonic crypt

9. ATP7B appears to partially co-localize with the Golgi in cells lining colonic crypts

10. Pre- incubation of anti-NTD of ATP7B antibody with 10-fold excess of purified NTD peptide changes the staining pattern previously observed

11. Incubation of colon tissue with an anti-rat 488 antibody, produced results that may indicate tissue autofluorescence

12. Without the use of antibodies imaging of human colon produces a faint haze throughout

13. Western blotting of mouse colon suggests ATP7B expression

14. Proposed mechanism of copper excretion

A1. ATP7B does not traffic when A2780-CP20 cells are treated with copper

A2. ATP7B does not re-localize from a peri-nuclear location when A2780-CP20 cells are treated with copper or CDDP

A3. ATP7A undergoes copper-dependent trafficking in A2780-CP20 cells

ABBREVIATIONS

AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride

ATOX1 Antioxidant protein homologue 1

ATP Adenine triphosphate

ATCC American Type Culture Collection

ATP7A Menkes disease protein

ATP7B Wilson's disease protein

BCS Bathocuproine disulfonate

BSA Bovine serum albumin

Caco-2 Colorectal adenocarcinoma cells

CAPS N-cyclohexyl-3-aminopropanesulfonic acid

CCS Copper chaperone for superoxide dismutase

CDDP Cisplatin, *Cis*-diamminedichloroplatinum(II)

$CuCl_2$ Copper Chloride

COX17 Cytochrome c oxidase copper chaperone

-COOH Carboxyl

CP Ceruloplsmin

CTR1 Copper transporter receptor 1

DAPI 4′,6-diamidino-2-phenylindole

DMEM Dulbecco's modified eagles medium

EDTA Ethylenediaminetetraacetic acid

FBS Fetal bovine serum

FITC Fluorescein isothiocyanate

Hek293 Human embryonic kidney cells

HepG2 Human hepatoma cells

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

H_2O_2 Hydrogen Peroxide

IF Immunofluorescence

Jeg-3 Human placental choriocarcinoma cell line

kDa Kilodalton

KO Knockout

MBDs Metal-binding domains

MD Menkes disease

MEM Minimal essential media

Mg Milligram

μg Microgram

μL Microliter

nm Nanometer

NaCl Sodium Chloride

NP-40 Nonyl phenoxylpolyethoxylethanol

NTD N-terminal domain

PBS Phosphate buffered solution

PAM Peptidylglycine α- amidating monooxygenase

PAGE Polyacrylamide gel electrophresis

ROS Reactive oxygen species

O_2^- Superoxide

SDS Sodium dodecyl sulphate

SOD1 Superoxide dismutase 1

TBST Tris-buffered saline-tween

TGN *Trans*-Golgi network

Tris-HCl Tris(hydroxymethyl)aminomethane-hydrochloric acid

WD Wilson’s disease

WT Wildtype

Acknowledgements

I wish to thank Svetlana Lutsenko for her caring, encouragement and guidance over the last few years. A good mentor is difficult to find, and Svetlana has been exceptional. I wish to thank Peter Rotwein, David Farrens, Linda Musil, and Richard Maurer for agreeing to give their time and advice to this project.

I dedicate this dissertation to my parents, Katherine Billington, and Mirko Zuzel. They supported my interest in science from an early age, and inspired me to pursue a career in research. I wish more than anything that they could be around to share in the celebration of this achievement.

ABSTRACT

All cells require copper as it is a co-factor for enzymes that catalyze essential reactions. A cell must balance its need for copper against the devastating oxidative damage caused by an excess of the metal. Cells express an array of copper-binding proteins that orchestrate its homeostasis and transport copper to various destinations within the cell. The consequences of copper misbalance can be observed in two diseases of copper metabolism known as Menkes and Wilson's disease; inactivating mutations in the copper ATPases ATP7A and ATP7B, respectively, causes these. Copper-ATPases are polytopic membrane proteins that catalyze the transfer of copper across biological membranes. ATP7B is expressed in the liver where it transports copper into the secretory pathway. It also traffics from the *Trans*-Golgi network towards the apical membrane of hepatocytes where it sequesters copper in exocytic vesicles for release into the bile. ATP7B is found in many other tissues, and in these cases it is co-expressed with ATP7A. While the role of ATP7B in the liver is understood, its function in other tissues remains elusive.

Here, the expression and trafficking behavior of ATP7B is investigated in the large intestine and the ovary. The results suggest that ATP7B is expressed apically in epithelia lining the lumen and crypts of the human colon. It is shown to undergo copper-dependent trafficking in Caco-2 cells, a human cell line modeling the intestine. With this information we propose a novel model of copper excretion. ATP7B functions (in part) to sequester excess copper in vesicles near the apical/luminal membrane of colonic epithelial cells. Subsequently, the metal is released from the cells into the lumen of the

large intestine where it is incorporated into the feces for removal from the body. This is hypothesized to be a physiological process secondary to biliary excretion.

Expression of copper-ATPases has been linked to resistance to the chemotherapeutic agent cisplatin in tumor cells. The mechanism of resistance is highly debated, and there is evidence both for and against direct transport of cisplatin by ATP7B. A2780-CP20 cells over-express ATP7B and are resistant to cisplatin. We show that ATP7B does not traffic when these cells are treated with either copper or cisplatin. These findings suggest that ATP7B binds to, and sequesters copper in these cells for fine-tuning its release when required. The lack of trafficking argues against ATP7B-mediated efflux of cisplatin across plasma membrane in these cells, and therefore resistance is caused by an alternate mechanism such as binding and sequestration.

CHAPTER I

Introduction

I. Copper is an essential component of metabolism

All cells have a metabolic requirement for copper. Therefore this micronutrient is essential to sustaining life in every organism. Copper ions readily change their ionization state under physiological conditions, and this high redox potential is utilized in many catalytic processes.

It is important to note that these redox properties can also be damaging to cells. Copper ions react with reduced oxygen species via Fenton-like reactions to produce hydroxyl-free radicals. These cause oxidative damage to proteins, lipids, and DNA (1). Therefore, to prevent cytotoxic effects, cells have developed mechanisms to sequester and remove excess copper (1).

II. Cuproenzyme catalysis is vital to many biochemical pathways

Cuproenzymes are copper-dependent enzymes that require a copper co-factor to catalyze reactions. Biosynthesis of several cuproenzymes is known to occur in the secretory pathway of cells. In the terminal portion of the Golgi apparatus, the *trans*-Golgi network (TGN), copper is inserted into newly synthesized proteins, such as tyrosinase and lysil oxidase. These are then exported to cell surface or excreted in vesicles, which 'bud off' the TGN, and are transported by trafficking machinery to their designated location.

Cuproenzyme mediated catalysis is essential to many physiological processes. Within specialized cells cuproenzymes catalyze the synthesis of specific compounds and individual reactions are part of complex biochemical pathways. For example, the pigment melanin is produced by melanocytes, and these reside in the epidermis of the skin. Within these cells tyrosinase (a cuproenzyme) catalyses the first step in the biosynthesis of this essential compound (2).

Lysyl oxidase is an enzyme involved in connective tissue formation. It catalyzes the oxidation of specific lysine residues within elastin and collagen polymers. This post-translational modification causes inter- and intra-peptide cross-linking. As a result of this process the extracellular matrix is stabilized enabling greater support and anchorage to surrounding cells (3).

Peptidylglycine α-amidating monooxygenase (PAM) catalyzes the conversion of peptides into α-amidated peptides. Many hormones, neuropeptides and other signaling molecules require an α-amide group at their COOH- terminus to become biologically active; examples of these are gastrin, vasopressin, oxytocin, and substance P (4,5).

Ceruloplasmin (CP) is a copper dependent ferroxidase that binds circulating copper. Oxidization of Fe (II) to Fe (III) by CP is essential for the metal to be loaded onto transferrin, an iron binding plasma protein. In this way CP is important for regulation of iron metabolism. CP is known to catalyze a range of reactions and has both pro- and anti- oxidative properties (6).

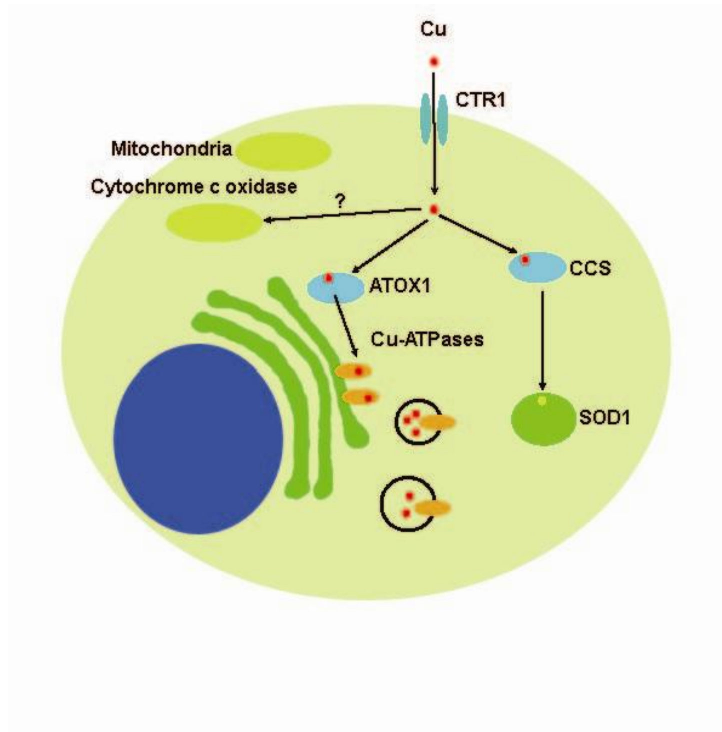
Zn, Cu-dependent Superoxide Dismutase1 (SOD1) is an example of a cytosolic cuproenzyme. Superoxide (O_2^-) is a damaging free radical that is produced by the one electron reduction of dioxygen (O_2), a commonly occurring reaction. SOD1 catalyzes the dismutation O_2^- to hydrogen peroxide (H_2O_2) and O_2. In this way SOD1 is an antioxidant enzyme. It is an essential component of the cellular machinery responsible for protecting cells from elevated levels of reactive oxygen species (ROS) (7).

III. An array of proteins orchestrate copper homeostasis

The high affinity copper-uptake protein CTR1 largely mediates copper entry into cells (Figure 1). This process does not require ATP due to a favorable concentration gradient. Once copper has entered a cell it binds to chaperone proteins, and these transport copper to designated sub-cellular locations. By binding copper upon cell entry, these chaperones prevent oxidative damage that would be caused by free copper ions. In general, the transfer of copper from chaperone to enzyme is mediated through protein-protein interactions (8).

ATOX1 (a copper chaperone) binds Cu (I) and transfers it to the N-terminal metal-binding domains of copper-transporting ATPases. These transporters regulate copper metabolism by donating copper to enzymes for biosynthesis in the TGN, and traffic to export excess copper from the cell (9,10). The copper chaperone for superoxide dismutase (CCS) delivers copper to apo-SOD1 in cells. The mechanism of copper delivery from chaperone to enzyme is complex, involving oxidative catalytic activity of copper-loaded CCS (11).

Figure 1



A simplified illustration of the pathways involved in copper homeostasis

Copper is delivered to the inner mitochondrial membrane for delivery to cytochrome c oxidase, but the mechanism of this is not well characterized. Cytochrome c oxidase is the final member of a sequential series of enzyme complexes known as the electron transport chain. These complexes are responsible for generating a protein gradient across the inner mitochondrial membrane, and this gradient drives oxidative phosphorylation/ATP production.

IV. Copper-ATPases catalyze the translocation of copper across biological membranes

Copper-transporting ATPases (Cu-ATPases) are multi-domain, polytopic membrane proteins. They allow copper to permeate cellular membranes and to enter luminal compartments of the TGN and vesicles. Cu-ATPases require energy from ATP hydrolysis for copper translocation, as concentration gradients are not conducive to passive transport. These transporters catalyze the breakdown of ATP, and this occurs as part of a multi-step catalytic cycle. Copper translocation is directly coupled to ATP hydrolysis, and a transient acyl-phosphorylated intermediate is formed as part of the cycle. This catalytic property categorizes Cu-ATPases as belonging to a family of transporters known as P-Type ATPases (see Voskoboinic *et al.,* 2001 for details of the catalytic cycle).

V. Mutations in ATP7A cause Menkes disease, and mutations in ATP7B cause Wilson's disease

In humans, two diseases of copper metabolism illustrate the necessity for fine-tuned copper balance and aid in understanding the physiological functions of Cu-ATPases. Wilson's disease (WD) and Menkes disease (MD) are both hereditary and occur due to mutations in the genes *ATP7A* and *ATP7B,* respectively.

WD is one of copper overload. Copper build up occurs in several tissues, and significant accumulation is observed in the hepatic tissue of the liver. This is due to impaired excretion of copper across apical membranes of hepatocytes. Normally, within hepatocytes ATP7B (Wilson's disease protein) donates copper to CP however when copper levels exceed a certain threshold, ATP7B traffics towards the apical membrane and sequesters excess copper into exocytic vesicles. Furthering this, vesicular copper is exocytosed from the cell and enters bile-canaliculi. Copper-stimulated trafficking of ATP7B towards the apical membranes of hepatocytes leads to excretion of copper on a whole body level. Removal of bile by contraction of the gall bladder enables an organism to excrete excess copper (and other waste materials) through the feces. Indeed, the liver is the main organ of copper homeostasis (13). In WD, copper delivery to CP and secretory vesicles is inactivated and copper accumulates in hepatocytes.

Copper accumulation is associated with significant impairment of liver function. Chronic hepatitis and liver cirrhosis are both known to occur. Copper is also elevated in other

organs/tissues such as the kidneys, brain, and eyes. Therefore, a range of disease phenotypes occurs, for example psychiatric abnormalities (14,15).

Copper is obtained from the diet; it is transported into enterocytes from the lumen of the small intestine across the brush border membrane. ATP7A performs a critical role in the absorption of dietary copper; it enables copper to be transported from the cytosol of enterocytes across basolateral membranes and into the circulation (15). ATP7A does not appear to be present at apical surface of the cells (16). Extensive immuno-fluorescent (IF) studies have shown it to undergo copper-dependent trafficking from the TGN to vesicular compartments near the baso-lateral membranes of epithelia. This allows copper to bind to serum proteins and be distributed to other tissues (17).

Generally speaking, MD is the disorder of copper deficiency, and this is due to defective transport of dietary copper by ATP7A. Many symptoms occur because the cuproenzymes that ATP7A serves are not appropriately metallated. Phenotypic manifestations include: mental retardation, neurodegeneration, hypopigmentation, and laxity of the skin and joints. These are a direct result of the inability of cuproenzymes to catalyze reactions (15,18).

Chapter II

Expression and trafficking of ATP7B in the intestine

Introduction

The functional significance of co-expression of ATP7A and ATP7B in certain tissues is poorly understood. ATP7A and ATP7B are highly homologous and, *in vitro*, can substitute for each other's functions. It is interesting therefore that ATP7A and ATP7B are co-expressed in some tissues. Examples of these tissues/cells are: the placenta (19,20), Purkinje neurons (21), the mammary gland (22), and the kidney (23).

This observation suggests that there could be differences in functional properties, regulation or intracellular localization of two transporters. Currently, little is known about the mechanistic differences between ATP7A and ATP7B. However, available data has provided some hypotheses. Kuo *et al,* 1997 were the first to study the expression of Cu-ATPases in developing mouse embryos. *In situ* hybridization showed that ATP7A is expressed ubiquitously during embryonic development. In contrast, ATP7B expression seems to be limited to certain tissues, suggesting a more specialized role. Further investigation of tissue-specific expression of Cu-ATPases, as well as situations of co-expression has lead to a clearer understanding of the differences between ATP7A and ATP7B (for review see Linz& Lutsenko, 2007).

ATP7A and ATP7B are not identical; however there is a high degree of sequence homology between the two transporters (about 60%) (26). ATP7A has faster kinetics than ATP7B. Both the phosphorylation and dephosphorylation steps of the catalytic cycle are faster for ATP7A, and therefore it seems that the turnover rate of ATP7A would be higher compared to ATP7B (21). It is likely that these characteristics allow ATP7A to act as the "housekeeping" ATPase. ATP7A also has a wide expression profile,

metallates several cuproenzymes, and its kinetic advantage means that it can remove excess copper from a cell more quickly, thus restoring homeostasis. In contrast, ATP7B expression appears to be tissue-specific, and its slower turnover rate suggests that its functions are more specialized, for example donating copper to CP (21, 24, 27-29).

Copper-dependent trafficking of Cu-ATPases results in vectorial transport of copper by ATP7A and ATP7B in polarized cells (outlined in Chapter I, and expanded upon in Appendix II). This is a distinct behavioral difference between the two transporters that is not fully understood but could be a critical rationale for co-expression. The distinct functional consequences of trafficking in polarized cells are apparent in the placenta. Hardman *et al.,* 2007 studied the trafficking of ATP7A and ATP7B in placental Jeg-3 cells. In these polarized epithelial cells ATP7B was shown to traffic to the apical membrane, and ATP7A to the basolateral. For these reasons ATP7A is believed to be responsible for delivering copper to the fetal circulation, whereas ATP7B is involved in the regulation of placental copper by facilitating excretion of excess copper back into the maternal circulation.

The research described in this thesis aims to investigate the specialized roles of ATP7B in cells and tissues. Basolateral excretion of copper into the circulation by MNKP is clearly described in literature (31,32). However, the functional consequences of ATP7B undergoing apical transport in many tissues remain unclear. Trafficking of ATP7B is tissue-specific. It appears that copper-dependent re-localization does not occur in some tissues, for example in the kidney (23) and the ovary (see Appendix and (33)). This difference in behavior indicates that the role of ATP7B is not uniform throughout tissues.

The purpose of the experiments described in this chapter is to investigate the expression and behavior of ATP7B in a novel cell line. If ATP7B is present (i.e. there is co-expression of ATP7A and ATP7B in these cells), then trafficking behavior will be investigated. Ultimately, the aim is to understand how maintaining copper homeostasis in a particular tissue may impact copper balance on a physiological level.

The initial part of this study focuses on the human colonic adenocarcinoma cell line (Caco-2) cells. The advantages of choosing these cells were the following:

- Using human cells is an immediate way to avoid discrepancies that occur due to obvious genetic differences between humans and other mammals.

- Caco-2 cells have gastrointestinal origin and therefore are useful for dissecting copper absorption/excretion mechanisms

- Caco-2 cells form monolayers in culture. This means that a single layer of cells can be viewed on the z-axis; therefore images are cleaner and easier to interpret. These cells can also be grown on a permeable support and became polarized; this allows for analysis of trafficking to basolateral and apical membranes

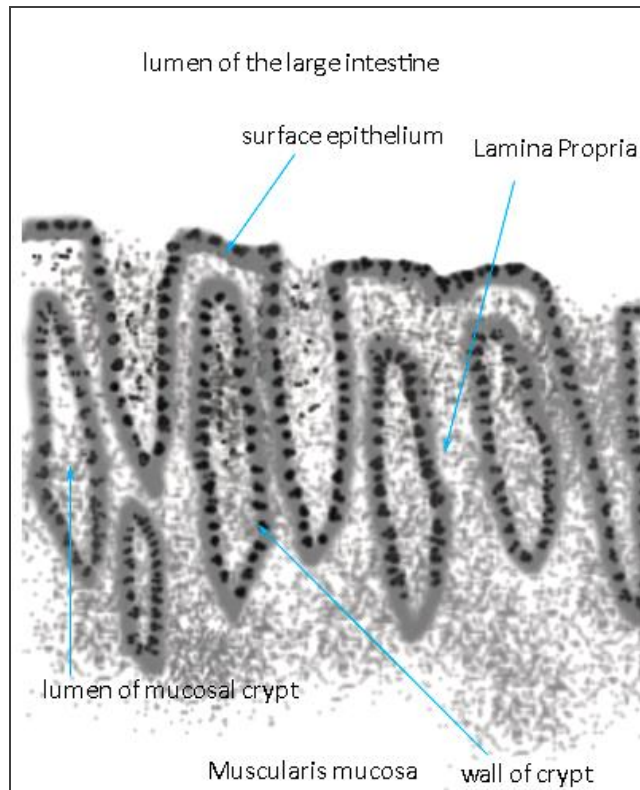
- Caco-2 cells are relatively large. The area between the peri-nuclear region and the cell surface enables clear visualization of intracellular proteins when conducting trafficking studies.

- The expression and trafficking of ATP7A in these cells have been previously reported (17).

The second part of this study focuses on ATP7B expression in the mucosal layer of the human colon.

It is important to understand the histology of the colon. The mucosal layer of the colon is comprised of surface columnar epithelium that contains both absorptive and mucous-producing goblet cells. Absorptive cells are responsible for water and ion transport and are known to contain large elongated nuclei that are parallel to the longer sides of these columnar cells. The mucosal surface invaginates to form colonic crypts that are also lined by epithelial and goblet cells. At the base of these crypts are precursor cells that are able to divide and thus allow for surface renewal. The lamina propria is composed of several different cell types embedded in strands of collagen. Both the lamina propria and muscularis mucosa contain immunological cells; these provide protection from toxic compounds that are found in the lumen of the colon. Capillary beds in these areas allow gas exchange and the removal of toxic by-products of cellular metabolism. The muscularis mucosa is comprised of a layer of muscle and separates the mucosal layer of the colon wall from the deeper submucosal tissue layer (34).

Figure 2



Representation of colon histology

Materials & Methods

Cell lines and cell culture. Immortalized cell lines obtained from ATCC were propagated in 10cm tissue culture dishes at 37°C in a tissue culture incubator. Caco-2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) and this was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomysin, and 1% nonessential amino acids (Gibco). HepG2 and Hek293 cells were cultured in Minimal essential media (MEM) (Gibco) and supplemented as above. Prior to culture cells were thawed (stocks maintained at (-120°) in 10% DMSO), propagated, and passaged until healthy cells were visible at approximately 80% confluency (passage number less than 20).

IF protocol. Cells were trypsinized (10% Trypsin/ EDTA, Gibco), diluted 1:10 and plated onto sterilized glass coverslips in a 12-well tissue culture plate. Cells were grown on glass for approximately 24 hrs to 85% confluency. For trafficking experiments, cells were treated with 50 μM BCS or 50 μM $CuCl_2$ for 1 hr. Regular media was removed, cells were washed with sterile PBS, and 1mL of a 50μM solution of either compound in cell media was added to each well. After 1 hour cells were removed from culture and supplemented media was removed. The 12-well plate was placed onto ice, and cells were fixed with acetone. Coverslips were rinsed with PBS to remove media and immersed into ice-cold acetone for 30 seconds and then rinsed with PBS. Fixation by this method enabled cells to retain their morphology while providing sufficient permeabilization for antibody entry.

Once cells were fixed coverslips were placed in a new 12-well tissue culture plate and covered with blocking solution (1% gelatin, 1% BSA, 0.02% sodium azide in PBS) overnight, at 4°C. Consequently, the plate was brought to room temperature, blocking buffer removed, and cells rinsed with PBS. Coverslips were placed facedown onto a parafilm (Pechiney Plastic Packaging Company) grid, where each square contained 50 μL of primary antibody diluted in blocking buffer (1:500). Incubation was performed in a humid environment for 1 hr. After this coverslips were washed in PBS (3x 10 mins) and then incubated with secondary antibodies in a dark humid environment. Secondary antibodies raised against the species of the first were diluted 1:1000 in blocking buffer. Anti-ATP7B N-Terminal domain (NTD) antibody was raised in rat, hence an anti-rat secondary antibody was used, and this was conjugated to the fluorophore (FITC) (Alexa Fluor Molecular Probes). Excitation occurred at 488 nm, producing emission in the visible light range (green). For Syntaxin-6 and ATP7A an anti-mouse antibody conjugated to Rhodamine was used. Excitation occurred at 555 nm, and red light was emitted. False coloring of pink/purple was utilized, so that those with red/green color blindness could interpret images. Coverslips were mounted onto slides using Vectashield with/DAPI (Vector Laboratories). For the purpose of focusing a 100x objective lens was used; details of subcellular regions were observed, and analyzed with a 1000x oil objective with a Zeiss Confocal Scanning microscope (Carl Zeiss).

Preparing cell lysates. Total cell lysate was obtained from cells in culture nearing confluency in a 10 cm tissue culture dish. Lysis Buffer (50 nM HEPES pH7.4, 0.1% NP-40 (Nonidet), 150 mM NaCl, ½ Protease inhibitor tablet (Complete Protease Inhibitor

Cocktail, Roche) and 1 mM AEBSF) was prepared and kept on ice. 800 μL of this buffer was added to each tissue culture dish and left on ice for 15 mins. Cells were scraped from surface of tissue culture plastic using a bent pipette tip. Lysate was transferred to a 1.5 mL Eppendorf tube and centrifuged at 600g for 15 mins at 4°C. Supernatant was collected; 120 μL was removed and added to 80 μL standard sample buffer (Tris-HCl pH 6.8, 8M Urea, 10% SDS in equal volumes). Remaining supernatant was used for protein quantification by a Bradford Assay (Thermo Scientific). Samples containing lysate and sample buffer were further centrifuged at 1600g for 5 mins at 4°C. Supernatant was loaded onto an SDS-PAGE gel.

SDS-PAGE and Western blotting. Cell lysates and 7μL of pre-stained protein ladder (Fermentas) were separated on a large 7.5% Laemmli gel, for approximately 4 hrs at 30 mA. Once optimal separation was observed, the gel was removed from electrophoresis equipment and prepared for Western blotting. Transfer buffer was 10% methanol in 10 mM CAPS, pH 11. Protein transfer was conducted for 1 hr at 180 mA. The blot was then blocked for 1 hr in 5% milk powder in TBST (10 mmol/L Tris (pH 8), 150 mmol/L NaCl, 0.05% Tween 20) on a shaking platform (as were all subsequent steps). Incubation with the primary antibody was performed overnight at 4°C (dilution 1:500 in 1% milk powder in TBST). The blot was washed in TBST (3x 10 mins) and incubated with an anti-rat antibody conjugated horseradish peroxidase (1 hr, 1:1000 in 1% milk powder in TBST). Once again the blot was washed in TBST (3x 10 mins). Chemiluminescent substrate (Pierce) was pipetted onto the surface of the blot in order to enable visualization of immunostained proteins as dark bands on photographic film.

Immunostaining of cryostat sections of human colon mucosa pre-mounted on slides. Frozen sections of human colon mucosa were obtained on pre-mounted slides by kind donation of the Corless laboratory (Oregon Health & Science University). These were thawed on ice, prior to acetone fixation (as before). The same IF protocol described for cells, was implemented for tissue sections.

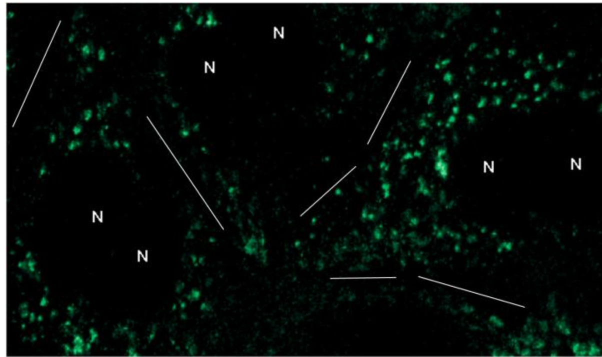
Western blot analysis of mouse tissue. Gastrointestinal tract was removed from sacrificed mice, and kept on ice. Colon was dissected away from esophagus, stomach, small intestine, spleen, and rectum. The contents of the colon was removed by washing with ice cold PBS. Care was taken to remove surrounding mesenchymal tissue without damaging intestine walls. Sections of cleaned colon approximating 4 mm in length were collected, and excess tissue was frozen (-80°C). For experimentation, colon sections (and a donated liver sample) were kept on ice and 1 mL of homogenization buffer was added (as above) to 5-10 mg of tissue. Tissue was homogenized extensively by douncing (on ice). The mixture was centrifuged at 600g for 15 mins, and the supernatant was collected for quantification and SDS-PAGE (see cell protocol).

Results

Immuno-staining of Caco-2 cells suggests ATP7B expression. The initial aim was to establish whether ATP7B is expressed in Caco-2 cells. To do this we used the antibody against N-terminal domain (NTD) of ATP7B, which detects ATP7B specifically in other cell lines. Cells were immuno-stained for ATP7B, and the staining was visualized by confocal microscopy (Fig.2a). Fluorescence staining of a punctate (vesicular) type was observed throughout the cytosol (Fig.2a).

Under basal conditions, ATP7B is expected to be located in the TGN and, to some degree, in vesicles. To determine whether the observed pattern represents the TGN staining, cells were co-stained for ATP7B and Syntaxin-6, a marker for the TGN. Co-localization with Syntaxin-6 was observed in the perinuclear space (Fig.2b). The co-localization was partial, suggesting that ATP7B is present in the TGN as well as in vesicular compartments under these conditions.

Figure 2a



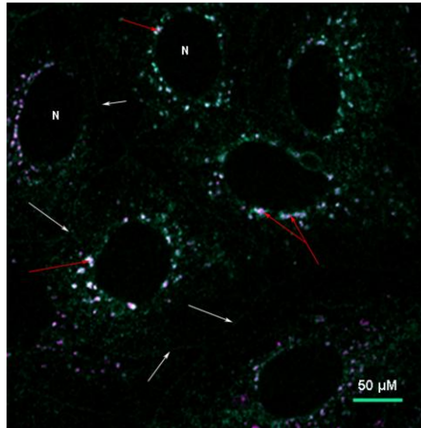
Immunostaining of ATP7B in Caco-2 cells

Cultured cells were incubated with anti-ATP7B N-terminal domain anti-serum (raised in rat) and then with an anti-rat secondary antibody conjugated to a fluorophore (FITC excitation at 488nm). Laser-scanning confocal microscopy was performed to visualize cells and collect images (Zeiss microscopy, and Zeiss LSM visualization software). Magnification 1000x, resolution 300 pixels/cm. ATP7B (green) staining appears to be in a vesicular pattern, and it is present near to nuclei, and within the cytosol. For orientation nuclei are labeled (N) and areas approximating cell outlines are shown (Adobe Photoshop). All cell and tissue images: Zeiss microscopy, Zeiss LSM software, 100x, 300 pixels/cm.

Images labeled with Adobe Photoshop.

Antibodies: anti-ATP7B NTD, Syntaxin-6, anti-ATP7A CTD, Alexa-Fluor Molecular Probes (unless stated otherwise).

Figure 2b



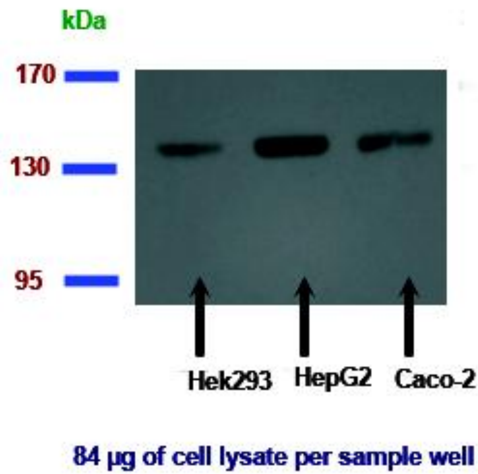
Caco-2 cells grown in basal media show partial co-localization of ATP7B and a TGN marker

Caco-2 cells were co-stained for WND and the TGN marker Syntaxin-6. ATP7B can be observed in cytosolic vesicles (green) and also within the TGN where co-localization with Syntaxin-6 is observed. Areas of co-localization appear white, and Syntaxin-6 staining is pink. Cell nuclei are clearly visible and illustrate a low level of background staining. Red arrows point to areas of co-localization and white arrows point to plasma membranes.

Detection of ATP7B expression in three different cell lines by Western blotting. In order to investigate ATP7B expression in Caco-2 cells lysates were prepared for SDS-PAGE, and Western blotting followed this. Expression was compared to that of two other cell lines that have been shown to express detectable levels of ATP7B by this method: human embryonic kidney (Hek293) and human hepatocellular liver carcinoma (HepG2) cells. The samples were prepared under the same conditions for the three cell lines.

A single band was observed corresponding to the molecular weight of ATP7B in Hek293 cells, HepG2 cells and Caco-2 cells (Fig.3). The level of expression of ATP7B in Caco-2 cells appears higher than in Hek293 cells but less than in HepG2 cells.

Figure 3



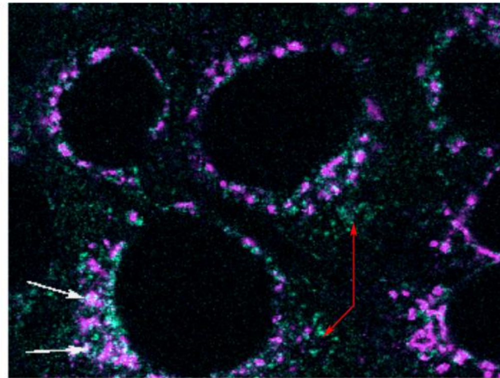
ATP7B protein expression in Caco-2 cells is visible by Western blotting. Caco-2, Hek293, and HepG2 cells appear to express varying levels of ATP7B

For each sample total protein was quantified by Bradford assay (Thermo Scientific), and 84 μg of cell lysate was loaded onto a Laemmli gel. The expected molecular weight of ATP7B is observed under these conditions.

Cell imaging reveals copper-dependent trafficking of ATP7B in Caco-2 cells. Having met the initial aim of the investigation by illustrating ATP7B expression, the next step was to determine whether ATP7B undergoes copper-dependent trafficking in Caco-2 cells. Trafficking was analyzed by comparing the localization of ATP7B and Syntaxin-6 in copper-treated cells. Fig.4 illustrates that following this treatment ATP7B is mostly present in vesicular compartments outside of the TGN. A very small amount of co-localization with Syntaxin-6 is observed. Although a difference in co-localization was detected in copper-treated cells, it was difficult to form firm conclusions because in both cases ATP7B was also present in cytosolic vesicles. Thus, to verify that trafficking is occurring in Caco-2 cells, it was important to compare images obtained from copper-treated cells to those cultured in basal media and to cells that have been treated with a copper chelator such as bathocuproine disulfonate (BCS). This latter treatment is useful, as copper is present in basal media and may induce some trafficking, which may account for partial localization observed in Fig.2b.

A time course experiment was performed where cells were treated with BCS for 1 hour, 6 hours, and 24hours. This enabled visualization of the subcellular localization of ATP7B in differing states of copper deprivation. Fig.5 illustrates a definite time-dependent increase in co-localization between ATP7B and Syntaxin-6.

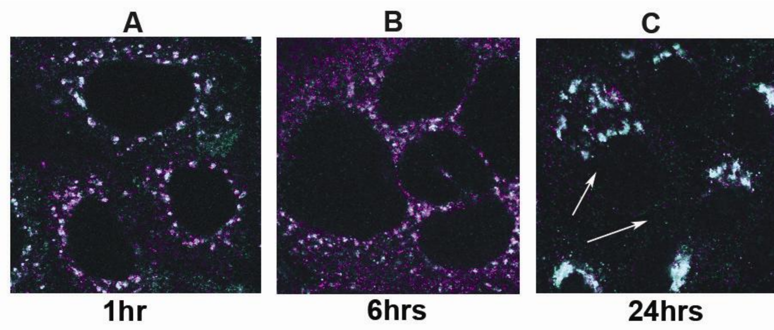
Figure 4



There is very little co-localization (white arrows) between ATP7B and Syntaxin-6 in copper-treated Caco-2 cells. ATPB is largely observed in cytosolic vesicular structures (red arrows)

Cells were grown on glass coverslips in regular media, and then in media supplemented with 50 μM $CuCl_2$ for 1 hour prior to acetone fixation.

Figure 5



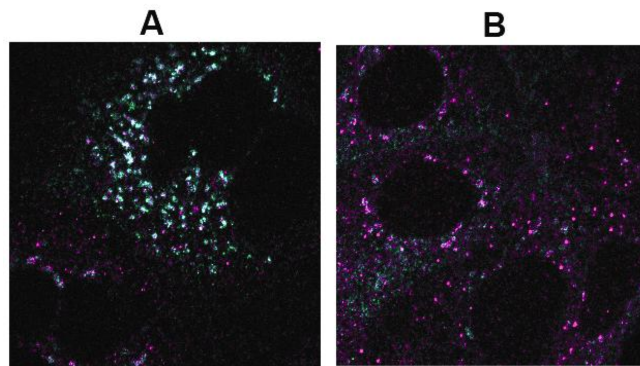
Caco-2 cells treated with 50 μM BCS for increasing lengths of time appear to show a sequential increase in the proportion of ATP7B located in the perinuclear region

White arrows in panel C illustrate the level of background staining by comparing nuclear and cytosolic regions. Cells were treated with 50 μM BCS for 1 hour, 6 hours, and 24 hours prior to acetone fixation.

Co-expression of ATP7A and ATP7B can be visualized in Caco-2 cells. Previous elegant imaging studies by Nyasae *et al.,* 2007 have illustrated the behavior of ATP7A in filter-grown Caco-2 cells. ATP7A was shown to undergo copper-dependent trafficking from a peri-nuclear region to the cytosol. Therefore, our next experiment was to determine whether ATP7A and ATP7B can be simultaneously detected in Caco-2 cells and whether they will co-localize in either copper limiting (BCS) and/or copper enriched conditions.

Fig.6 illustrates partial co-staining of ATP7A and ATP7B. Under copper limiting conditions, both transporters are more abundant in a peri-nuclear compartment, where they co-localized. In addition, a punctate pattern of ATP7A and ATP7B staining is present throughout the cytosol. In this location the transporters do not co-localize. As mentioned previously, treating for 1 hour with BCS was not long enough to observe ATP7B in a tight peri-nuclear location in Caco-2 cells. Upon the addition of copper there is very little or no co-localization between ATP7A and ATP7B, and both transporters appear to be present in cytosolic vesicles. Therefore, ATP7A and ATP7B traffic in 50 μM $CuCl_2$, and they move to different vesicular compartments To visualize proximity to the plasma membrane one would need to polarize cells and co-stain with membrane specific markers (see Nyasae et al, 2006).

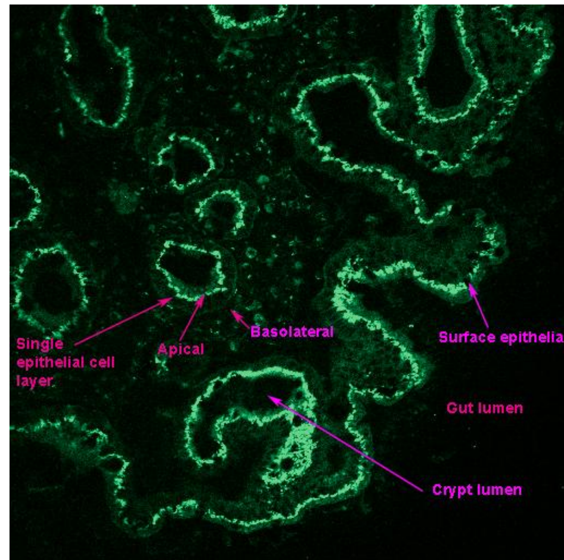
Figure 6



There is partial co-localization between ATP7A and ATP7B in BCS-treated Caco-2 cells (Panel A) and this is lost when cells are treated with $CuCl_2$ (Panel B) (50 μM, 1 hr)

ATP7B appears to be expressed in human colon mucosa. With a solid platform of data from the cell work, the next step was to study ATP7B expression in colon tissue. Microscope slides pre-mounted with section of human colon mucosa were obtained. These were fixed, and stained with the antibody against the NTD of ATP7B; the staining was visualized by confocal microscopy.

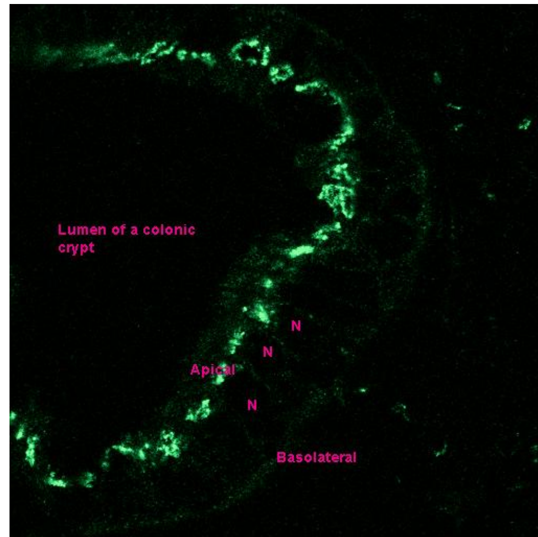
Figure 7



ATP7B appears to be expressed in human colon mucosa, and tissue morphology is identifiable

Frozen cryostat sections were allowed to thaw prior to being fixed in acetone and stained for ATP7B (100x).

Figure 8



Higher magnification (1000x) enables visualization of sub cellular regions of epithelial cells lining a colonic crypt

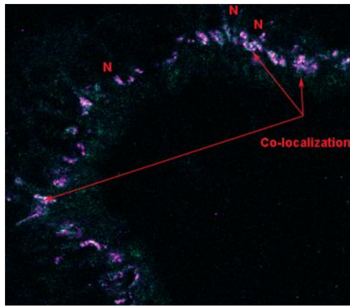
Notice the elongated nuclei and the apical expression of ATP7B.

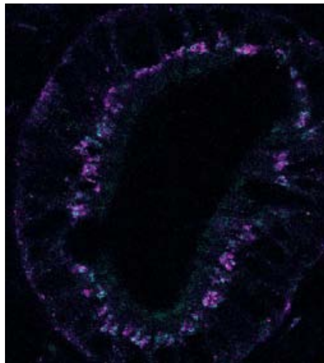
In Figs 7&8 tissue morphology and cellular orientation can be observed. Surface cells and those lining colonic crypts are clearly defined. Areas that lack staining are identifiable as luminal, and therefore the orientation of cell polarity can be deduced. A distinct pattern of bright immunostaining suggests that ATP7B is expressed in the mucosal layer of human colon. The apical region of epithelial cells are brightly stained, basolateral areas are weakly stained, and nuclei are not labeled.

Partial co-localization of ATP7B with a Golgi marker. Tight perinuclear staining oriented towards the apical membrane was suggestive of Golgi localization of ATP7B. To verify this, co-staining with a TGN marker was performed. Labeling with Syntaxin-6 did not produce a signal in tissue sections (data not shown). Therefore, a different marker of the Golgi apparatus (Golgin) was used, and in this case labeling was successful.

As with the previous figures tissue morphology is visible in Fig.9. However, it should be noted that staining is not as "clean" as in Figs 7&8, and this was observed over multiple experiments. The loss of acuity may be a phenomenon of double labeling of tissue sections. Co-localization between ATP7B and Golgin is evident however both proteins also appear to localize in separate vesicles in close proximity to each other.

Figure 9





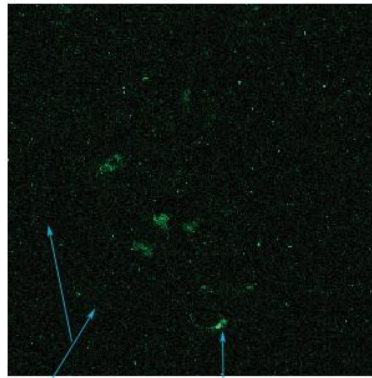
ATP7B appears to partially co-localize with the Golgi in cells lining colonic crypts

Slides were prepared as before but immunolabled for both ATP7B and Golgin.

Pre-incubation with the antigen diminishes staining. Linz et al, 2008 successfully illustrated specificity of the anti-ATP7B NTD antibody by pre-incubation with the recombinant peptide containing the epitope for that particular antibody. Staining was reduced to a faint haze, which was indicative of antibody specificity. The following experiments used the same approach to test specificity of immunostaining in colon tissue.

The ATP7B primary antibody was pre-incubated with a 10-fold excess of recombinant ATP7B NTD peptide (Fig.10). Tissue morphology is no longer identifiable, and staining appears to be of a punctate/hazy nature. Therefore, upon pre-incubation staining is distinctly diminished, suggesting antibody specificity.

Figure 10



High level of what appears to be non-specific staining

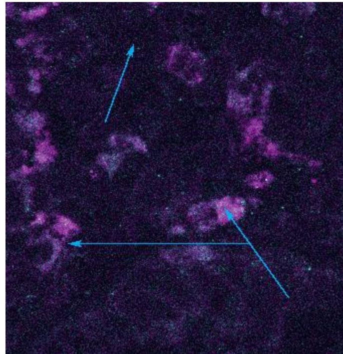
Brighter staining may correspond to cellular structures

Preincubation with the antigen markedly decreased the intensity of staining, suggesting that the pattern observed prior to incubation was specific

Another method to investigate tissue staining was to omit the use of the ATP7B primary antibody and stain only with the secondary antibody (FITC 488nm). It is expected that the secondary antibody will be washed off during the standard IF procedure as its species-specific epitope region should not be present. Fig.11 illustrates staining, but tissue morphology is not identifiable. Therefore, staining is likely to be due to unexpected non-specific labeling by the secondary antibody. Although the staining in Fig.11 is of similar brightness to the previous figures it is not comparable. This is because when using the microscope and computer software the aperture size was automatically optimized for each experiment. In this experiment the level of staining is likely to have been considerably lower than that of previous ones. To test this, the aperture size would need to be fixed and the experiment repeated.

When slides were prepared without the use of any antibodies a faint haze was observed, as expected (Fig.12).

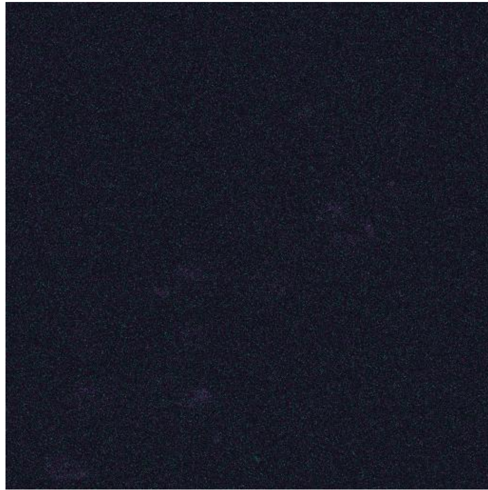
Figure 11



Incubation of colon tissue with an anti-rat 488 antibody produced results that indicate non-specific labeling

Two areas that are clearly stained but unidentifiable are labeled with double arrows and hazy background-like staining with the single arrow. Slides were prepared as previously except that incubation was performed solely with the secondary antibody used for visualization of ATP7B. The image is an overlay of emission caused by excitation of FITC and Rhodamine.

Figure 12

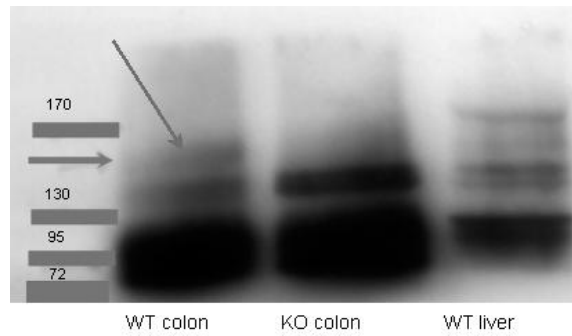


Without the use of antibodies imaging of human colon produces a faint haze throughout

Slides were fixed in the normal manner but the staining steps were omitted. The image is an overlay of excitation at 488 and 555nm.

Western blotting from mouse colon tissue suggests ATP7B expression. In order to illustrate that ATP7B is present in mouse colon tissue Western blotting was performed. With access to WT and KO (ATP7B $^{-/-,}$ ATP7B $^{+/-}$) mice a logical progression was to perform a Western blot from tissue samples derived from these mice. The hypothesis was that a band at 165kDa would be observed in the WT sample but not the knockout. Liver tissue was used as a positive control for ATP7B in mouse tissue. Fig.13 illustrates the presence of the band of appropriate size in the WT sample and the absence of the 165kDa band in the KO. However, the difference between WT and KO was not consistently observed and other cross-reacting bands were also evident. Therefore, further experiments are needed to verify staining specificity.

Figure 13



Western blotting of mouse colon suggests ATP7B expression

Colon derived from WT and ATP7B knockout mice was prepared for Western blotting. Liver from WT mouse was used as a positive control. The arrows in ‘A’ indicate a faint band in WT colon that is not observed KO colon that may possibly correspond to ATP7B.

Discussion

ATP7A, one of the two copper-ATPases found in human cells, is relatively well characterized. It has a wide expression profile, undergoes rapid enzyme cycling, metalates a range cuproenzymes, and re-localizes to the basolateral membrane for the export of excess copper into the bloodstream. Its homologue ATP7B is also expressed in a wide array of tissues. It enables biliary excretion of copper from the liver and donates copper to ceruloplasmin, but its role outside of this remains elusive.

The long-term aim of this investigation is to characterize the role of ATP7B in maintaining copper balance within intestinal epithelial cells and to understand the impact of this mechanism on a physiological level.

Our results demonstrate that ATP7B is expressed in Caco-2 cells. Upon the addition of copper, ATP7B traffics from the TGN to cytosolic vesicles where it sequesters copper for release from the cell. ATP7B appears to be expressed in epithelia lining the lumen and crypts of the human colon. This finding has interesting physiological implications, and we propose a novel mechanism for the excretion of excess copper from the body as a whole. Our data further suggests that the role of ATP7B is tissue specific, and that the transporter performs specialized roles in cells where it is co-expressed with ATP7A.

At the time of this research Weiss and colleagues (35) published their studies on characterization of ATP7B expression and trafficking in intestine. They found ATP7B to be expressed in Caco-2 cells and demonstrated that it re-localizes upon the addition of

copper; ATP7B was found to traffic from a perinuclear localization to cytosolic vesicles. These results are in full agreement with our observations.

Weiss *et al*. did not observe co-localization with either an apical or a basolateral marker when trafficking was investigated in polarized cells. Similarly, in our experiments excess copper also caused ATP7B to re-localize to vesicles, as evident from a cytosolic punctuate-vesicular staining pattern (Fig.4). Thus, it can be concluded that ATP7B is present in an unknown vesicular compartment and not at the plasma membrane. To characterize this compartment, co-localization studies with vesicular-specific marker proteins would be required (as in Nysae *et al.,* 2007). The presence of ATP7B in cytosolic vesicles suggests that it serves to sequester excess copper, which is then released from the cell by exocytosis when appropriate.

Caco-2 cells originate from different clonal populations and are known to differentiate in culture. Therefore, there is heterogeneity both between clonal populations and within the same population (36). Despite this, similar data was obtained from two different laboratories investigating the trafficking behavior of ATP7B in these cells. This suggests that Caco-2 cells were a useful model in this investigation.

Weiss et al. illustrated ATP7B mRNA and protein expression from various sections of murine alimentary canal. From their data it can be concluded that ATP7B appears to be expressed in sections of the small intestine but is absent from the large intestine. In contrast, our data suggest that ATP7B is expressed in human colon (Figs 7, 8, and 9). Although antibody specificity was not firmly proven, the obliteration of a clean staining

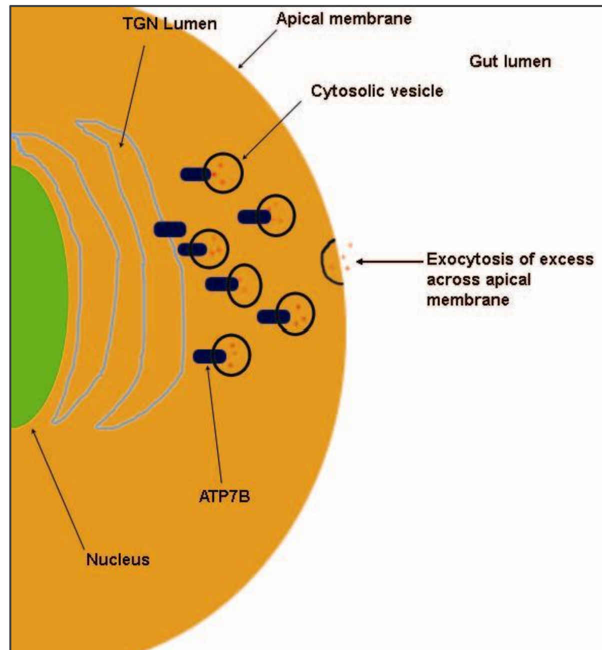
pattern when the ATP7B anti-NTD antibody was pre-incubated with recombinant NTD peptide suggests that this may be so. It is important to note that Weiss *et al.* studied ATP7B expression in murine tissue, whereas human tissue was immunostained in this investigation. This should be considered when comparing data and may account for the differences observed in the large intestine.

In both investigations ATP7B staining is found to be apically orientated around the nucleus, but present in different parts on the intestine; compare Fig.3b middle panel (35), and Fig.8 of this investigation. Weiss and colleagues co-stained images using apical and basolateral markers as well as a Golgi marker. They did not observe co-localization of ATP7B with either membrane marker, but observed a strong degree of co-localization at the Golgi. This Golgi localization was also observed in this investigation (compare Fig.3C right hand panel (35) to Fig.9).

To increase the validity of colon imaging data in this investigation, controls are required. Currently, Western blotting of mouse tissue (Fig.13) illustrates the presence of ATP7B from the colon of *WT* but not from ATP7B$^{-/-}$ mice; however further studies are needed to get reproducible data and confirm antibody specificity. Ideally, the experiment would include images of colon mucosa from ATP7B$^{-/-}$ mice. To support the theory that the staining observed in Figs 7, 8, and 9 is specific to ATP7B one would expect an absence of staining in tissue sections from ATP7B$^{-/-}$ mouse colon and a high degree of apically orientated staining in hepatic tissue of the *WT*.

Our data provides a platform for further investigation of the role of ATP7B in the intestine. The presence of apically orientated ATP7B in the human colon has interesting physiological implications. One explanation for this is that ATP7B functions (at least in part) to enable the release of excess copper across the apical membranes of epithelial cells lining the gut. The Caco-2 cell data suggests that the mechanism is one of cytosolic sequestration prior to release by exocytosis across the apical membrane. This would be similar to the proposed role of ATP7A at the opposite pole of the cell (see Nyasae *et al.,* 2007). Exocytosed copper would enter the lumen of the intestine; consequently it would be incorporated into fecal matter and released from the body. Secondary to biliary excretion this mechanism would provide an alternate route for the removal of excess (toxic) levels of copper from the organism as a whole.

Figure 14



Proposed mechanism of copper excretion

ATP7B sequesters copper in vesicles near the TGN for exocytosis across the apical membrane of a colonic epithelial cell. Once released from the cell, copper is incorporated into the feces in the lumen of the large intestine, and is consequently excreted from the body.

Appendix I

ATP7B expression and trafficking in the ovary, and cisplatin resistance

Published as part of:

"Therapeutic Targeting of ATP7B in Ovarian Carcinoma."

Mangala LS, Zuzel V, Schmandt R, Leshane ES, Halder JB, Armaiz-Pena GN, Spannuth WA, Tanaka T, Shahzad MM, Lin YG, Nick AM, Danes CG, Lee JW, Jennings NB, Vivas-Mejia PE, Wolf JK, Coleman RL, Siddik ZH, Lopez-Berestein G, Lutsenko S, Sood AK.

Clin Cancer Res. **2009** Jun 1;15(11):3770-80.

Summary

A2780-CP20 cells are ovarian cancer cells that express high levels of ATP7B and are resistant to the cytotoxic effects of cisplatin (CDDP). In this investigation the trafficking behavior of ATP7B was studied in these cells. ATP7B has been shown to undergo copper-dependent relocalization from the TGN to cytosolic vesicular compartments in certain cell types such as hepatocytes. This ligand-induced trafficking mechanism allows the transporter to facilitate excretion of excess metal from the cell.

Overexpression of ATP7B is known to correlate with increased CDDP resistance in many tumor cells, but the mechanism by which this occurs remains unclear. One proposed mechanism suggests a “copper-mimic” role for CDDP and predicts that the drug is transported by ATP7B and induces ATP7B trafficking. The experiments described in this chapter tested the ability of CDDP to induce trafficking of endogenous ATP7B by treating A2780-CP20 cells with the drug, followed by immunocytochemistry.

It was observed that ATP7B did not re-localize from the TGN when A2780-CP20 cells were treated with either copper or CDDP. This result suggests that ATP7B does not play a major role in copper/CDDP efflux in these cells, but rather performs a more specialized function. This role may be to sequester copper in or near the TGN for fine-tuning its release when required.

The lack of CDDP-induced trafficking of ATP7B in A2780-CP20 cells argues against vesicle-mediated exocytosis as a mechanism through which ATP7B increases cell

resistance to the drug, and therefore resistance is caused by another mechanism. This may also be one of binding and sequestration, in this case serving to prevent nuclear translocation.

Background information

Cisplatin is an anti-tumor agent whose effectiveness is hindered by acquired resistance. CDDP and other platinum based drugs are widely used chemotherapeutic agents. They are used to treat a variety of cancers, for example those of the head, neck, testis, colon and ovary. When tumors are treated with CDDP, the drug forms adducts with DNA and stimulates crosslinking; as a result the cells can no longer synthesize or repair DNA. This leads to activation of the cell's apoptotic pathways.

Unfortunately, treatment with CDDP becomes less effective over time. This occurs as tumor cells acquire resistance to the drug's cytotoxic effects. The mechanism behind this resistance has been an intense topic of research, as preventing or reversing the process has major clinical implications.

Sensitivity to a drug such as CDDP is dependent upon it being retained inside a cell so that it can translocate to the nucleus, and mediate its function. Therefore, if cells were to acquire/activate a mechanism of decreased uptake, and/or increased efflux of CDDP, this is likely to result in a resistance phenotype (37).

There is a body of evidence linking ATP7B expression with CDDP resistance. In 2000, human epidermoid carcinoma (KB-3-1) cells made to overexpress ATP7B were shown to have an 8.9-fold higher resistance to the cytotoxic effects of CDDP compared to mock transfected controls. Also, prostate cancer cells with increased resistance to cisplatin have been found to express a higher level of ATP7B than their sensitive parental cell line (38).

In 2002 Katano *et al.* performed a series of experiments in ovarian carcinoma cell lines. CDDP sensitive parental cells and daughter cell lines stably expressing a resistant phenotype were compared in various ways. The results of these experiments illustrated that CDDP resistant cells were also resistant to copper. There was decreased accumulation and increased efflux of both copper and CDDP in the resistant cells compared to sensitive controls. This cross-resistance to copper and CDDP suggested that the same transporters might be responsible for the movement of copper and CDDP. Importantly, the resistant cells were found to over-express either ATP7A or ATP7B.

In 2003 the same group further explored the link between ATP7B expression and CDDP resistance by over-expressing ATP7B in 3 different CDDP sensitive carcinoma cell lines (of which 2 were ovarian). All three ATP7B-expressing cell lines showed significant resistance to CDDP, and resistance was shown to be a product of decreased drug accumulation.

The mechanism by which ATP7B causes CDDP resistance is not fully understood. Early studies described a correlation between Cu-ATPase expression and CDDP resistance.

However, resistance was thought to be multi-factorial with several biochemical alterations proposed to be contributing to the phenomenon (37). More recently, a mechanism of direct transport by Cu-ATPases has been proposed. Several investigations suggest that when cells are treated with CDDP, ATP7B responds by trafficking out of the TGN, and actively transporting CDDP into exocytic vesicles, for release from the cell.

Arguments that favor transport. Safaei *et al.* proposed that CDDP might be a substrate for ATP7B. By isolating vesicles from insect cells infected with either *WT* or a mutant form of ATP7B (that was unable to transport copper) they created a cell-free system to assay CDDP transport by the Cu-ATPase. Vesicles containing *WT* ATP7B were shown to accumulate CDDP, but this was not the case for vesicles containing the mutant transporter or for untransfected controls.

A series of experiments indicated that exogenously expressed ATP7B traffics in ovary cells. Cells were transfected with ECFP tagged protein for visualization. Both copper and CDDP appeared to induce trafficking of the transporter out of the TGN and into dispersed cytosolic vesicles. Co-localization between ECFP-ATP7B and a fluorescein labeled form of CDDP was observed. The latter data suggests that there is direct interaction between ATP7B and CDDP (42).

Alternative mechanisms. Leonhardt *et al.* investigated the role of ATP7B in resistance of hepatoma cells to treatment with CDDP. Hepatic tumors are difficult to treat, and ATP7B is highly expressed in these cells. The group did not observe a correlation between

ATP7B expression and CDDP resistance. Furthermore, unlike copper, CDDP did not induce trafficking of endogenous ATP7B in hepatic cells. CDDP was however shown to bind to the N-Terminal domain (NTD) of ATP7B. Mangala *et al*. also showed that CDDP binds to the NTD of ATP7B. Importantly, overexpression of recombinant NTD protein caused DDP sensitive ovary cells to show resistance to the drug's effects. Experiments by Dolgova *et al*. illustrated that cells can be made resistant to CDDP by overexpression of a fragment of the NTD of ATP7B. Together these results support that CDDP binds to the NTD of ATP7B, and this binding contributes to resistance.

In addition, when ATP7B is up regulated copper pharmacology is altered (39). This phenomenon may affect the cell in a number of ways. Gene transcription (the cell's trascriptome) may be altered. Leonhardt *et al.* suggested that this might switch on other drug resistance pathways.

Purpose of research

The aim of this research is to investigate whether copper and/or CDDP induce trafficking of ATP7B in A2780-CP20 cells. These are ovarian carcinoma cells that have been selected for CDDP resistance (Behrens *et al.,* 1987). Thus far, there is no data indicating whether endogenous ATP7B traffics in CDDP resistant cells.

By investigating intracellular localization and trafficking response of ATP7B, the studies were expected to yield the following information:

1. A better understanding of the role of ATP7B in cellular copper homeostasis when there is co-expression with ATP7A. Depending on its intracellular localization, ATP7B can perform several functions: copper transport to the lumen of the TGN for biosynthesis of copper-dependent enzymes, transport of excess copper into exocytic vesicles for export, or sequestration of copper in vesicles for storage. ATP7B trafficking behavior in ovary cells in response to copper will suggest which function, if any, dominates. A2780-CP20 cells are useful for immunofluorescence investigations because they express high levels of endogenous protein. This aids in antibody detection.

2. The effect of CDDP on the intracellular localization of ATP7B in A2780-CP20 cells. As is discussed above, there is much debate as to whether ATP7B actively transports CDPP into secretory vesicles as with copper. If ATP7B traffics out of

the TGN and into cytosolic vesicles when CDDP is added to cells then a model of direct export is favored.

Materials and methods

Cell culture. A2780-CP20, and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco), and this was supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomysin, and 1% nonessential amino acids (Gibco).

IF protocol. Cells were plated onto glass coverslips and maintained in culture until approximately 85% confluent (24 to 48 hrs at 37°C). For trafficking experiments cells were treated with 50 μM BSC, 50 μM $CuCl_2$, 10 μM $CuCl_2$, 10 μM CDDP, or 20 μM CDDP at 37°C for 1hr. Cells were fixed and stained as previously described (Chapter II).

Results

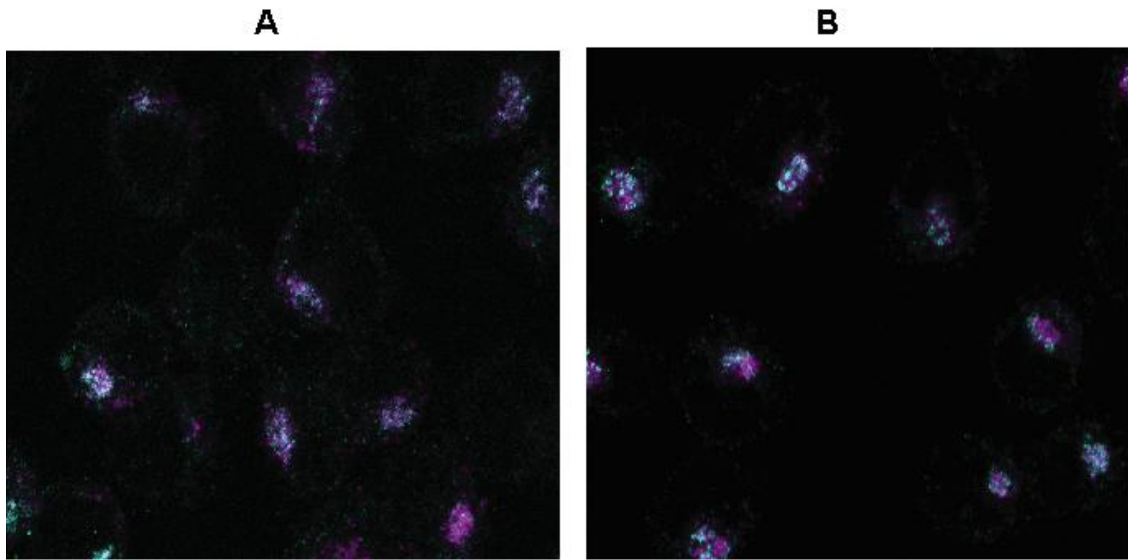
Treatment of A2780-CP20 cells with copper or CDDP does not result in noticeable trafficking of ATP7B. We first determined the localization of ATP7B in cells depleted of copper by treatment with the copper chelator BCS. Under these conditions, the expected location of ATP7B is in the TGN (see Chapter I) therefore we used an antibody against the TGN-resident protein Syntaxin-6 as a marker. As illustrated by Fig.15 (panel A), in A2780-CP20 cells the TGN is small and oval in shape; and in Fig.15 there is visible co-localization between ATP7B and syntaxin-6 in this compartment. To determine whether ATP7B traffics in response to copper elevation cells were treated with 50 μM $CuCl_2$. The image shows a large degree of co-localization between ATP7B and Syntaxin-6, as depicted by white/blue staining. Comparison with the BCS pattern illustrates that localization of ATP7B in copper-treated cells did not change significantly.

Fig.16 illustrates that treating cells with CDDP does not induce trafficking of ATP7B (panels A and B). Staining of BCS and CDDP treated cells is similar, with no evidence of ATP7B being present in cytosolic vesicular structures in either case. This can be compared to the top row of panel C in the figure. HepG2 cells were used as a positive control to illustrate the difference in ATP7B staining in BCS and copper treated cells due to trafficking. The figure shows a distinct loss in co-localization (white) upon copper treatment. Furthermore, ATP7B staining appears to be cytosolic and punctate in nature when HepG2 cells are treated with copper.

ATP7A appears to undergo copper-dependent trafficking in A2780-CP20 cells. To investigate the role of ATP7A in copper homeostasis in these cells, treatment with BCS and copper was performed and trafficking was investigated as described for ATP7B.

As shown in Fig.17 there is a distinct difference in staining pattern between BCS and copper treated cells. In copper limiting conditions ATP7A is found in the TGN, as is evident by its co-localization with Syntaxin-6. When excess copper is added to cells ATP7A traffics out of the TGN and is present in vesicular structures. This difference in ATP7A localization was consistently observed; only a couple of cells are shown for clarity.

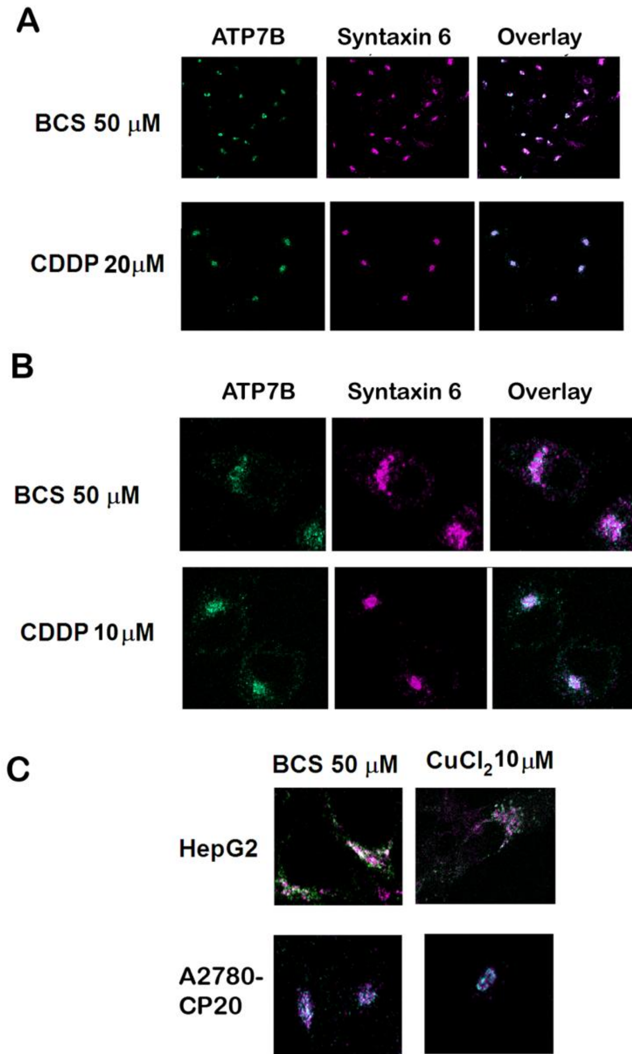
Figure A1



ATP7B does not traffic when A2780-CP20 cells are treated with copper

A2780-CP20 cells were plated onto glass coverslips as previously described and treated with 50 μM BCS (panel A) and 50 μM $CuCl_2$ (panel B) for an hour prior to being fixed and co-stained for ATP7B (green) and sytaxin-6 (purple) (antibodies as before). Co-localization can be observed in overlay images as blue/white staining as before.

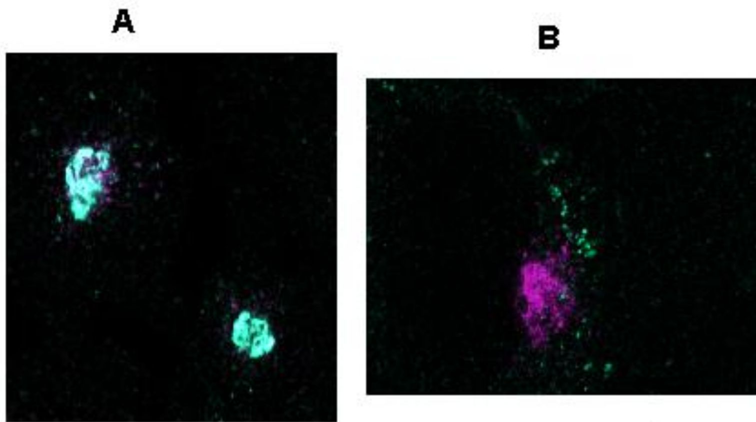
Figure A2



ATP7B does not re-localize from a peri-nuclear location when A2780-CP20 cells are treated with copper or CDDP

A2780-CP20 cells were treated with 50 μM BCS, 10 μM CDDP (panel A), or 20 μM of CDDP (panel B). In panel C HepG2, and A2780-CP20 cells were treated with either 50 μM BCS or 10 μM BCS or 50μM $CuCl_2$.

Figure A3



ATP7A undergoes copper-dependent trafficking in A2780-CP20 cells

A2780-CP20 cells were treated with 50 μM BCS (panel A) and 50 μM $CuCl_2$ (panel B) prior to being fixed and co-stained for ATP7A (green) (ATP7A CTD antibody as before) and syntaxin-6 (purple). Here, the zoom function in Adobe Photoshop was utilized for clear visualization of vesicular structures in panel B.

Discussion

Our study demonstrates that neither copper nor CDDP induce trafficking of ATP7B in CDDP resistant ovarian cancer cells. The lack of trafficking in the presence to CDDP is similar to findings by Lehonhardt et al. where ATP7B did not relocalize in hepatoma cells treated with the drug. If ATP7B were causing A2780-CP20 cells to be resistant to CDDP by a mechanism of active transport, it would traffic out of the TGN upon the addition of CDDP. The drug would be pumped into vesicles and released from the cell by exocytosis. However this is not the case, and the findings in this investigation suggest that ATP7B causes CDDP resistance in A2780-CP20 cells by a different mechanism. As suggested by Lehonhardt et al. this may be via binding and sequestration of the compound.

From the data in this investigation and that of others it seems that over expression of ATP7B causes tumor cells to become resistant to CDDP by a complex and as yet unknown mechanism and that this may vary across cell types. From a clinical standpoint this complexity illustrates one of the many barriers faced by scientists and clinicians when treating malignancy.

Interestingly, ATP7B in A2780-CP20 cells does not relocalize in response to elevated copper. This is different to what was observed in Caco-2 cells in the previous section where copper dependent trafficking was evident. The fact that ATP7B traffics in some cell types but not others further points towards it having specialized, cell-specific functions.

The data in this investigation exemplify the different roles of ATP7A and ATP7B in cells where they are co-expressed. When Figs 15 and 17 are compared it is clear that ATP7A undergoes copper-dependent trafficking, but ATP7B does not. This is similar to the situation in renal cells. Hek293 endogenously express both copper ATPases. In these cells ATP7A undergoes copper dependent trafficking when copper is added to extracellular media, but ATP7B remains in a peri-nuclear location. This was also found to be the case in primary kidney cells (Barnes *et al.,* 2009).

It has been proposed that endogenous ATP7B protein in the kidney and liver are not identical. Renal ATP7B mRNA is modified in its exon 1 region, resulting in a protein with an altered N-terminal structure. The importance of specific N-terminal motifs for the trafficking of copper ATPases has been widely studied and is described in the next chapter. It is believed that renal ATP7B does not traffic because of the modification of exon 1 (Barnes *et al.,* 2009). A similar scenario may be occurring in A2780-CP20 cells.

Appendix II

Intracellular trafficking of ATP7A and ATP7B

Published as part of:

"*Cellular multitasking: the dual role of human Cu-ATPases in cofactor delivery and intracellular copper balance.*"

Lutsenko S, Gupta A, Burkhead JL, Zuzel V.

Arch Biochem Biophys. **2008** Aug 1;476(1):22-32

Summary

Copper-ATPases traffic out the TGN to vesicular compartments at or near the plasma membrane. This property enables the transporters to perform both a biosynthetic and protective functions; they donate copper to cuproenzymes in the TGN and transport copper into exocytic vesicles for removal from the cell. Despite trafficking being so fundamental to the role of Cu-ATPases in maintaining cellular homeostasis, the details of this complex phenomenon are just beginning to be understood.

Introduction

Cu-ATPases deliver copper to cuproenzymes in the secretory pathway and maintain the intracellular concentration of copper in a cell by exporting excess of copper either into circulation for further distribution between tissues or into the bile for removal with feces. This dual function is made possible by the ability of the transporters to traffic between intracellular compartments.

It is well established that under basal (low copper) conditions both ATP7A and ATP7B reside in the TGN, where they display a characteristic perinuclear staining pattern. It is also well established that both ATP7A and ATP7B respond to an increase in copper by trafficking from the TGN towards the plasma membrane. In polarized cells ATP7A traffics to the basolateral membrane to release copper into the circulation, while ATP7B traffics apically. In hepatocytes the purpose of this apical transport is to release excess copper across canalicular membranes into the bile (46). ATP7B is hypothesized to

perform cell-specific functions, and this is reflected in its trafficking behavior. In the kidney (23) and ovary (33) ATP7B does not re-localize in conditions of excess copper instead it is believed to sequester copper near the TGN for fine-tuning its release.

A review of trafficking

Understanding the basic elements of trafficking. To determine whether the relocalization of ATP7A to the cell surface is due to a copper-stimulated forward traffic of ATP7A from the TGN to the cell surface (exocytosis) or due to the decreased retrieval at the plasma membrane (endocytosis), Petris and Mercer introduced an epitope tag for the antibody (myc-tag) to the first lumenal/extracellular loop of ATP7A (47). Immunofluorescence imaging of this ATP7A variant in non-permeabilized cells showed that the internalization of the myc-tagged ATP7A occurred under both basal and elevated copper conditions, supporting the model that copper stimulates anterograde trafficking from the TGN (47). The presence of ATP7A at the plasma membrane was confirmed by cell-surface protein biotinylation (31, 48), suggesting that ATP7A may function at this location and export copper directly across the plasma membrane. However, these studies also revealed the presence of a recycling pool of ATP7A (48). In addition, recent quantitative analysis of ATP7A distribution in intestinal cells (31) as well as immunocytochemistry of ATP7A in tissues (49, 27) convincingly demonstrates that the plasma membrane represents a minor and transient location for Cu-ATPases.

The vast majority of protein in high copper condition resides in vesicles in close vicinity to the plasma membrane. Altogether, these observations lead to a current model of

constitutive cycling of ATP7A between the TGN, vesicles, and the plasma membrane under basal conditions. When copper is added, the steady state distribution of ATP7A is shifted towards the vesicles and cell surface (see (50, 51)).

Mechanism of trafficking. ATP7A mediates copper export from the cell in at least two steps. As copper levels increase ATP7A traffics to vesicles in close proximity to the plasma membrane where excess copper is sequestered into the lumen of vesicles. These vesicles fuse with the plasma membrane, and copper is released into the extracellular milieu. During this process the Cu-ATPase appears at the cell surface, prior to being endocytosed, where it returns to a recycling vesicular compartment. Intracellular copper levels determine whether it continues to transport copper to exocytic vesicles or whether it returns to the TGN. How these intracellular levels are "sensed" by the Cu-ATPase is currently unknown.

ATP7B trafficking was investigated in hepatic cells where it is primarily expressed and where ATP7A is absent. Both *in vitro* and *in vivo* experiments have illustrated copper dependent trafficking of ATP7B in hepatocytes (13, 51, 52). Current data suggest a mechanism of the ATP7B trafficking that is very similar to the one described for ATP7A.

Other factors to consider. Despite it being repeatedly shown that the addition of copper to cell culture medium results in trafficking of ATP7A and ATP7B, the underlying mechanism for the exit of Cu-ATPases from the TGN is not well understood. A scenario where the Cu-ATPase trafficking is due to increased cellular copper is the most probable

and currently accepted. However, research has shown there to be other factors to consider.

Signaling at the cell surface. A model that involves receptor/kinase-mediated signaling from the cell surface cannot be excluded. This is supported by the recent demonstration of the ATP7A relocalization in response to changes in calcium concentration (induced by the opening of the NMDA receptors (54)) or in response to hormonal signaling (55); both these trafficking events occur without extracellular additions of copper.

Kinase-mediated phosphorylation of ATP7A and ATP7B. Elevated copper has been shown to cause kinase-mediated phosphorylation of ATP7A (56) and ATP7B (57) although the identity of these kinase(s) is unknown. The change in the phosphorylation state of Cu-ATPases is linked to the change in subcellular localization. Specifically, their re-localization from the TGN to vesicles is associated with a hyper-phosphorylated state while recycling back to the TGN with dephosphorylation to the basal level. Kinase-mediated phosphorylation may provide a link between various signaling events and Cu-ATPase trafficking. The precise mechanism and the role of kinase-mediated phosphorylation in regulating the Cu-ATPase trafficking awaits further exploration.

Conformational changes are linked to trafficking. It has been shown that the copper-transport activity is necessary for the ability of Cu-ATPases to leave the TGN. Mutations that prevent copper binding within the transmembrane portion or those that disrupt the formation of phosphorylated intermediate also prevent trafficking of Cu-ATPase from the TGN (for review see (50)). The mutation of the phosphatase motif (TGE>AAA) in

ATP7A, which inhibits copper transport but stabilizes the phosphorylated intermediate, resulted in vesicular localization under both basal and high copper conditions (58). These experiments provided convincing evidence for the existence of a conformational state of Cu-ATPases that is preferentially recognized by cellular trafficking machinery.

Further evidence for the important of Cu-ATPase conformation came from the studies on ATP7B, in which the deletion of the part of the N-terminal domain including the first 63 residues also led to relocalization from the TGN even in the basal copper (59).

Trafficking in polarized cells. Another set of interesting observations was revealed by the experiments trying to determine the molecular basis of Cu-ATPase trafficking to different membranes in polarized cells. As described previously, ATP7A traffics towards the basolateral membrane, while ATP7B moves to the apical membrane. Subsequent studies demonstrated that the very N-terminal 63 amino acids of ATP7B are absent in ATP7A. These are not only important for the copper-dependence of ATP7B trafficking, but also are essential for the apical delivery of the transporter in polarized cells (59). If this segment is kept intact, then the deletion of the N-terminal copper-binding sub-domains 1–4 does not affect its trafficking in polarized hepatic cells (59). Deletion of metal-binding domains (MBSs) 1–4 together with the most N-terminal 63 amino acids results in a functional transporter that traffics towards the basolateral membrane and vesicles even when copper is low (59). This observation suggests that copper sensing and subsequent trafficking are intrinsic properties of the transporter (rather than being determined by copper-binding proteins in the cytosol). Furthermore it seems clear that

the cell’s trafficking machinery recognizes specific signal sequences within ATP7B to direct it to the appropriate membrane.

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