Molecular Pathogenesis of Genetic and Inherited Diseases

Copper-Induced Translocation of the Wilson Disease Protein ATP7B Independent of Murr1/COMMD1 and Rab7

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Wilson disease is a genetic disorder of copper metabolism. Impaired biliary excretion results in a gradual accumulation of copper, which leads to severe disease. The specific gene defect lies in the Wilson disease protein, ATP7B, a copper-transporting ATPase that is highly active in hepatocytes. The two major functions of ATP7B in the liver are the copper loading of ceruloplasmin in the Golgi apparatus, and the excretion of excess copper into the bile. In response to elevated copper levels, ATP7B shows a unique intracellular trafficking pattern that is required for copper excretion from the Golgi apparatus into dispersed vesicles. We analyzed the translocation of ATP7B by both confocal microscopy and RNA interference, testing current models that suggest the involvement of Murr1/COMMD1 and Rab7 in this pathway. We found that although the ATP7B translocation is conserved among nonhepatic cell lines, there is no co-localization with Murr1/COMMD1 or the Rab marker proteins of the endolysosomal system. Consistent with this finding, the translocation of ATP7B was not impaired by the depletion of either Murr1/COMMD1 or Rab7, or by a dominant-negative Rab7 mutant. In conclusion, our data suggest that the translocation of ATP7B takes place independently of Rab7-regulated endosomal traffic events. Murr1/COMMD1 plays a role in a later step of the copper excretion pathway but is not involved in the translocation of the Wilson disease protein. (*Am J Pathol* 2008, 173:1783–1794; DOI: 10.2353/ajpath.2008.071134)

Copper is a trace element in the diet, but is required as a protein co-factor for basic cellular processes and there-

fore essential for all living organisms. However, too much intracellular copper is cytotoxic, leading to the formation of reactive oxygen species. In mammals, intestinal copper uptake does not seem to be regulated, and homeostasis is achieved primarily by adjusting biliary excretion of copper.^{1,2}

Wilson disease is characterized by gradual accumulation of copper in tissues, manifested by liver disease and/or neurological symptoms.^{3,4} This autosomal recessive disorder of copper homeostasis in humans is caused by a functional deficiency of ATP7B, the Wilson disease protein (WDP).^{5,6} Many different mutations distributed along the whole *ATP7B* gene lead to Wilson disease.^{7,8} The WDP is critical for biliary excretion of copper⁹ but also supplies copper ions for the ferroxidase ceruloplasmin,¹⁰ which is the main copper containing protein of serum.¹¹ Copper transport defects may also lead to systemic copper deficiency when ATP7A, a related intestinal P-type ATPase, is mutated.^{12–14}

The WDP is a copper-translocating ATPase highly expressed in hepatocytes. ATP7B features eight transmembrane domains forming a channel, and shows unidirectional ATP-dependent transport of cytoplasmic copper ions across lipid bilayers. In hepatocytes, translocated copper is secreted on the apical side into the bile or is transferred to the ferroxidase ceruloplasmin at a late Golgi compartment,^{3,10} and is then secreted to the basolateral side (serum).

Supported by the Dietmar Hopp Foundation (grant to W.S.), the Medical Faculty of the University of Heidelberg (young investigator grant to K.H.W.), and by the Stiftung Nephrologie (to R.E. and J.F.).

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Accepted for publication September 9, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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ATP7B shows striking changes in its subcellular localization when copper concentrations are manipulated. At low copper levels, ATP7B is localized to the late Golgi compartment^{15,16} where presumably copper loading of ceruloplasmin takes place. However, when copper levels are high, ATP7B shifts away from the Golgi apparatus to cytoplasmically dispersed vesicles, which in polarized liver cells accumulate subapically.^{16–20} It is not clear how copper transported into these vesicles would finally reach the bile, but it is generally assumed that the translocation of ATP7B is a necessary precondition for copper excretion.^{3,21,22}

Certainly other proteins than ATP7B contribute to the molecular mechanism of copper excretion, and mutations or polymorphisms of these proteins might contribute to Wilson disease, maybe explaining the highly variable clinical presentation^{23–25} and course of this disease. Canine copper toxicosis of Bedlington terriers²⁶ is caused by a deficiency of Murr1/COMMD1^{27,28} and resembles Wilson disease, although ceruloplasmin levels are not decreased²⁹ and there are no evident neurological symptoms. Murr1/COMMD1 has been reported to interact physically with ATP7B.^{30–32} Based on these observations, a role of Murr1/COMMD1 in the biliary excretion of copper downstream of Golgi-localized ATP7B has been suggested.^{2,3,33} Consistent with this, Murr1/COMMD1 was found on endolysosomal membranes but also in the cytosol²⁸ and in the nucleus.³⁴ Depletion of Murr1/COMMD1 by RNA interference results in an intracellular copper accumulation.^{35,36} The lack of exon 2 of the Murr1 gene leads to copper toxicosis in dogs, but the same deletion is embryonically lethal in mice.³⁷ Murr1 is part of a larger protein family sharing a C-terminal leucine-rich domain termed the copper metabolism Murr1 domain (COMMD),^{34,38} and there is evidence that the other members of this family also bind to ATP7B.³¹ Murr1 is involved in NF- κ B-mediated regulation of gene transcription,^{39,40} which has been recently reviewed together with other possible functions of the COMMD family.³⁸ XIAP is another protein interacting with Murr1, and enhanced degradation of XIAP mediated by copper binding sensitizes hepatocytes for apoptosis,⁴¹ providing an unexpected new angle for copper-induced cell damage.

Here, we applied confocal microscopy and RNA interference to investigate the copper-induced translocation of the WDP, testing current models^{2,3,33} that hypothesize an interaction of Murr1 and ATP7B during this intracellular trafficking step. We found no evidence for a role of Murr1, suggesting that this protein is involved in a different step of copper excretion. Furthermore, we analyzed the reported relationship between ATP7B and Rab7-positive endosomes.⁴² Although we could document a partial co-localization between Murr1 and Rab7, the WDP was not observed in Rab7-positive endosomes, even after copper-induced translocation to cytoplasmically dispersed vesicles.

Materials and Methods

Antibodies

The antibody against ATP7B was essentially prepared as described.¹⁸ Oligonucleotides were designed and used to amplify the region of the Wilson protein encoding amino acids 325 to 635. This region was amplified by polymerase chain reaction (PCR) and subcloned in the pGEX-2T vector (Amersham Pharmacia Biotech, Uppsala, Sweden). *Escherichia coli* BL21 cells harboring the expression plasmid were grown to an optical density of 1.5 at 600 nm at 31°C and induced with isopropyl 1-thio- β -D-galactopyranoside. Cultures were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) containing 1% Triton X-100, and lysed using a high-pressure cell crusher. The supernatant was incubated with glutathione-agarose beads. Bound glutathione S-transferase (GST) fusion protein was thrombin-cleaved to obtain the ATP7B-fragment. New Zealand White rabbits were immunized with 4 $\times$ 100 mg of this recombinant protein (immunization was performed according to standard procedures by EuroGentec, Seraing, Belgium).

Affinity purification of the antiserum was performed using AminoLink Plus columns as recommended by the manufacturer (Pierce, Rockford, IL). Recombinant protein was coupled to the columns using 0.1 mol/L sodium citrate, 0.05 mol/L sodium carbonate, pH 10. Remaining active sites were blocked by sodium cyanoborohydride solution (5 mol/L NaCNBH₃ in 0.01 mol/L NaOH). An additional pre-elution step using elution buffer (0.2 mol/L glycine-HCl, pH 2.6) was performed to remove noncovalently bound recombinant protein. After washing the column with PBS (pH 7.2), the antiserum was loaded to the column. After repeated washing, the bound antibodies were eluted from the column using the elution buffer. Protein concentration of the eluate was determined using a mini Bradford assay. The eluted antibody solution was rebuffed in PBS and 5% bovine serum albumin using PD-10 columns. For long-term storage 50% glycerol was added to the rebuffed antibody solution. The monoclonal COMMD1/Murr1 antibody (M01, clone 2A12) was obtained from Abnova Corporation (Jhongli City, Taiwan). The goat anti-Rab7 antibody (Sc6563) was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti β -actin antibody (clone AC-15) was obtained from Sigma (St. Louis, MO).

Cell Culture

HeLa [American Type Culture Collection (ATCC) no.: CCL-2], Ptk2 (ATCC no.: CCL-56; derived from normal kidney of *Potorous tridactylis*), CaCo-2 (ATCC no.: HTB-37), MDCK (ATCC no.: CCL-34) cells were cultured according to the ATCC protocols. The immortalized human keratinocyte cell line, HaCaT, and the human hepatoma cell line, HuH7, were cultured in Dulbecco's modified Eagle's medium (high glucose 4.5 g/L), supplemented with glutamine (2 mmol/L), penicillin (400 U/ml), streptomycin (50 ng/ml), and 10% fetal bovine serum in a hu-

modified 5% CO₂ atmosphere at 37°C. The phoenix-gp packaging cell line was cultured as previously described.⁴³

Expression Plasmids

Rab7-GFP,⁴⁴ GFP-Rab7-DN(T22N),⁴⁴ Rab11-GFP,⁴⁵ Rab9-YFP,⁴⁶ and caveolin-1-GFP⁴⁷ were provided by Marino Zerial (MPI Molecular Cell Biology and Genetics, Dresden, Germany) and Lucas Pelkmans (ETH, Zurich, Switzerland), respectively. The T2-GFP plasmid⁴⁸ was supplied by Jamie White (EMBL, Heidelberg, Germany). The full-length ATP7B-cDNA⁵ was provided by John R. Forbes (Department of Medical Genetics, University of Alberta, Edmonton, Canada) in a yeast vector. ATP7B-cDNA was subcloned using *Bam*HI into pcDNA3 (Invitrogen, Carlsbad, CA), resulting in a mammalian expression vector. Generation of fluorescent protein-MURR1 fusion constructs was performed as follows: the complete coding region of human MURR1 was amplified from the total cDNA of the human CaCo-2 cells by PCR using sense primer (5'-ACGTAAGCTTACCATGGCGGCGGGCGAGCTTG-3') and antisense primer (5'-CAC TGATCAGCCAGCCTAACGCGGATCCACGT-3'). The sense primer introduced a *Hind*III site and the consensus Kozak sequence ACC in front of the starter ATG, the antisense primer covered a *Bam*HI restriction site and the stop codon. The *Hind*III to *Bam*HI fragment of whole-length Murr1 cDNA was cloned upstream of green fluorescent protein (GFP) cDNA in the mammalian expression vector pEGFP-N1 (BD Biosciences Clontech, Mountain View, CA). The correct sequence was confirmed by bidirectional sequencing. Murr1-RFP was derived from the Murr1-GFP plasmid by replacing GFP (*Bam*HI, *Not*I) with PCR-modified monomeric RFP.⁴⁹ Murr1-GFP and Murr1-mRFP had identical localizations when co-expressed (see Supplemental Figure 1 at <http://ajp.amjpathol.org>).

Immunoblotting

Equal amounts of protein (50 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% gels followed by electrophoretic transfer to nitrocellulose membranes, incubated with the primary antibody and visualized using enhanced chemiluminescence detection. Immunostaining of β-actin served as a further loading control.

Transfection, Copper Exposure, and Immunofluorescence

For transient transfection cells were grown on coverslips in a six-well plate (10 cm²/well) to a density of ~80% and transfected using 4 µg of plasmid DNA and 10 µl of Lipofectamine 2000 reagent (Invitrogen) per well according to the manufacturer's protocol. Six hours after transfection, cells were washed in prewarmed PBS (pH 7.2) and normal culture medium was added to the cells. Copper exposure experiments were started 46 hours after

transfection. Before copper exposure, cells were washed twice in prewarmed PBS (pH 7.2) to remove culture medium. Cells were then either incubated with prewarmed Dulbecco's modified Eagle's medium containing 100 µmol/L of the copper chelator bathocuproinedisulfonic acid (BCS) or 100 µmol/L copper sulfate (CuSO₄) for 2 hours.

After repeated washing in PBS, cells grown on coverslips were fixed with methanol (−20°C) for 3 minutes. With this fixation procedure Murr1-GFP and Murr1-RFP showed a clean vesicular distribution. Use of paraformaldehyde emphasized the cytosolic and nuclear localization of Murr1 (see Supplemental Figure 1, a and b, at <http://ajp.amjpathol.org>). Antibodies were incubated in PBS containing 0.1% saponin (Sigma), 0.5% gelatin (Teleostan gelatin, Sigma), and 5 mg/ml bovine serum albumin. Corresponding secondary antibodies (donkey anti-rabbit/mouse; Dianova, Hamburg, Germany) were used conjugated to Cy3 or fluorescein isothiocyanate. Coverslips were mounted in Prolong Gold antifade mounting medium (Molecular Probes, Leiden, The Netherlands) and confocal images were taken on a TCS SP2 microscope (objective: ×63 magnification, oil immersion, NA 1.32; Leica Microsystems, Wetzlar, Germany). Double-immunofluorescence images were taken sequentially, and parameters were adjusted so that all light intensities were in the recording range. Intensity of the laser beam and photo multiplier levels were adjusted for each slide and each fluorophore (Cy3, fluorescein isothiocyanate, GFP, RFP, YFP, lysotracker); line averaging was set to 4 to optimize signal to noise ratio. Images shown were derived from representative single confocal planes and arranged with Adobe Photoshop and Adobe Illustrator (Adobe, San Jose, CA).

RNA Interference

Target sequences for human Murr1/COMMD1³⁹ and Rab7⁵⁰ were 5'-AAGUCUAUUGCGUCUGCAGAC-3' and 5'-CGGUUCCAGUCUCGUG-3', respectively. Oligonucleotides encoding the corresponding shRNAs were designed and cloned into pSuper as described⁵¹ except that *Bgl*II and *Xho*I were used. For confirmation, plasmids were sequenced in both directions.

Cloning of the retroviral plasmids, transfection of the packaging cell line, collection of pseudotyped retrovirus particles, and transduction of HuH7 cells were as published.⁴³ Briefly, the shRNA expression cassette was transferred by subcloning into the *Xho*I/*Eco*RI site of pRVH1-puro. Cells of the human phoenix gag-pol packaging cell line were grown in 10-cm dishes to near confluence. Using Lipofectamine 2000 reagent, phoenix-gp cells were co-transfected with 2.5 µg of DNA of the pVSV-G-plasmid (pseudotyping; enhanced stability and efficient infection) and 24 µg of DNA of the pRVH1-puro plasmid containing the Murr1, Rab7, or control shRNA expression cassette, respectively. Twenty-four hours after transfection, medium was changed from high glucose (4.5 g/L) to low glucose (1.0 g/L) and cells were placed at 32°C. Batches of virus-containing supernatants were collected every 24 hours for up to 6 days.

For target cell transduction, 1.5×10^5 HuH7 cells were mixed with 450 μ l of virus-containing supernatant in the presence of 4 μ g/ml polybrene (Sigma) and seeded into a well of a six-well plate and incubated at 32°C. Twenty-four hours after infection, the medium was changed to normal culture medium and cells were returned to 37°C. From 48 hours to 144 hours after infection, cells were grown in the presence of 2 μ g/ml of puromycin (BD Biosciences) to eliminate nontransduced cells. Cells were used for experiments from 14 to 18 days after infection. The RNAi-mediated depletion of target mRNA by Murr1-shRNAi and Rab7-shRNAi was measured by reverse transcriptase (RT)-PCR and visualized by immunofluorescence staining and Western blot. Results were compared to HuH7 cells infected with the control virus containing no shRNA sequence.

Quantification of ATP7B Translocation

After copper exposure and ATP7B immunofluorescence, coverslips were analyzed with an Olympus IX50 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a $\times 60$ oil immersion objective. Counting was blinded because the examiner did not know which treatment the cells had received. A cell was classified as translocated when a diffuse vesicular staining was observed. Cells showing a tight perinuclear ATP7B immunoreactivity without a vesicular pattern were classified as not translocated. Translocated cells were divided by the total number of cells (~ 200 cells were counted for each coverslip, translocation ratio was calculated per coverslip, two coverslips were counted per experiment). SD was calculated from three independent experiments.

Real-Time PCR

Total RNA was isolated from tissue culture cells with the RNeasy mini kit including DNase digestion. The Omniscript RT kit and random N6 primers were used for reverse transcription. All reagents and procedures for cDNA synthesis were from Qiagen, Hilden, Germany. Real-time PCR was performed using the LightCycler system (Roche, Mannheim, Germany). Transcripts were detected with SYBR Green I and normalized to β -actin as internal control. LightCycler Relative Quantification Software Version 1.0 together with calibration plasmids [pRRRL-PGK-ATP7B,⁵² Murr1-GFP (this study), Rab7-GFP (this study), EST-actin (RZPD IOH27856, Berlin, Germany)] was used for the calculation of ATP7B/actin and Murr1/actin ratios and Rab7/actin ratios (efficiency corrected relative quantification). Primer sequences human ATP7B: 5'-CCA-CATGAAGCCCTGAC,GTACTGCTCCTCATCCCTGC-3'; human COMMD1/Murr1: 5'-GGGATTCTTAAGTCTATTGCG-TCTTCT,GTCTGTCAGATGTGATCCCACCTTGCTT-3'; human Rab7: 5'-TGTTGGGAAACAAGATTGACCACC,CTTCTTAAGTGATTCCGTGCAA-3'; human β -actin: 5'-AGGATGCAAGAGGAGATCACTGCTG,GGGGGGTGTAAACGCAACT-AAGTCATAG-3'.

Results

Copper-Dependent Translocation of the WDP ATP7B in Ptk2 Cells

To study the intracellular trafficking of the WDP we raised and affinity-purified an antiserum against the N-terminal domain and screened different cell lines for expression. Although high levels of ATP7B were found in hepatocyte-derived cell lines, these cells were not easy to evaluate morphologically even by confocal microscopy. HeLa cells were suitable for analysis when ATP7B was ectopically expressed (Figure 1A). Kidney-derived Ptk2 cells are, because of their flat and extended cell body, an excellent model system to study intracellular trafficking by light microscopy, and were found to express significant amounts of endogenous WDP (Figure 1A).

Copper concentrations different from standard culture medium (MEM) were achieved using either incubation with 100 μ mol/L copper sulfate or the copper-chelating reagent BCS. Comparison to a Golgi-GFP marker protein⁴⁸ demonstrated that the WDP showed a Golgi pattern when copper concentrations were low, but a diffuse vesicular pattern under high copper levels (Figure 1B). This is in line with reports on primary hepatocytes or liver-derived cell lines.^{16–18} Withdrawal of copper leads to a return of WDP to the Golgi apparatus (not shown).

Analyzing this apparent translocation event at different time points, it became clear that this intracellular movement is not synchronized across the whole cell population. In fact, 60% of cells showed translocation after 1 hour, but an additional 20% changed their WDP localization only within the second hour (Figure 1C). Both the copper-dependent translocation of endogenous WDP and the time scale proved to be essentially the same in hepatocyte-derived and other cell lines (HuH7, Caco-2, MDCK; data not shown). Although the physiological role of ATP7B in copper excretion has been suggested to be essential only in the liver (summarized by J.D. Gitlin³), we concluded that the copper-dependent trafficking step is remarkably conserved across very different cell types.

Comparison of ATP7B to Rab Marker Proteins of the Endosomal Pathway

Although the Golgi localization of the WDP under standard conditions (ie, low copper concentration) has been documented extensively,^{16–19} the nature of the dispersed vesicular structures after copper exposure is not clear and no co-localizing proteins have been reported to date. Based on the recent report that overexpressed GFP-ATP7B resides in late endosomes together with Rab7,⁴² we analyzed the distribution of endogenous WDP in relation to different rab marker proteins of the endosomal pathway, again in Ptk2 cells. However, we did not observe any overlap between Rab7 and ATP7B, regardless of the copper concentration (BCS, Figure 2A; MEM, Figure 2B; added copper, Figure 2C). Rab7 and Rab9 both localize to late endosomes but occupy distinct

Figure 1. **A:** Endogenous expression of the WDP ATP7B in Ptk2 cells. Cell lysates (50 μ g of protein) were analyzed by Western blotting with the affinity-purified rabbit antibody against ATP7B (molecular weight of ATP7B: 157 kDa). Kidney-derived Ptk2 cells express endogenous ATP7B (**lane 1**) comparable to the human hepatoma cell line HuH7 (**lane 4**). The level of endogenous ATP7B in HeLa cells was low (**lane 2**), and transient expression of the WDP was necessary for analysis (**lane 3**, HeLa+). Molecular weight standards are indicated in kDa. **B:** Copper-dependent translocation of endogenous ATP7B in Ptk2 cells. Ptk2 cells expressing the Golgi marker protein T2-GFP were incubated for 2 hours either with the copper-chelating agent BCS (**a**), in standard culture medium (MEM, **b**), or with 100 μ mol/L CuSO_4 (+Cu, **c**). Cells were fixed and endogenous ATP7B stained by indirect immunofluorescence. Overlap between ATP7B (WDP, red) and the Golgi marker protein in the basal and copper-chelated state (**a** and **b**, overlay) is indicated by **arrowheads**. No co-localization was observed after copper treatment (**c**). **C:** Time course of translocation of endogenous ATP7B in Ptk2 cells. After an initial treatment with the copper chelator BCS for 2 hours, Ptk2 cells were exposed to 100 μ mol/L copper ions for variable amounts of time. Microscopy evaluation was done after indirect immunofluorescence staining of ATP7B, scoring Golgi pattern (not translocated) versus dispersed staining (translocated). Data are expressed as mean with SD. The percentage of translocated ATP7B for cells in basal medium was 21% versus 18% for BCS-treated cells. This difference was not statistically significant. Scale bars = 10 μ m.

subdomains with little co-localization,⁴⁶ but again Rab9 was present in different structures when compared to ATP7B (Figure 2). Recycling endosomes marked by Rab11 were also distinct from endogenous WDP staining (Figure 2). In addition labeling with the lysotracker dye marking less specifically the late endosomal/lysosomal pathway did not show any overlap with ATP7B (see Supplemental Figure 2 at <http://ajp.amjpathol.org>). We repeated the comparison between WDP and Rab7, Rab9, and Rab11 in HeLa cells transiently expressing ectopic WDP but results were unchanged (see Supplemental Figure 3 at <http://ajp.amjpathol.org>). Endosomal structures termed caveosomes⁵³ were also investigated but did not overlap with ATP7B (see Supplemental Figure 4 at <http://ajp.amjpathol.org>).

Because the copper-induced redistribution of the WDP from the *trans*-Golgi compartment to dispersed vesicles is of major functional importance especially in liver cells, we further evaluated the hepatoma cell line HuH7. ATP7B and Rab7 (Figure 3, a–c), Rab9,

or Rab11 (see Supplemental Figure 5 at <http://ajp.amjpathol.org>) remained segregated in HuH7 cells regardless of the copper concentration, consistent with the data obtained for Ptk2 and HeLa cells. An overlap between Rab7 and overexpressed ATP7B has been reported in MDCK cells.⁴² Therefore we repeated our localization analysis in this canine kidney cell line, but again no co-localization between endogenous WDP and Rab7-GFP could be observed (Figure 3d).

Murr1/COMMD1 Localization to Intracellular Vesicles Distinct from ATP7B

Murr1 has been suggested to participate in the excretion of copper by interacting with ATP7B during or after copper-triggered translocation away from the Golgi apparatus.^{2,3,33} The reported vesicular localization of Murr1 in HeLa cells²⁸ was consistent with the idea that there could

Figure 3. ATP7B and Rab7 are segregated in the hepatoma cell line HuH7 and in MDCK cells. HuH7 cells (**a–c**) and MDCK cells (**d**) were transiently transfected with Rab7-GFP. Two days after transfection cells were either treated with BCS (**a**) or cultured in basal medium (**b, d**) or treated with copper (**c**) for 2 hours, fixed, and processed for indirect immunofluorescence staining of ATP7B (WDP, shown in red). Scale bars = 10 μ m.

be a direct interaction with translocated ATP7B on membrane structures. Therefore we constructed a Murr1-GFP fusion protein, which indeed gave a convincing punctate membrane pattern when expressed in HeLa cells. Surprisingly, there was no significant overlap with the WDP, neither under low, basal (MEM) or high copper concentrations (Figure 4A). Moreover, although ATP7B shifted its localization in response to copper, we did not observe any change in the pattern for Murr1/COMMD1. Murr1-GFP also gave a punctate pattern in Ptk2 cells (data not shown). An antibody against Murr1 became recently available so that we were able to confirm the segregation of Murr1 and ATP7B by immunofluorescence staining of endogenous proteins in Ptk2 cells (Figure 4B). We again confirmed with the HuH7 cells that our findings are also relevant for endogenous WDP and Murr1/COMMD1 in hepatocyte-derived cells (Figure 4C). The proposed interaction between Murr1 and the WDP^{2,30,33} implies that there should be corresponding expression patterns. The relative quantification of mRNA expression levels by efficiency corrected real-time PCR of human cell lines was generally consistent with this idea (Figure 4D), however

the keratinocyte HaCaT cell line only expressed Murr1/COMMD1 but not the WDP.

Partial Localization of Murr1/COMMD1 to Late Endosomes

We observed significant co-localization of punctate structures between Murr1 and Rab7, which was obvious even without overlaying the images (Figure 5a). This suggests that although neither Murr1/COMMD1 nor Rab7 overlap with the WDP, they do share some common vesicular compartment, presumably of endosomal origin. Rab7 is involved in the regulation of vesicular traffic and has been localized to late endosomes and lysosomes.^{44,54} Rab9, which regulates transport between late endosomes and the *trans*-Golgi network,⁵⁵ also gave a partial overlap with Murr1 although in general less extensive (Figure 5b). No co-localization was evident when Murr1/COMMD1 was compared to the recycling endosome marker Rab11 (Figure 5c). We did not see any pattern changes when extracellular copper concentrations were manipulated (data not shown).

The Translocation of ATP7B Is Not Impaired by Knockdown of Either Murr1 or Rab7

Although we did not detect any co-localization between the WDP and either Murr1 or Rab7, this did not rule out a functional contribution of these proteins to the copper-induced trafficking of ATP7B. To directly test this, we developed human hepatoma cell lines (HuH7) depleted for Murr1 or Rab7 by RNA interference (RNAi). This was based on validated RNAi target sequences,^{39,50} which we designed into small hairpin RNA templates (shRNA). The shRNA coding sequences were incorporated into retroviruses that were then used for stable transduction of the target cells.⁴³ Murr1/COMMD1 and Rab7 were efficiently depleted from HuH7 cells as confirmed by immunofluorescence, RT-PCR quantification, and Western blotting (Figure 6, A–C). HuH7 cells depleted for Murr1 or Rab7 were treated with copper as before, stained for the WDP, and blinded before translocation efficiency was scored (Figure 6E). An additional, independent line of experiments used the well-described dominant-negative Rab7 mutant.⁴⁴ Translocation of the WDP still occurred as assessed by the localization pattern of ATP7B after copper treatment, even in cells highly expressing the dominant-negative Rab7 mutant (Figure 6D). Summarizing, all cell lines had a translocation efficiency similar to control cells (Figure 6E) suggesting that neither Murr1 nor Rab7 are critically involved in the movement of the WDP from the Golgi apparatus to the copper-induced vesicular structures.

Figure 2. ATP7B and endolysosomal rab marker proteins remain segregated after copper-dependent translocation. Ptk2 cells were transiently transfected with Rab7-GFP (late endosomes, lysosomes), Rab9-YFP (late endosomes), or Rab11-GFP (recycling endosomes) as indicated in the figures. Two days after transfection, cells were either treated with BCS (**A**) or cultured in basal medium (**B**) or incubated with copper (**C**) for 2 hours, fixed, and processed for indirect immunofluorescence staining of ATP7B (WDP, shown in red). Scale bars = 10 μ m.

Figure 4. **A:** ATP7B/WDP and Murr1/COMMD1 do not co-localize in HeLa cells. HeLa cells were transiently transfected with Murr1-GFP and ATP7B and processed for indirect immunofluorescence of ATP7B (WDP, shown in red). Before fixation with methanol, cells were either treated with BCS (**a**), cultured in basal medium (**b**), or treated with copper (**c**) for 2 hours. No co-localization was observed. **B:** No overlap between endogenous ATP7B/WDP and Murr1/COMMD1 in Ptk2 cells. Ptk2 cells were processed for indirect immunofluorescence of endogenous Murr1/COMMD1 (red) and ATP7B (WDP, green). Before fixation with methanol, cells were either treated with BCS (**a**), cultured in basal medium (**b**), or treated with copper (**c**) for 2 hours. No co-localization was observed. **C:** No co-localization between endogenous ATP7B/WDP and Murr1/COMMD1 in HuH7 cells. HuH7 cells cultured in standard medium were processed for indirect immunofluorescence of endogenous Murr1/COMMD1 (red) and ATP7B (WDP, green). **D:** RT-PCR quantification of ATP7B and Murr1 in human cell lines. HuH7 (hepatoma), CaCo-2 (intestine), HeLa (cervical), and HaCaT (keratinocytes) cells were evaluated for their mRNA content of ATP7B/WDP and Murr1/COMMD1. *HaCaT cells did not show any signal above background for ATP7B. The results confirmed a low expression of ATP7B in HeLa cells. Arbitrary units were obtained by normalizing the signal to the internal control (values shown are 10^{-3} * signal ATP7B, Murr1/signal actin). Scale bars = 10 μ m.

Figure 5. Murr1 is partially localized to late endosomes. HeLa cells co-expressing Murr1/COMMD1-mRFP and either Rab7-GFP (**a**; late endosomes, lysosomes), Rab9-YFP (**b**; late endosomes, *trans*-Golgi network), or Rab11-GFP (**c**; recycling endosomes) were fixed with methanol and analyzed by confocal microscopy. Overlapping structures are marked by **arrowheads**. Scale bars = 10 μ m.

Discussion

The WDP shows a copper-dependent subcellular localization, and it is thought that the translocation from the Golgi apparatus to subapical vesicles is a necessary step before systemic copper excretion into the

bile.^{3,14,20–22} The nature of these vesicles still represent an enigma, and no other protein components have been identified to date.^{14,56} The molecular mechanism of copper-dependent translocation of ATP7B is not restricted to hepatocytes and hepatoma-derived cell lines; it appears to be a broadly conserved feature of many different cell types eg, CHO cells,^{21,57} HeLa, Ptk2 (this study), MDCK, Caco-2 (K.H.W. and J.F., unpublished). In some cell lines where the Golgi apparatus has a more fragmented appearance as opposed to a tight perinuclear localization, translocation of ATP7B is more difficult to assess but these problems are greatly reduced when co-evaluating a Golgi marker protein. Although the translocation itself might be easier to score in liver-derived cells, kidney-derived Ptk2 cells offer a remarkable spatial resolution and were chosen as the main model system for this study. Although the first cells showed a dispersed vesicular ATP7B staining pattern already after 15 to 20 minutes, many cells took longer times and some did not respond at all; our data correspond well to an earlier examination of HepG2 cells.¹⁶

Rab proteins are small GTPases involved in the regulation of vesicular trafficking, and they are localized to specific intracellular sites.^{58,59} The fact that Rab-GFP proteins faithfully mimic their endogenous counterparts has been characterized extensively.^{46,58,60,61} Because endosomes and lysosomes have been implicated in the pathway of copper excretion,^{9,42,62–64} we compared ATP7B to a subset of well-established rab marker proteins of the endolysosomal pathway. Rab7 localized to late endosomes^{46,54} was of special interest because a co-localization with WDP/ATP7B had been claimed.⁴² However we did not observe any overlap between the

Figure 6. Copper-dependent translocation of ATP7B functions independent of Murr1 and Rab7. **A:** Immunofluorescence of RNAi knockdown cells. HuH7 cells were stably transduced with a control retrovirus (**a**, **c**) or the shRNAi retrovirus specific for Murr1 (**b**) or specific for Rab7 (**d**). Endogenous Murr1 (**a**, **b**) and Rab7 (**c**, **d**) were stained by indirect immunofluorescence (red). Microscopy exposure times and other settings were identical when comparing **a** and **b** and **c** and **d**, respectively. All cells were also stained with Hoechst dye to visualize cell nuclei. **B:** RT-PCR quantification of Murr1 and Rab7 in stably transduced HuH7-RNAi knockdown cells. Arbitrary units were obtained by normalizing the signal to the internal control (values shown are 10^{-4} * signal Murr1/signal actin and 10^{-1} * signal Rab7/signal actin, respectively). **C:** Western blot of Murr1 and Rab7 in stably transduced HuH7-RNAi knockdown cells. Protein lysates were analyzed by gel electrophoresis and Western blotting. Blots were stripped and reprobed with β -actin. **D:** Dominant-negative Rab7 does not impair the translocation of the WDP. HuH7 cells transfected with a dominant-negative Rab7 mutant (GFP-Rab7DN) were treated for 2 hours either with BCS or copper ions and processed for indirect immunofluorescence staining for ATP7B. ATP7B (red) showed a perinuclear Golgi pattern when copper concentrations were low, but a diffuse vesicular pattern with high copper levels. **E:** Translocation is not impaired after Murr1 or Rab7 knockdown. Stably transduced RNAi-HuH7 cells or HuH7 cells expressing the dominant-negative Rab7 mutant (Rab7-DN) were treated for 2 hours either with BCS, basal medium, or copper ions. After fixation and indirect immunofluorescence staining for ATP7B, slides were blinded and scored for translocation. Scale bars = 20 μ m.

WDP and either Rab7, Rab9 (late endosomes but different from Rab7^{46,54,55}), Rab11 (recycling endosomes^{65–67}), or a more general marker of the endolysosomal pathway, the lysotracker dye (Figure 2; and see Supplemental Figure 2 at <http://ajp.amjpathol.org>). This held true for basal (standard tissue culture medium) as well as low and high copper concentrations.

Rab11 had been described to give a partial overlap with ATP7B in copper-treated WIF-B cells.¹⁹ This could be attributable to the limitations of light microscopy because an overlap does not necessarily mean co-localization. With this in mind, segregation between two proteins is easier to interpret than an overlap, especially in the perinuclear region where recycling endosomes, Golgi apparatus, microtubule organizing center, and lysosomes tend to occupy the same space.

Transiently expressed GFP-ATP7B was reported to reside in late endosomes marked by Rab7, regardless of the copper concentration.⁴² However the cells shown in the micrographs⁴² display a diffuse but strong signal for Rab7 throughout the whole cell, with little subcellular resolution. This together with a perinuclear signal for GFP-ATP7B gives a yellow color in the merged picture, but it seems appropriate to exert some caution when interpreting this as the sole evidence for a co-localization. GFP-ATP7B did not overlap completely with ATP7B-DsRed in the same study,⁴² which might indicate that the GFP or DsRed modification could change the trafficking/localization properties of ATP7B. We were unable to see any co-localization between endogenous ATP7B and Rab7, despite trying many different conditions and cell lines. In addition to Ptk2 cells (Figure 2), we also analyzed endogenous WDP in the hepatoma cell line HuH7 (Figure 3, a–c), and overexpressed WDP in HeLa cells (see Supplemental Figure 3 at <http://ajp.amjpathol.org>). Finally, mimicking previous conditions,⁴² we confirmed our finding by staining endogenous WDP in MDCK cells cultured with standard medium (without any manipulation of copper concentrations; Figure 3d).

Vast overexpression of ATP7B in HeLa cells was the only condition in which a marginal overlap could be observed (K.H.W. and J.F., unpublished). However these cells in general did not appear healthy and had lost the translocation response to elevated copper. In principle, this could suggest that ATP7B cycles through late endosomes as part of its trafficking itinerary and is made visible only under these conditions of high overexpression. A more likely explanation is simply an overflow phenomenon after saturation of internal membranes with ATP7B, compromising the targeting/sorting capacity. In line with this hypothesis, Hubbard and Braiterman⁶⁸ recently pointed out that higher expression of GFP-ATP7B yielded protein aggregates that were unresponsive to changes in copper.

In an independent approach, we depleted Rab7 by RNA interference and analyzed the distribution of endogenous ATP7B in hepatoma cells (Figure 6). Because neither the steady state localization nor the copper-induced translocation of ATP7B was impaired, this suggested that Rab7 is not functionally required for the copper-induced translocation of the WDP. Finally, over-

expression of a dominant-negative Rab7 mutant protein was also without any discernible effect on the localization of ATP7B. We have to conclude that regulation of membrane traffic by the GTPase Rab7 is not relevant for the intracellular movement of the WDP in response to elevated copper concentrations.

Deficiency of Murr1 is the main cause for copper toxicosis in dogs,^{1,26,69,70} and co-precipitation experiments suggest a direct interaction with the WDP.^{30–32} In our hands, there was no co-localization between Murr1/COMMD1 on membrane vesicular structures and ATP7B (Figure 4, A–C). However, an interaction between cytosolic Murr1/COMMD1 and the WDP cannot be excluded by our immunofluorescence data, especially if only part of the total cellular Murr1 would bind to ATP7B. Because several other interaction partners for Murr1 have already been put forward (HIF- α ,³⁷ XIAP,³⁵ RelA/NF κ B,^{34,39,40} other COMMD proteins,³⁴ Δ ENaC⁷¹), and Murr1 has also been localized to the nucleus,³⁴ it seems reasonable to assume that only a fraction of Murr1 at any given time may interact with ATP7B.

We were expecting that depletion of Murr1/COMMD1 (by RNAi, Figure 6) would impair the first step of copper excretion from the cell, the translocation of ATP7B away from the Golgi apparatus toward the dispersed vesicles. However we could not find any difference to control cells in any parameter. In line with this, the localization of Murr1/COMMD1 was not influenced by copper treatment. The obvious conclusion is that Murr1/COMMD1 is required only at a later step of copper excretion, maybe in guiding copper-loaded subapical vesicles to the apical membrane, where hepatocytes can finally discharge copper into the bile. An earlier interference with copper transport seems unlikely because the loading of ceruloplasmin by ATP7B in the late Golgi is not affected even in knockout animals (Bedlington terriers^{28,29}). In a side observation, even high overexpression of Murr1/COMMD1 in HeLa cells would neither change the steady state localization of the WDP nor the copper-dependent translocation (K.H.W. and J.F., unpublished).

Interestingly, we did find a partial overlap between Murr1 and rab marker proteins of the late endosomal pathway. It could be that this is the pool of Murr1 involved in copper excretion, but this speculation would need to be supported by future studies; eg, addressing precisely the partitioning of Murr1 between membranes, cytosol, and the nucleus and the relevance of these subpopulations for copper homeostasis. Alternatively, the influence of Murr1/COMMD1 on ubiquitination³⁸ might solve the puzzle how exactly Murr1 is involved in copper secretion from cells.

In conclusion, our data imply that the copper-induced translocation of the WDP is a widely conserved mechanism that functions independently of Rab7-regulated late endosomal pathways. The Murr1/COMMD1 protein presumably has a function later in the pathway of copper excretion than currently considered^{2,3,14,33} because it is not involved in copper-mediated translocation of ATP7B.

Acknowledgments

We thank Uta Merle and Karin Bents for help with the RT-PCR quantifications; Celine Johanssen and Mark Schäfer for helpful comments on the manuscript; Marino Zerial, Lucas Pelkmans, and Jamie White for the generous supply of reagents; and Günther Giese at the microscopy facility of the MPI for Medical Research, Heidelberg.

References

- Wijmenga C, Klomp LW: Molecular regulation of copper excretion in the liver. *Proc Nutr Soc* 2004, 63:31–39
- Prohaska JR, Gybina AA: Intracellular copper transport in mammals. *J Nutr* 2004, 134:1003–1006
- Gitlin JD: Wilson disease. *Gastroenterology* 2003, 125:1868–1877
- Ala A, Walker AP, Ashkan K, Dooley JS, Schilsky ML: Wilson's disease. *Lancet* 2007, 369:397–408
- Bull PC, Thomas GR, Rommens JM, Forbes JR, Cox DW: The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. *Nat Genet* 1993, 5:327–337
- Tanzi RE, Petrukhin K, Chernov I, Pellequer JL, Wasco W, Ross B, Romano DM, Parano E, Pavone L, Brzustowicz LM: The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. *Nat Genet* 1993, 5:344–350
- Thomas GR, Forbes JR, Roberts EA, Walshe JM, Cox DW: The Wilson disease gene: spectrum of mutations and their consequences. *Nat Genet* 1995, 9:210–217
- Ferenci P, Caca K, Loudianos G, Mieli-Vergani G, Tanner S, Sternlieb I, Schilsky M, Cox D, Berr F: Diagnosis and phenotypic classification of Wilson disease. *Liver Int* 2003, 23:139–142
- Terada K, Aiba N, Yang XL, Iida M, Nakai M, Miura N, Sugiyama T: Biliary excretion of copper in LEC rat after introduction of copper transporting P-type ATPase, ATP7B. *FEBS Lett* 1999, 448:53–56
- Terada K, Nakako T, Yang X-L, Iida M, Aiba N, Minamiya Y, Nakai M, Sakaki T, Miura N, Sugiyama T: Restoration of holoceruloplasmin synthesis in LEC rat after infusion of recombinant adenovirus bearing WND cDNA. *J Biol Chem* 1998, 273:1815–1820
- Hellman NE, Gitlin JD: Ceruloplasmin metabolism and function. *Annu Rev Nutr* 2002, 22:439–458
- Cox DW, Moore SD: Copper transporting P-type ATPases and human disease. *J Bioenerg Biomembr* 2002, 34:333–338
- Mercer JF, Llanos RM: Molecular and cellular aspects of copper transport in developing mammals. *J Nutr* 2003, 133:1481S–1484S
- Lutsenko S, Barnes NL, Bartee MY, Dmitriev OY: Function and regulation of human copper-transporting ATPases. *Physiol Rev* 2007, 87:1011–1046
- Huster D, Hoppert M, Lutsenko S, Zinke J, Lehmann C, Mossner J, Berr F, Caca K: Defective cellular localization of mutant ATP7B in Wilson's disease patients and hepatoma cell lines. *Gastroenterology* 2003, 124:335–345
- Roelofs H, Wolters H, Van Luyn M, Miura N, Kuipers F, Vonk R: Copper-induced apical trafficking of ATP7B in polarized hepatoma cells provides a mechanism for biliary copper excretion. *Gastroenterology* 2000, 119:782–793
- Schaefer M, Hopkins RG, Failla ML, Gitlin JD: Hepatocyte-specific localization and copper-dependent trafficking of the Wilson's disease protein in the liver. *Am J Physiol* 1999, 276:G639–G646
- Hung IH, Suzuki M, Yamaguchi Y, Yuan DS, Klausner RD, Gitlin JD: Biochemical characterization of the Wilson disease protein and functional expression in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 1997, 272:21461–21466
- Guo Y, Nyasae L, Braiterman LT, Hubbard AL: N-terminal signals in ATP7B Cu-ATPase mediate its Cu-dependent anterograde traffic in polarized hepatic cells. *Am J Physiol* 2005, 289:G904–G916
- Cater MA, La Fontaine S, Shield K, Deal Y, Mercer JF: ATP7B mediates vesicular sequestration of copper: insight into biliary copper excretion. *Gastroenterology* 2006, 130:493–506
- Forbes JR, Cox DW: Copper-dependent trafficking of Wilson disease mutant ATP7B proteins. *Hum Mol Genet* 2000, 9:1927–1935
- Cater MA, La Fontaine S, Mercer JF: Copper binding to the N-terminal metal-binding sites or the CPC motif is not essential for copper-induced trafficking of the human Wilson protein (ATP7B). *Biochem J* 2007, 401:143–153
- Riordan SM, Williams R: The Wilson's disease gene and phenotypic diversity. *J Hepatol* 2001, 34:165–171
- Steindl P, Ferenci P, Dienes HP, Grimm G, Pabinger I, Madl C, Maier-Dobersberger T, Herneth A, Dragosics B, Meryn S, Knoflach P, Granditsch G, Gangl A: Wilson's disease in patients presenting with liver disease: a diagnostic challenge. *Gastroenterology* 1997, 113:212–218
- Ala A, Borjigin J, Rochwarger A, Schilsky M: Wilson disease in septuagenarian siblings: raising the bar for diagnosis. *Hepatology* 2005, 41:668–670
- Twedt DC, Sternlieb I, Gilbertson SR: Clinical, morphologic, and chemical studies on copper toxicosis of Bedlington terriers. *J Am Vet Med Assoc* 1979, 175:269–275
- van De Sluis B, Rothuizen J, Pearson PL, van Oost BA, Wijmenga C: Identification of a new copper metabolism gene by positional cloning in a purebred dog population. *Hum Mol Genet* 2002, 11:165–173
- Klomp AE, van de Sluis B, Klomp LW, Wijmenga C: The ubiquitously expressed MURR1 protein is absent in canine copper toxicosis. *J Hepatol* 2003, 39:703–709
- Su LC, Ravanshad S, Owen Jr CA, McCall JT, Zollman PE, Hardy RM: A comparison of copper-loading disease in Bedlington terriers and Wilson's disease in humans. *Am J Physiol* 1982, 243:G226–G230
- Tao TY, Liu F, Klomp L, Wijmenga C, Gitlin JD: The copper toxicosis gene product Murr1 directly interacts with the Wilson disease protein. *J Biol Chem* 2003, 278:41593–41596
- de Bie P, Burstein E, van De Sluis B, Berger R, Wijmenga C, Duckett CS, Klomp LWJ: Several COMMD proteins interact with ATP7B; possible candidate genes for hepatic copper overload disorders with unknown etiology. *Eur J Gastroenterol Hepatol* 2006, 18:A52
- Donadio S, De Keukeleire J, Micoud J, Piccarreta F, Benharouga M: Cellular localization and trafficking of endogenous ATP7B and COMMD1 proteins. *FEBS J* 2007, 274:106 Abstract B1-40
- de Bie P, van de Sluis B, Klomp L, Wijmenga C: The many faces of the copper metabolism protein MURR1/COMMD1. *J Hered* 2005, 96:803–811
- Burstein E, Hoberg JE, Wilkinson AS, Rumble JM, Csomos RA, Komarck CM, Maine GN, Wilkinson JC, Mayo MW, Duckett CS: COMMD proteins: a novel family of structural and functional homologs of MURR1. *J Biol Chem* 2005, 280:22222–22232
- Burstein E, Ganesh L, Dick RD, van De Sluis B, Wilkinson JC, Klomp LW, Wijmenga C, Brewer GJ, Nabel GJ, Duckett CS: A novel role for XIAP in copper homeostasis through regulation of MURR1. *EMBO J* 2004, 23:244–254
- Spee B, Arends B, van Wees AM, Bode P, Penning LC, Rothuizen J: Functional consequences of RNA interference targeting COMMD1 in a canine hepatic cell line in relation to copper toxicosis. *Anim Genet* 2007, 38:168–170
- van de Sluis B, Muller P, Duran K, Chen A, Groot AJ, Klomp LW, Liu PP, Wijmenga C: Increased activity of hypoxia-inducible factor 1 is associated with early embryonic lethality in *Commd1* null mice. *Mol Cell Biol* 2007, 27:4142–4156
- Maine GN, Burstein E: COMMD proteins: COMMMing to the scene. *Cell Mol Life Sci* 2007, 64:1997–2005
- Ganesh L, Burstein E, Guha-Niyogi A, Louder MK, Mascola JR, Klomp LW, Wijmenga C, Duckett CS, Nabel GJ: The gene product Murr1 restricts HIV-1 replication in resting CD4+ lymphocytes. *Nature* 2003, 426:853–857
- Maine GN, Mao X, Komarck CM, Burstein E: COMMD1 promotes the ubiquitination of NF-kappaB subunits through a cullin-containing ubiquitin ligase. *EMBO J* 2007, 26:436–447
- Mufti AR, Burstein E, Csomos RA, Graf PCF, Wilkinson JC, Dick RD, Challa M, Son J-K, Bratton SB, Su GL: XIAP is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders. *Mol Cell* 2006, 21:775–785
- Harada M, Kawaguchi T, Kumemura H, Terada K, Ninomiya H, Taniguchi E, Hanada S, Baba S, Maeyama M, Koga H, Ueno T, Furuta K, Suganuma T, Sugiyama T, Sata M: The Wilson disease protein ATP7B resides in the late endosomes with Rab7 and the Niemann-Pick C1 protein. *Am J Pathol* 2005, 166:499–510
- Schuck S, Manninen A, Honsho M, Fullekrug J, Simons K: Generation of single and double knockdowns in polarized epithelial cells by

- retrovirus-mediated RNA interference. *Proc Natl Acad Sci USA* 2004, 101:4912–4917
44. Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B: Rab7: a key to lysosome biogenesis. *Mol Biol Cell* 2000, 11:467–480
45. Sönnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M: Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J Cell Biol* 2000, 149:901–914
46. Barbero P, Bittova L, Pfeffer SR: Visualization of Rab9-mediated vesicle transport from endosomes to the trans-Golgi in living cells. *J Cell Biol* 2002, 156:511–518
47. Pelkmans L, Kartenbeck J, Helenius A: Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nat Cell Biol* 2001, 3:473–483
48. Storrie B, White J, Rottger S, Stelzer EH, Suganuma T, Nilsson T: Recycling of Golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. *J Cell Biol* 1998, 143:1505–1521
49. Campbell RE, Tour O, Palmer AE, Steinbach PA, Baird GS, Zacharias DA, Tsien RY: A monomeric red fluorescent protein. *Proc Natl Acad Sci USA* 2002, 99:7877–7882
50. Jäger S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen EL: Role for Rab7 in maturation of late autophagic vacuoles. *J Cell Sci* 2004, 117:4837–4848
51. Brummelkamp TR, Bernards R, Agami R: A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002, 296:550–553
52. Merle U, Encke J, Tuma S, Volkmann M, Naldini L, Stremmel W: Lentiviral gene transfer ameliorates disease progression in Long-Evans cinnamon rats: an animal model for Wilson disease. *Scand J Gastroenterol* 2006, 41:974–982
53. Nichols B: Caveosomes and endocytosis of lipid rafts. *J Cell Sci* 2003, 116:4707–4714
54. Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M: Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. *Cell* 1990, 62:317–329
55. Lombardi D, Soldati T, Riederer MA, Goda Y, Zerial M, Pfeffer SR: Rab9 functions in transport between late endosomes and the trans Golgi network. *EMBO J* 1993, 12:677–682
56. La Fontaine S, Mercer JF: Trafficking of the copper-ATPases, ATP7A and ATP7B: role in copper homeostasis. *Arch Biochem Biophys* 2007, 463:149–167
57. La Fontaine S, Theophilus MB, Firth SD, Gould R, Parton RG, Mercer JF: Effect of the toxic milk mutation (tx) on the function and intracellular localization of Wnd, the murine homologue of the Wilson copper ATPase. *Hum Mol Genet* 2001, 10:361–370
58. Zerial M, McBride H: Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* 2001, 2:107–117
59. Pfeffer S, Aivazian D: Targeting Rab GTPases to distinct membrane compartments. *Nat Rev Mol Cell Biol* 2004, 5:886–896
60. Hoepfner S, Severin F, Cabezas A, Habermann B, Runge A, Gillooly D, Stenmark H, Zerial M: Modulation of receptor recycling and degradation by the endosomal kinesin KIF16B. *Cell* 2005, 121:437–450
61. Rink J, Ghigo E, Kalaidzidis Y, Zerial M: Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 2005, 122:735–749
62. Vulpe CD, Packman S: Cellular copper transport. *Annu Rev Nutr* 1995, 15:293–322
63. Klein D, Lichtmanegger J, Heinzmann U, Muller-Hocker J, Michaelson S, Summer KH: Association of copper to metallothionein in hepatic lysosomes of Long-Evans cinnamon (LEC) rats during the development of hepatitis. *Eur J Clin Invest* 1998, 28:302–310
64. Gross Jr JB, Myers BM, Kost LJ, Kuntz SM, LaRusso NF: Biliary copper excretion by hepatocyte lysosomes in the rat. Major excretory pathway in experimental copper overload. *J Clin Invest* 1989, 83:30–39
65. Ullrich O, Reinsch S, Urbe S, Zerial M, Parton RG: Rab11 regulates recycling through the pericentriolar recycling endosome. *J Cell Biol* 1996, 135:913–924
66. Calhoun BC, Goldenring JR: Rab proteins in gastric parietal cells: evidence for the membrane recycling hypothesis. *Yale J Biol Med* 1996, 69:1–8
67. Goldenring JR, Smith J, Vaughan HD, Cameron P, Hawkins W, Navarre J: Rab11 is an apically located small GTP-binding protein in epithelial tissues. *Am J Physiol* 1996, 270:G515–G525
68. Hubbard AL, Braiterman LT: Could ATP7B export Cu(I) at the tight junctions and the apical membrane? *Gastroenterology* 2008, 134:1255–1257
69. Hyun C, Lavulo LT, Filippich LJ: Evaluation of haplotypes associated with copper toxicosis in Bedlington terriers in Australia. *Am J Vet Res* 2004, 65:1573–1579
70. Coronado VA, Damaraju D, Kohjoki R, Cox DW: New haplotypes in the Bedlington terrier indicate complexity in copper toxicosis. *Mamm Genome* 2003, 14:483–491
71. Biasio W, Chang T, McIntosh CJ, McDonald FJ: Identification of Murr1 as a regulator of the human delta epithelial sodium channel. *J Biol Chem* 2004, 279:5429–5434