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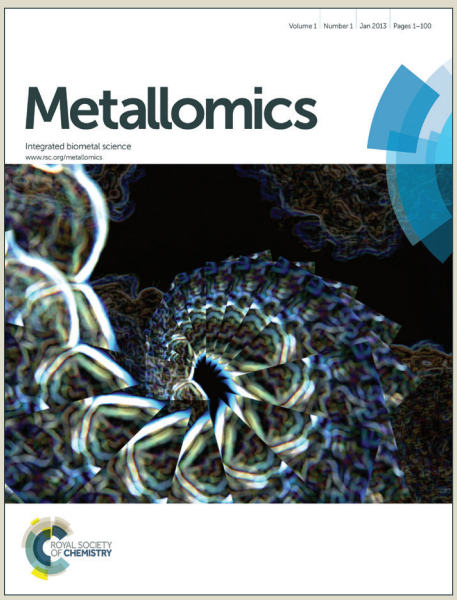

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Understanding copper sensitivity in zebrafish (*Danio rerio*) through the intracellular localization of copper transporters in a hepatocyte cell-line ZFL and the tissue expression profiles of copper transporters

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Abstract

Zebrafish (*Danio rerio*) is a freshwater fish species of Cyprinidae known for its copper (Cu) intolerance, yet the underlying mechanisms of the sensitivity remain unclear. In this study, we examined the highly conserved molecular machineries in the copper handling system, namely ATOX1, ATP7A, ATP7B, and CTR1, by profiling their gene expression patterns among tissues before and after acute waterborne Cu exposure, and investigating their intracellular localization patterns using a zebrafish hepatocyte cell line, ZFL. We found that ATP7B was weak in responding toward Cu exposure to elicit its copper efflux function. Tissue distribution of these Cu transporters, however, revealed a distinct expression profile compared with mammals and other fish, particularly ATP7A, which unlike ATP7B was highly expressed in the liver, while ATP7B, not ATP7A, was specifically expressed in the intestine. ATOX1 transcript expression was also found to be significantly up-regulated with acute waterborne Cu, in contrast to the decreased expression found in other fish. A possible explanation for the Cu sensitivity in zebrafish is discussed.

Keywords: *Copper homeostasis, ATOX1, ATP7A, ATP7B, CTR1*

Abbreviations

A, absorbance; B2M – Beta-2-microglobulin; Cu – Copper; DEPC, ditheylpyrocarbonate; EF1a – Elongation factor 1-alpha; GAPDH – Glyceraldehyde-3-phosphate-dehydrogenase; h, hour; HMA – Heavy metal-associated domain; LC_{50} – Median lethal concentration; MBD – Metal binding domain; PBS, phosphate-buffered saline; YBX1 – Y box binding protein 1.

1. Introduction

An essential micronutrient, copper (Cu) serves as a catalytic cofactor in enzymes involved in various vital processes, such as cytochrome c oxidase (EC 1.9.3.1), which plays a key role in the electron transport chain for cellular respiration, and Cu/Zn superoxide dismutase (EC 1.15.1.1), which is crucial for the removal of free radicals.[1] While its wide range of interactions with amino acids, such as cysteine and histidine,[2] and its active redox activity allow Cu to be an excellent enzymatic cofactor, rigorous control of the amount of intracellular labile Cu is needed. Cu ions exist in two forms in water, cuprous (Cu^{+}) and cupric (Cu^{2+}), and the exchange between these two forms in aqueous solution constitutes the active redox activity of Cu. Excessive free Cu ions cause cellular toxicity, as Cu ions catalyze the formation of free radicals, which confers oxidative stress and causes cellular damage via a Fenton reaction.[3] Ectopic binding of Cu on proteins can also disturb their structures and even induce protein aggregation. For example, labile Cu^{2+} was shown to promote and accelerate amyloid-beta peptide oligomerization in Alzheimer's disease.[4] Therefore, a well-established system for Cu uptake, distribution, detoxification, and elimination is developed and highly conserved in various organisms.[2]

The molecular machineries involved in Cu metabolism were first discovered and investigated in two human genetic disorders, Menkes syndrome (MNK) and Wilson's disease (WD). Mutations in genes encoding the two isoforms of Cu ATPases, namely ATP7A and ATP7B, were found to be the cause of MNK and WD, respectively.[5,6] ATP7A and ATP7B are P-type ATPases in the P_{1B} subfamily,[7] which are differentially expressed among the tissues of mammals and other organisms studied. ATP7A expresses in many tissues except the liver, whereas ATP7B expresses strictly or mainly in liver tissue.[8] This different tissue expression leads to different symptoms in MNK and WD patients: a

systemic Cu deficiency with entrapment of Cu in intestinal enterocytes is observed in MNK patients[9] who have ATP7A gene mutations. ATP7B gene mutations in WD patients result in hepatic and neuronal Cu toxicities that cause critical liver damage, and neurological or psychiatric symptoms are exhibited due to the accumulation of Cu in the brain and liver.[10] Cu-ATPases deliver Cu at both functional and subcellular levels, for example to nascent proteins synthesized in the Golgi apparatus, in Cu transfer to ceruloplasmin via ATP7A for Cu uptake in enterocytes, and in eliminating excess intracellular Cu from the liver using ATP7B in hepatocytes.[11] These functional roles are conferred by the metal binding domains (MBDs) present in the heavy metal-associated (HMA) domains. The sixth MBD, adjacent to the trans-membrane domain, has been shown to be particularly important for Cu transport.[12] Although they are isoforms of each other, the functional roles of ATP7A and ATP7B appear to be non-redundant. It has been demonstrated that ATP7A can replace ATP7B when the latter is inactivated,[11] but a lack of ATP7A cannot be compensated by ATP7B. [13]

The high affinity Cu transporter 1 (CTR1) is the machinery responsible for Cu entry in eukaryotes for the cellular uptake of Cu. This membrane-bound Cu importer was initially identified in yeast,[14] and its homologue was subsequently found in humans.[15] Biochemical characterization of CTR1 revealed that it specifically imports Cu^{+} with a high affinity in mammalian cells.[16] This Cu transporter is vital in developmental processes, as embryonic lethality resulted when CTR1 knockout and knockdown were carried out in mice[17] and in zebrafish,[18] respectively.

As the level of the labile form of Cu should be kept to a minimum to avoid Cu toxicity, intracellular Cu trafficking is conducted by a cytoplasmic chaperone, ATOX1, to transfer

Cu from the entry site on a plasma membrane to machineries localized on the Golgi apparatus. ATOX1 is a mammalian homologue of ATX1 found in yeast,[19] which interacts with both ATP7A and ATP7B.[20,21] Knockout of this chaperone leads to poor development in mouse pups, due to impaired Cu efflux.[22] It has also been demonstrated that ATOX1 is involved in cell proliferation by acting as a Cu-dependent transcription factor.[23]

The study of the Cu homeostatic mechanism of aquatic species, especially of fish that possess two Cu uptake pathways via the gills and intestine, is particularly important, due to the concern over levels of Cu water pollution resulting from the extensive use of Cu-made pipes and Cu-based products such as fertilizers and fungicides. Some freshwater fish species are highly tolerant to Cu, such as the Nile tilapia *Oreochromis mossambicus* (96-h LC_{50} of Cu^{2+} equals 1.52 ppm, or 23.9 mM) [24], while others are highly sensitive to it, such as the zebrafish *Danio rerio* larvae, which has a 96-h LC_{50} of 85.7 ppb, or 1.35 μM.[25,26] As very little research work has been previously carried out to examine the molecular mechanism of Cu handling processes in fish under acute waterborne Cu exposure, we investigated the tissue expression profiles of Cu transporters and their intracellular locations in ZFL cells, a zebrafish hepatic cell line, to understand the Cu sensitivity of zebrafish. These fish of Cyprinadae have been shown to be Cu intolerant, or sensitive, yet the underlying mechanisms accounting for this sensitivity remain unclear. Our study of zebrafish as a model provides some insights to explain this phenomenon through tissue expression profiles of Cu transporters, namely ATOX1, ATP7A, ATP7B and CTR1, and their intracellular localization in ZFL cells.

2. Materials and methods

2.1 Zebrafish maintenance and treatments[27]

Adult zebrafish (*Danio rerio*) of mixed sex were obtained from a local pet shop and acclimated in 60 L glass aquaria supplied with dechlorinated, circulated, and aerated local tap water at 26 – 28°C, and exposed to a 14 h light and 10 h dark photoperiod for 2 – 3 weeks. For the Cu exposure experiment, wild-type adult zebrafish of mixed sex were administered with 0, 1, 5 and 10 ppb of Cu^{2+} solution (equivalent to 0, 15.7, 78.7 and 157 nM; Cu (II) chloride, $CuCl_2 \cdot 2H_2O$, Sigma-Aldrich, St Louis, USA) for 24 h. Prior to the dissection, the fish were anesthetized with tricaine (0.2 g/L) (Finquel, Argent Chemical Laboratories, Redmond, WA) and decapitated. Liver, gill, intestine, heart, and kidney tissues were immediately isolated from zebrafish exposed to different Cu dosages and homogenized in TRIzol reagent (Invitrogen, Carlsbad, USA). Three biological replicates were collected per Cu^{2+} concentration.

2.2 Determination of LC_{50} values of $CuCl_2$ for zebrafish larvae and adult

Adult zebrafish were used for the determination of the toxicities of $CuCl_2$. For each group, twenty zebrafish adults or larvae were exposed to different concentrations of $CuCl_2$ for 96 h, with each dosage administered in triplicate. Exposure conditions were 25 ± 0.5 °C with a photoperiod of 14 h light and 10 h dark. The lethal percentage of adults was calculated by observing the death of the adults by the naked eye at 96 h. The 96-h LC_{50} values were calculated using GraphPad Prism 5.0 software (San Diego, USA) with non-linear regression.

2.3 Cell culture[28] and transient transfection

ZFL (American Typed Culture Collection, ATCC, CRL-2643TM), an adherent hepatocyte

cell line with epithelial-like morphology isolated from zebrafish, was purchased from ATCC and maintained in a complete culture medium consisting of 50% L-15 medium, 35% DMEM, and 15% Hams F-12, supplemented with 0.15 g/L of sodium bicarbonate, 15 mM of HEPES, 0.01 mg/ml of bovine insulin, 50 ng/ml of mouse EGF, 5% heat-inactivated fetal bovine serum (FBS), and 1% penicillin/streptomycin. The culture was incubated at 28°C with 5% CO_2. The cells were routinely sub-cultured once a week by washing with phosphate-buffered saline (PBS), trypsinized with 1 ml trypsin-EDTA (0.25%, 1 mM) for 5 min., addition with complete medium, centrifuged at 1,000 rpm for 5 min. and further suspended in complete medium. Cultured cells were counted for cell number using Trypan Blue exclusion method under a microscope for Cu exposure experiments or transfection studies with plasmids. Cells in 24-well plates at 80% confluence were transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) as suggested by the manufacturer; after 4 - 6 h, the medium was removed and transfected cells were washed with PBS, and finally in complete medium to recover for Cu exposures.

2.4 Bioinformatics analysis

ATP7B peptide sequences from zebrafish (*Danio rerio*; ENSDARP00000029666), stickleback (*Gasterosteus aculeatus*; ENSGACP00000019725), tetraodon (*Tetraodon nigroviridis*; ENSTNIP00000002634), fugu (*Takifugu rubripes*; ENSTRUP00000034454), Nile tilapia (*Oreochromis niloticus.*; XP_003446605), and seabream (*Sparus aurata*; ACX37120) were obtained from the NCBI Genbank and the Ensembl database. These sequences were retrieved and analyzed in the NCBI Conserved Domains database with default parameters (version 3.02; data retrieved on 26 Feb 2012). The HMA domain sequences from each protein sequence were obtained, and a phylogenetic analysis of these domain sequences was conducted on the web server Phylogeny.fr, using default parameters

(the MUSCLE algorithm for multiple sequence alignment and the PhyML algorithm for phylogenetic tree generation).[29]

2.5 First-strand cDNA synthesis[30]

For the cloning of the full-length cDNAs of the Cu transporters, the total RNA was extracted from a sample of intestine tissues from adult zebrafish, using TRIzol reagent (Invitrogen, Carlsbad, CA), as per the manufacturer's instructions. After phenol-chloroform extractions and isopropanol precipitation, pelleted RNA samples were suspended in diehtylpyrocarbonate (DEPC)-treated deionized-Nano pure water. Using spectrophotometry in a quartz cuvette of 1 cm path, and reading absorbance (A) at 260 and 280 nm, the quality and quantity of RNA samples were examined. The ratio of A_{260}/A_{280} should be > 1.8 or best equal to 2. After the DNase I treatment, random hexamer-primed first-strand cDNA was reverse-transcribed from 1 μg of the total RNA by M-MLV reverse transcriptase (RNase H^-) (TaKaRa Bio, Shiga, Japan) in a 20 μl reaction, according to the manufacturer's manual. For qPCR gene expression analysis after Cu exposure, first-strand cDNA was synthesized using a PrimeScript RT reagent kit (Perfect Real Time) (TaKaRa Bio, Shiga, Japan), as per the manufacturer's instructions. All first-strand cDNA samples obtained were stored at -38 °C for further study and analysis.

2.6 Plasmid construction and site-directed mutagenesis

The full-length cDNAs of the Cu transporters (ATP7B, CTR1 and ATOX1) were amplified from zebrafish liver tissues, with corresponding primers (Tech Dragon, Hong Kong). The nucleotide sequences of these primers are listed in **Table 1**. Amplifications were performed using the Hot Start Version of LA Taq (TaKaRa Bio, Shiga, Japan), with 3% DMSO added, using first strand cDNAs. The reactions were subjected to a three-step cyclic program

(94°C for 1 min, followed by 35 cycles of 98 °C for 10 s, 55°C for 30 s and 72°C for 2.5 min). The resulting fragments were then subjected to purification, 3'-A overhangs addition, sub-cloned into pMD18-T vector (TaKaRa Bio, Shiga, Japan), and verification by DNA sequencing conducted at the Beijing Genomics Institute (Hong Kong).

pXFP-N1 (XFP = mCherry, mCitrine, mCerulean) were generated from pmCherry_a_tubulin_IRES_puro2, pLM-mCitrine-Sox2, and CMV-Brainbow-1.0 L respectively (Addgene plasmid #21043, 323242, #18721, Cambridge, USA), with pEGFP-N1 (Clonetech Laboratories Inc., USA) as the vector backbone. EYFP-GalT was obtained from Addgene, Cambridge, USA (plasmid #11936). The amplified fragments of Cu transporters were cloned into the pXFP-N1 vectors to produce pzATP7B-mCherry-N1, pzCTR1-mCerulean-N1 and pzATOX1-mCitrine-N1 constructs respectively, using specific restriction sites for transient expression in ZFL cells.

To generate ATP7B with a non-functional MBD2, pzATP7B-MBD2-C/S-mCherry-N1, a pair of complementary primers was designed to introduce amino acid substitutions of C192S/C195S by PCR. The primer sequences are available in **Table 1**. Amplifications were performed using the Phusion DNA polymerase (Finnzymes, Espoo, Finland). The reactions were subjected to a three-step cyclic program (94°C for 2 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min). The PCR products were purified and *Dpn*I digestion was conducted for parental DNA template removal. Correct mutations in the mutants were confirmed by nucleotide sequencing conducted at the Beijing Genomics Institute (Hong Kong) or Tech Dragon Limited (Hong Kong).

2.7 Confocal microscopy

ZFL cells were seeded into 35 mm glass bottom culture dishes at a density of 1×10^5 and transfected with either a mix of 0.3 μg of pzATP7B-mCherry-N1 and 0.3 μg of EYFP-GalT plasmids, 0.5 μg of pzCTR1-mCerulean-N1 plasmid, or 0.5 μg of pzATOX1-mCitrine-N1 plasmid, using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) for 1 day. The transiently transfected ZFL cells were analyzed and photographed using a FluoView FV1000 Confocal Microscope (Olympus, Tokyo, Japan) with a 40X lens. The cells were then exposed to 150 μM of Cu^{2+} solution (equivalent to 50% of 24 h-LC_{50} in ZFL) for 4 h and again photographed.

2.8 Alamar blue assay[25]

For the assessment of cell viability after inactivating MBD2 in ATP7B, ZFL cells were seeded into 96-well microplates at a density of 1×10^5 per well, and transfected with water (blank control), 320 ng of pzATP7B-mCherry-N1, or pzATP7B-MBD2-C/S-mCherry-N1 plasmids by using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) for 1 day, and then exposed to water, 77 μM, 154.1 μM, 231.1 μM, or 308.1 μM Cu^{2+} solution (equivalent to 25%, 50%, 75% and 100% of 24 h-LC_{50} in ZFL) for 1 day. Alamar Blue (10% final concentration; Invitrogen) was added 3 h prior to the end of the treatment. Absorbance values were measured at 570 nm with a reference wavelength at 600 nm on a Tecan Sunrise microplate reader (Männedorf, Switzerland). Relative viability was expressed as percentage survivorship relative to blank controls.

2.9 Housekeeping gene analysis

Reference genes (B2M, EF1a, GAPDH, and YBX1) were chosen for characterization for qPCR analysis in zebrafish from previous studies.[31,32] To identify and validate the housekeeping genes with the greatest expression stability under different Cu treatments and

in different tissue types, the expression profiles of B2M, EF1a, GAPDH, and YBX1 were determined via qPCR. It was also confirmed that these housekeeping genes' protein expressions do not change significantly upon Cu exposure.[25] The primer sequences and accession numbers for these reference genes are listed in **Table 1**.

The expression of these four housekeeping genes was determined using 60 RNA samples isolated from organs (gill, heart, intestine, kidney, and liver) of zebrafish that had been treated with different Cu dosages. The normalized expression levels for these genes were evaluated with geNorm software, version 3.5, as described elsewhere.[33] It was found that EF1a and YBX1 exhibited the highest expression stability with the lowest M-value (**Figure S2**). Therefore, both EF1a and YBX1 were selected as the best constitutively expressed housekeeping genes for the subsequent qPCR experiments.

2.10 Real-time quantitative PCR (qPCR)

Real time qPCR was performed using Power SYBR Green master mix (Applied Biosystems, Forster City, CA), amplified and detected by using the ABI 7500 Fast Real-time PCR System (Applied Biosystems, Forster City, CA). Primers were designed using PrimerExpress software (Applied Biosystems, Forster City, CA) (**Table 1**) and synthesized by Tech Dragon (Hong Kong). The PCR efficiencies of the primers were evaluated by a four-point standard curve, prepared by a 1:10 serial dilution of cDNA samples. The amplification of specific transcripts was confirmed by analyzing dissociation curves and resolving the PCR products on 4.0% agarose gel. All gene expression data obtained was normalized against EF1a and YBX1 by geometric averaging using geNorm as previously described.[33]

2.11 Statistical analysis

The statistical significance of differences (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$) was assessed by one-way ANOVA followed by Dunnett's or Tukey's *post hoc* tests using GraphPad Prism 5.0 software, with $p \leq 0.05$ considered statistically significant. All of the data were expressed as the mean ± SEM.

3. Results

3.1 Lethal concentrations of $CuCl_2$

We first determined the lethal concentrations of the adults of zebrafish exposed to $CuCl_2$ to further confirm the sensitivity of zebrafish to Cu (**Figure 1**). **Table 2** lists the LC_{50} values of $CuCl_2$ for different fish species, tilapia and seabream were reported to have ppm levels at 24 h or 96 h, however carps, trout, and zebrafish were determined at ppb levels. LC_{50} of $CuCl_2$ was determined as 85.7 ppb (1.34 μM) for zebrafish larvae, the 95% confidence interval was from 41.2 to 178.5 ppb (0.64 to 2.79 μM).[26]

3.2 Tissue distribution of Cu transporters and corresponding change in expression levels upon Cu exposure

The expression profiles of ATOX1, ATP7A, ATP7B, and CTR1 in the gill, heart, intestine, kidney, and liver are shown in **Figure 2A**. Although there was no significant difference in the ATOX1 expression levels of these tissues, ATP7A expressed most abundantly in the kidney, showing an eight-fold higher expression than that in the gill and heart, a five-fold higher expression than that of the liver, and a 55-fold higher expression than in the intestine tissue. The highest expression levels of both ATP7B and CTR1 were found in the intestine, where other tissues expressed these genes at similar levels. To evaluate the relative abundance of the two Cu-ATPases, ATP7A and ATP7B, among the tissues, relative expression ratios were calculated as shown in **Figure 2B**. Although ATP7B expressed specifically in the intestine tissue at levels around 32 times greater than the ATP7A expression, however, higher ATP7A expressions were found in other tissues, with about 140-fold more ATP7A expressed in the liver, and ranging from a 4-(heart) to 14-fold (gill and kidney) higher than ATP7B in the expression level.

We also investigated the change in the mRNA expression of these Cu transporters in different tissues when the zebrafish were exposed to different doses of Cu. In the gills, ATOX1 expression was up-regulated at least five-fold in response to a low (1 ppb, 15.7 nM) and high (10 ppb, 157 nM) Cu dosage (**Figure 3A**). There was at least a 7.5-fold, and up to 13.6-fold, up-regulation in ATP7A expression upon Cu exposure. The other Cu-ATPase, ATP7B, was found to be up-regulated by about 30 times at a medium Cu dosage (5 ppb, 78.7 nM), but appeared to be down-regulated after treatment with a higher Cu dosage. CTR1 was slightly up-regulated with a 1 ppb (15.7 nM) Cu exposure, and then returned to normal expression level after treatment with higher Cu concentrations.

There was significant up-regulation of ATOX1 (about 4-fold) and ATP7A (at least 13-fold) expression in the heart tissue as Cu concentration increased, but no significant change in ATP7B and CTR1 gene expression was observed in the heart (**Figure 3B**).

A similar expression pattern to that of the heart was observed in the intestine tissue, with ATOX1 and ATP7A expression levels significantly up-regulated by about 6-fold and 40-fold, respectively (**Figure 3C**). It appears that ATP7B expression also increased in response to Cu challenge, thus both ATP7A and B might be working together to moderate Cu uptake from gut. A slight increase in CTR1 expression was observed at low and medium Cu dosages, followed by a 0.5-fold down-regulation when exposed to a higher Cu dosage.

In kidney tissue, ATOX1 was up-regulated by about 3-fold upon Cu exposure (**Figure 3D**). A 12.4- to 18-fold induction in ATP7A expression was observed. ATP7B was also up-regulated when exposed to Cu. There was no significant change in CTR1 expression

level as the Cu concentration increased.

At least a 5-fold induction in ATOX1 mRNA expression was observed upon Cu challenge in liver tissue (**Figure 3E**), and ATP7A up-regulation showed a 6-fold expression at a high Cu dosage. No significant change in ATP7B and CTR1 gene expression levels was observed, however ATP7B gave the highest levels (~ 10- 30 folds) of fold induction in the liver of zebrafish after Cu exposures.

3.3 Second metal binding domain in ATP7B slightly contribute to Cu sensitivity in zebrafish

While ATOX1, ATP7A, and CTR1 were found highly conserved in other fish species, we observed from the cDNA clones we obtained that there is an extra metal binding domain (MBD) in the ATP7B of zebrafish (**Figure 4A**). There are typically 4 MBDs in the ATP7B of fish, but an additional MBD (MBD2) in zebrafish, ATP7B, was found, and is unique in its amino acid sequence (**Figure 4B** and **4C**). We reasoned that this extra MBD could be a possible cause of Cu sensitivity in zebrafish relative to other fish, and therefore we generated a mutant ATP7B with non-functional MBD2 to evaluate whether the Cu sensitivity could be alleviated. ZFL cell viability was evaluated after overexpressing the wild type and mutant ATP7B, and carrying out Cu treatments with Alamar Blue assay (**Figure 5**). A significant increase in survivorship (~52% compared with blank control) was observed in the mutant ATP7B (carrying a non-functional MBD, similar to other cloned fish ATP7B) as the Cu concentration increased, but the wild type ATP7B with 5 MBDs provided only around 40% survivorship relative to blank control without any Cu added.

3.4 CTR1 and ATOX1, but not ATP7B, translocate in response to Cu exposure

We transiently transfected the ATOX1, ATP7B, and CTR1 individually into ZFL cells to observe their localizations with and without Cu challenge. ATP7B, one of the Cu efflux pumps, was found to be localized on the trans-Golgi network, as suggested by the high co-localization with GalT, a trans-Golgi network marker (**Figure 6A**). The same co-localization pattern was still observed even after exposure to 150 μM of Cu^{2+} solution for 4 h. CTR1, the Cu importer, initially expressed in some part of the cytoplasm but did not spread throughout the cytosol (**Figure 6B**). Upon Cu exposure, CTR1 transited to the plasma membrane. ATOX1, the cytosolic chaperone, expressed in cytosol as expected, and some localized inside the nucleus (**Figure 6C**), it was then further trans-located into the nucleus upon Cu challenge.

4. Discussion

4.1 Expression profiles of ATOX1, ATP7A, ATP7B, and CTR1 among different tissues

Based on the assumption that zebrafish shares similar expression profiles with humans and other fish such as seabream (*Sparus aurata*),[37] we initially chose to investigate ATP7B instead of ATP7A in ZFL cells. However, to our surprise, zebrafish was found to have distinct Cu-ATPase expression profiles. ATP7B was found to be specifically expressed in the intestine, in contrast to mammals, where it is expressed in the liver.[7] ATP7A was also widely expressed throughout the tissues in zebrafish, which is similar to mammals. However, zebrafish liver tissue expressed ATP7A about 140 times more than ATP7B, whereas ATP7B is the major Cu-ATPase expressed in mammalian liver. Nevertheless, both these Cu-ATPases were highly expressed in the liver and intestine, suggesting they could have non-overlapping functional roles, as in mammals.

Other insights gained from the expression profiles include a greater ATP7A expression in the kidney, both in normal conditions and under Cu challenge, a greater CTR1 expression in the intestine, and a similar expression level of ATOX1 among the tissues. A previous study showed that ATP7A has a major role in Cu export, protecting renal tissue from Cu overload in mice,[38] which may also hold true in zebrafish. Our observation was also consistent with the study of chronic Cu exposure in zebrafish.[39] For CTR1, it appears that the intestine, but not the gill, is an important Cu entrance pathway in zebrafish, as a greater CTR1 expression was found in the intestine (about 14-fold greater; see **Figure 2A iv**), which agrees with the tissue distribution of CTR1 in seabream.[40] No significant difference in ATOX1 expression level was observed among tissues, which may have been due to a sufficient basal expression for intracellular Cu trafficking in all tissues when there was no

Cu stress.

4.2 Alterations in transcript expressions under Cu challenge

A general induction of ATOX1 expression and up-regulation of Cu-ATPases expression was observed in all of the tissues investigated. This suggested that appropriate Cu homeostatic responses were elicited in these organs, as Cu efflux pumps were highly expressed, corresponding to increasing exposed Cu concentrations. In particular, some tissues showed a greater fold induction in ATOX1 expression, such as the gill and liver, than others. This result may indicate that these organs require more frequent Cu trafficking in cells or more chaperones for Cu capturing. The up-regulation of ATOX1 implied a build-up of intracellular Cu upon Cu challenge, which may be related to the aforementioned Cu sensitivity in zebrafish. Indeed, Cu accumulation in gill, intestine and liver was observed in zebrafish exposed to 8 ppb Cu.[39] In the same study, expression of metallothionein 2, another major intracellular Cu-capturing protein, was up-regulated in gill and liver, but not intestine.

In general, there was no significant change in CTR1 expression level in the tissues on exposure to Cu, except in the gill and intestine, where there was a slight increase when exposed to a lower Cu dosage, followed by a drop to the control expression level upon greater Cu dosage (**Figure 3A iv** and **3C iv**). While more CTR1 was expressed for Cu uptake when there was a slight increase in Cu availability, a down-regulated expression of CTR1 under Cu exposure prevented the cells from further Cu uptake, lowering the potential cytotoxic effects associated with Cu.

4.3 Localization patterns of ATOX1, ATP7B and CTR1 in ZFL cells

A typical localization pattern of ATOX1 was observed with confocal microscopy. As a cytosolic chaperone, ATOX1 was found in the cytosol in normal conditions. Upon Cu exposure, a nuclear translocation was observed. This result agrees with the finding of a previous study that used mouse embryonic fibroblast cells.[23] However, CTR1, which functions as a Cu importer, only localized on the plasma membrane upon Cu challenge for Cu uptake. In the absence of Cu, CTR1 localized on structures in the cytoplasm, but did not spread throughout the cytosol (**Figure 6B**). These structures could possibly be vesicles on the cytoskeleton that wait for translocation towards the plasma membrane upon Cu exposure. In contrast to the constitutive localization on the plasma membrane in humans,[41] it seems that plasma membrane localization of zebrafish CTR1 occurs only in the presence of Cu stimulation. We demonstrated that despite a normal trans-Golgi network localization conferred by the Golgi retention signals (**Figure 6A**), ATP7B did not translocate to the plasma membrane under a sub-lethal Cu concentration, even after 4 h. Together with the results from the functional assay using ZFL cells transfected with wild-type or mutant ATP7B (**Figure 5**), these findings may provide some clues to Cu intolerance in zebrafish. It is anticipated that an extra MBD may help in transporting Cu ions, however the ATP7B mutant with MBD2 mutated did provide higher survivorship over the wild-type ATP7B in ZFL exposed to low and medium levels of Cu ions. With higher concentrations of Cu ions, > 300 nM, over-expression of either wild type or mutant of ATP7B could not save ZFL from being killed by Cu exposures.

4.4 Possible explanations for Cu intolerance in zebrafish

Cu intolerance in zebrafish could be explained in terms of transporter expression levels as simply the result of significantly enhanced CTR1 expression or a reduction in Cu-ATPase expression. However, neither case matches our observation.

Although ATP7B, rather than the mainly expressed ATP7A, was chosen to study both intracellular localization and the functional role of MBD2 in ZFL cells, our results still provide some insights into Cu sensitivity in zebrafish. We demonstrated that Cu stimulated the translocation of ATOX1 and CTR1, but ATP7B did not translocate toward the plasma membrane for Cu efflux under Cu challenge. ATP7B acquires a Golgi retention signal and so it localized on the trans-Golgi network as expected. Although ATP7B shares a high homology with its isoform ATP7A to serve as a Cu efflux pump in the tissues, it is possible that ATP7B is non-functional in the liver, which accounts for the Golgi retention, even when exposed to Cu. However, overexpressing ATP7B in ZFL cells appeared to enhance the 24-h LC_{50} of Cu^{2+}, as no significant drop in survivability percentage was observed when ZFL cells were exposed up to 100% 24-h LC_{50} of Cu^{2+} (**Figure 5**), suggesting that ATP7B might still acquire its Cu efflux function in ZFL cells. Another possible explanation would then be an insensitive response to Cu for ATP7B to translocate toward the plasma membrane for Cu removal. Co-transfection of ATP7A and ATP7B in ZFL cells should be conducted to demonstrate whether they share similar functions in Cu efflux in the liver, and more importantly, whether ATP7A has an insensitive response as observed in ATP7B, which could explain the Cu sensitivity in zebrafish better with just solely using ATP7B.

Other evidence further supports the hypothesis of Cu transporters affect Cu intolerance. Although there was significant up-regulation of the Cu-ATPases transcript expression in the tissues to deal with the Cu load, it was still insufficient to eliminate intracellular Cu effectively. Craig *et al.*[39] showed that when zebrafish were exposed to 8 ppb (125.6 nM) of Cu^{2+}, there were significant Cu loads in the gill, intestine, and liver tissues compared with specimens not exposed to Cu. This phenomenon could be explained by the elevation in

ATOX1 mRNA expression, and no significant down-regulation in CTR1 expression, in all of the tissues we investigated. Given that ATOX1 is a cytoplasmic chaperone that binds Cu, more ATOX1 would be required to capture the accumulated Cu. Together with a constant expression of the Cu importer, the intracellular Cu level could build up until the capturing capacity of ATOX1 and other metal-binding proteins such as metallothionein saturates is reached, resulting in a leakage of labile Cu and subsequently giving rise to Cu toxicity. Ineffective Cu transfer from ATOX1 to Cu-ATPases in zebrafish could also be one of the causes. Further structural analysis of the interactions between ATOX1 and Cu-ATPases, and the evaluation of Cu loads in Cu-binding proteins and the amount of labile Cu present in the cytosol under Cu stress would also be needed.

5. Conclusion

Very little research has been carried out to investigate the molecular mechanisms of Cu intolerance or sensitivity in zebrafish, particularly under acute waterborne Cu exposure. We present some insights to help explain such Cu intolerance, which we conclude could possibly be a leakage of labile Cu due to an insensitive or less sensitive response of Cu-ATPases (focusing ATP7B in this paper) to eliminate intracellular Cu upon Cu challenge or the ineffective Cu delivery from ATOX1 to Cu-ATPases. This hypothesis requires further experiments to confirm and warrants further investigations.

Disclosure

The authors report no conflicts of interest.

Acknowledgements

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Figure Legend

Figure 1 Lethal concentrations of $CuCl_2$ were determined for zebrafish adults. The 96-h LC_{50} value of $CuCl_2$ was 63.6 ppb (0.99 μM) for zebrafish adults, with 95 % confidence interval ranging from 60.8 to 66.5 ppb (0.95 to 1.04 μM).

Figure 2 Tissue expression profiles of ATOX1, ATP7A, ATP7B, and CTR1 in zebrafish. (A) The relative mRNA expression levels of the Cu transporters **(i)** ATOX1, **(ii)** ATP7A, **(iii)** ATP7B, and **(iv)** CTR1 were evaluated in the gill, heart, intestine, kidney and liver tissues via qPCR. * denotes a significant difference compared with the other tissues (one-way ANOVA with Tukey's test, $p \leq 0.05$, $n = 3$). The bars bearing different lettering are significantly different (one-way ANOVA with Tukey's test, $p \leq 0.001$, $n = 3$). **(B)** Relative expression ratio of ATP7A and ATP7B in the tissues. The normalized gene expression levels of ATP7A and ATP7B were corrected to the smaller gene expression level.

Figure 3 Alterations in the expression levels in ATOX1, ATP7A, ATP7B, and CTR1 in response to Cu challenge. Fold induction of **(i)** ATOX1, **(ii)** ATP7A, **(iii)** ATP7B, and **(iv)** CTR1 was evaluated in **(A)** gill, **(B)** heart, **(C)** intestine, **(D)** kidney, and **(E)** liver tissues via qPCR. Significant differences compared with a 0 ppb control are denoted by * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($p \leq 0.001$), respectively (one-way ANOVA with Dunnett's test, $n = 3$).

Figure 4 The second metal binding domain (MBD) of ATP7B in zebrafish is unique. (A) Schematic diagram of the primary structures of ATP7B in zebrafish and other fish. Conserved structural motifs are indicated, with the residues essential for domain functioning included. Modified from Minghetti *et al*.[24] **(B)** Multiple sequence alignment of HMA domains from the ATP7Bs of various fish. The groups of HMA domains indicated from **i** to **v** correspond to the HMA domains in **(A)**, with MBDs indicated by boxes. MBDs of ATP7B from zebrafish (*Danio rerio*; ENSDARP00000029666), stickleback (*Gasterosteus aculeatus*; ENSGACP00000019725), tetraodon (*Tetraodon nigroviridis*; ENSTNIP00000002634), fugu (*Takifugu rubripes*; ENSTRUP00000034454), Nile tilapia (*Oreochromis niloticus*; XP_003446605), and seabream (*Sparus aurata*; ACX37120) were analyzed against the NCBI Conserved Domain database and used to generate a phylogenetic tree based on the multiple alignment result from MUSCLE. Branch support values (indicated as red) represent the percentage of frequency returning the associated branch topology after a maximum of 16 iterations of tree generation. Similar residues are colored as the most conserved according to BLOSUM62, where light blue and gray highlights represent an average BLOSUM62 score of a maximum of 3.0 and a lower score of 0.5. Lower case residues represent unmatched residues among the sequences. MBDs with similar sequences were classified into different groups. For the full sequence alignments, see **Figure S1**. **(C)** Phylogenetic tree generated according to the multiple alignment shown in **(B)**. MBDs with similar sequences were classified into different groups. The numbers on the branches indicate branch support values. Branches with less than 50% of support values were collapsed.

Figure 5 MBD2 in ATP7B does not contribute to the Cu sensitivity of zebrafish. Change in viability upon overexpression of wild-type and MBD2-non-functional ATP7B in response to Cu exposure was determined by Alamar Blue assay. Significant differences compared with a 0 μM control are denoted by * ($p \leq 0.05$), and *** ($p \leq 0.001$)

respectively, derived from one-way ANOVA with Dunnett's test, $n = 3$.

Figure 6 Localization of Cu transporters in ZFL cells with or without Cu exposure. ZFL cells were transfected with **(A)** pzATP7B-mCherry-N1 and EYFP-GalT (encoding the trans-Golgi network marker), **(B)** pzCTR1-mCerulean-N1 and **(C)** pzATOX1-mCitrine-N1, and treated with water or 150 μM of Cu^{2+} solution for 4 h before confocal image collection. Bar = 10 μm.

Supporting Information

Figure S1 Global sequence alignment of MBD-containing the N-terminus region of ATP7B in various fish. ATP7B from zebrafish (*Danio rerio*; ENSDARP00000029666), stickleback (*Gasterosteus aculeatus*; ENSGACP00000019725), tetraodon (*Tetraodon nigroviridis*; ENSTNIP00000002634), fugu (*Takifugu rubripes*; ENSTRUP00000034454), Nile tilapia (*Oreochromis niloticus.*; XP_003446605) and seabream (*Sparus aurata*; ACX37120) were used to generate a phylogenetic tree based on the multiple alignment results from MUSCLE. Branch support values (indicated in red) represent the percentage of frequency returning the associated branch topology after a maximum of 16 iterations of tree generation. Boxes indicate the MBDs. Similar residues are colored as most conserved according to BLOSUM62, where light blue and gray highlights represent an average BLOSUM62 score of a maximum of 3.0 and a lower score of 0.5. Lower case residues represent unmatched residues among the sequences.

Figure S2 Expression stability of the housekeeping genes. Calculation of the stability of housekeeping genes (B2M, EF1a, GAPDH and YBX1) using the qPCR results generated from different tissue types and Cu concentrations exposed using geNorm.[33] For every control gene, the pairwise variation with all other control genes was determined. An average pairwise variation of a particular gene with other control genes was defined as the M-value, which is a measure of gene expression stability. A greater M-value represents less stability. Two constitutively expressed housekeeping genes (in this case, EF1a and YBX) among all of the tested samples were determined by the stepwise exclusion of the gene with the highest M-value.

Table

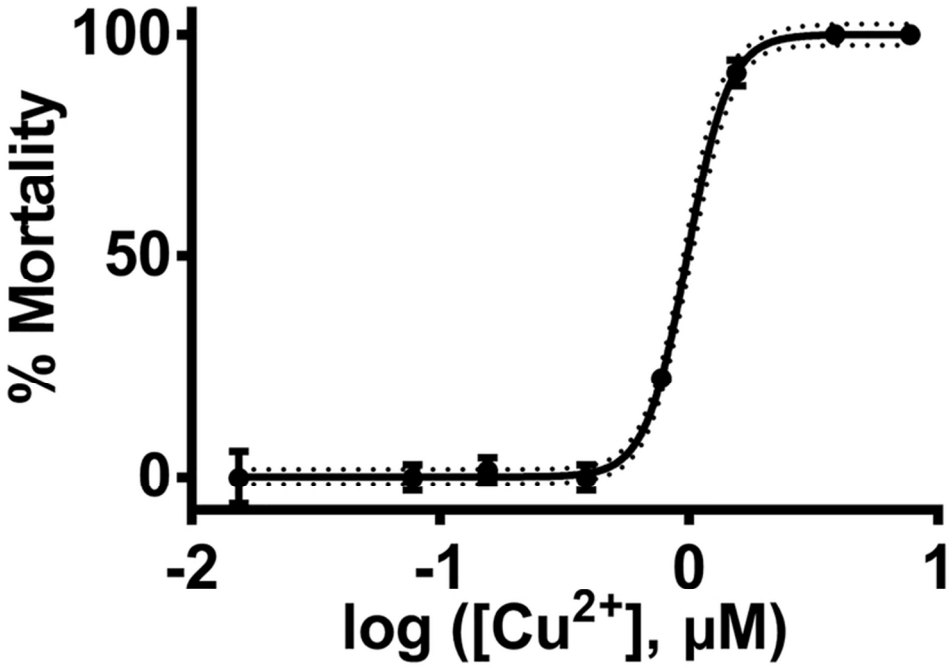
Table 1 Oligonucleotides used in this study. The underscored portions indicate the restriction sites. The amplicon sizes of the qPCR products are indicated in square brackets. HKG: Housekeeping gene.

Gene (Accession Number)	*Primer Name*	*Sequence (5' – 3')*	*Remarks*
ATP7A (NM_001042720)	zATP7A qF	TGGTGCTTATCAGGAATGATCTTC	qPCR [127 bp]
	zATP7A qR	GCAATGGGAATTCCTACAAGATTG	
ATP7B (ENSDART00000030246; Ensembl)	zATP7B HindIII F	CCCAAGCTTGGGATGAATAAACTCAGTCCTTTCAGTAATCTT	Full-length amplification
	zATP7B KpnI R	GGGGTACCCCCACAATGCTCCTTCCTGAGGTGTTGTATGG	
	zATP7B qF	GTTGTGGGACCGAGCGAG	qPCR [119 bp]
	zATP7B qR	CCAACTCCTCTTTGCAGTGCT	
	zATP7B MBD2 C/S F	GAAGGGATGCATTCCGGGTCATCTGTGAAGAACATA	Site-directed mutagenesis
	zATP7B MBD2 C/S R	TATGTTCTTCACAGATGACCCGGAATGCATCCCTTC	
CTR1 (NM_205717)	zCTR1 HindIII F	CCCAAGCTTGGGATGGATTCGTCACATTCACATCACC	Full-length amplification
	zCTR1 KpnI R	GGGGTACCCCATGACAGTGTTCTGTAATATCCACC	
	zCTR1 qF	GGCCACGGAGATCACATGAT	qPCR [63 bp]

	zCTR1 qR	CTCCACATTTTTGTAGCCGAAGT	
ATOX1 (NM_001256633)	zATOX1 HindIII F	CCCAAGCTTGGGATGACGACTCACGAGTTTTTTGTTG	Full-length amplification
	zATOX1 KpnI R	GGGGTACCCCTTTTGTACCAATGTAGGTAACAGTT	
	zATOX1 qF	TCGATCATGACGACTCACGAG	qPCR [68 bp]
	zATOX1 qR	CCTTCTTGTTGGGAAGATCGAT	
B2M (NM_131163)	zB2M qF	ATGGAGCGATGGATTCGTG	qPCR (HKG)
	zB2M qR	ACAGGCCTTAATTTGGACTCAGTAG	[67 bp]
EF1a (NM_131263)	zEF1a qF	GCTCAAACATGGGCTGGTTC	qPCR (HKG)
	zEF1a qR	AGGGCATCAAGAAGAGTAGTACCG	[82 bp]
GAPDH (BC083506)	zGAPDH qF	GGATTGCCGTTCATCCATCT	qPCR (HKG)
	zGAPDH qR	GTCATACCATGTGACCAGCTTGAC	[82 bp]
YBX1 (NM_131620)	zYBX1 qF	CCGGCCGGTTTTGTCA	qPCR (HKG)
	zYBX1 qR	TTATTGCTCAGATGTTGGATGTTGT	[48 bp]

Table 2 LC_{50} values of Cu^{2+} for different teleost species

Fish Species	***LC_{50} Values***	***References***
Tilapia (*Oreochromis mossambicus*)	2.8 ppm (24 h) and 1.5 ppm (96 h)	Lam *et al.*[34]
Common Carp (*Cyprinus carpio*)	200 ppb (24 h) and 50 ppb (96 h)	Lam *et al.*[34]
Rainbow trout (*Oncorhynchus mykiss*)	20 ppb (96 h)	Eyckmans *et al.*[35]
Gibel carp (*Carassius auratus gibelio*)	150 ppb (96 h)	Eyckmans *et al.*[35]
Sea bream (*Sparus sarba*)	2.36 ppm (24 h) and 1.24 ppm (96 h)	Wong *et al.*[36]
Zebrafish (*Danio rerio*)	64 ppb (96 h)	This study.



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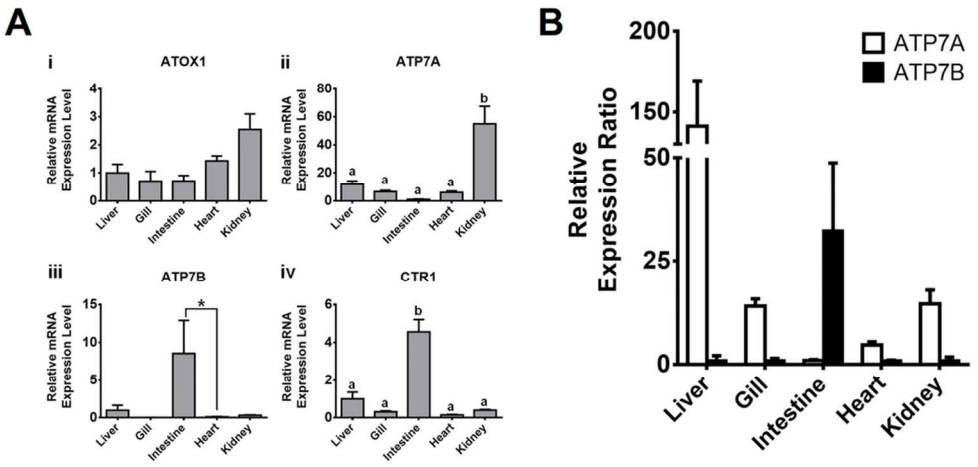

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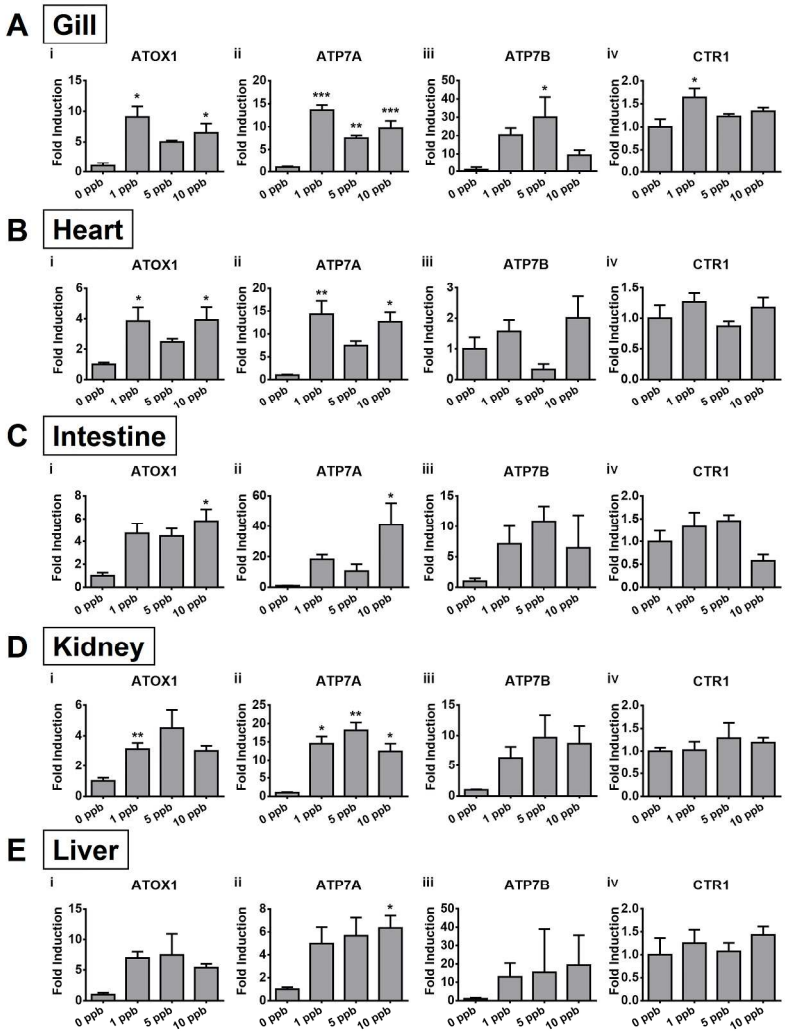

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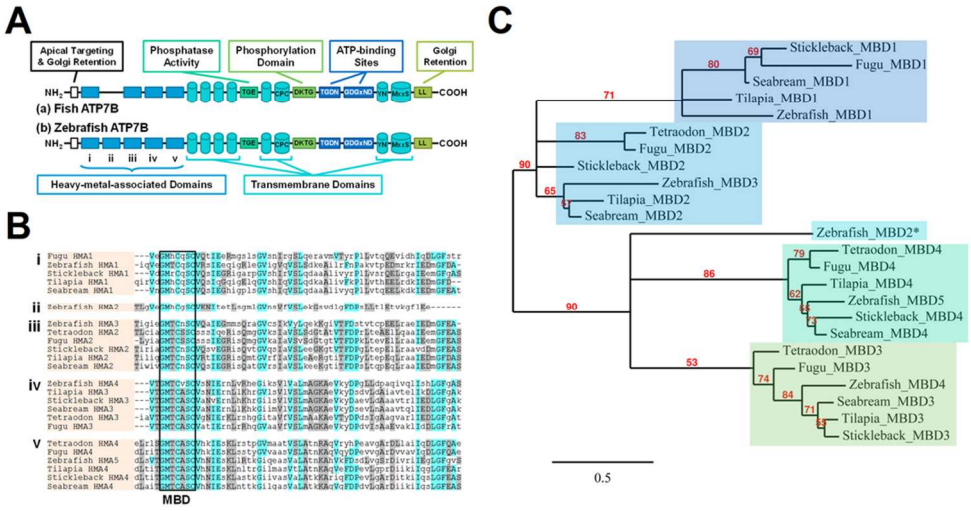

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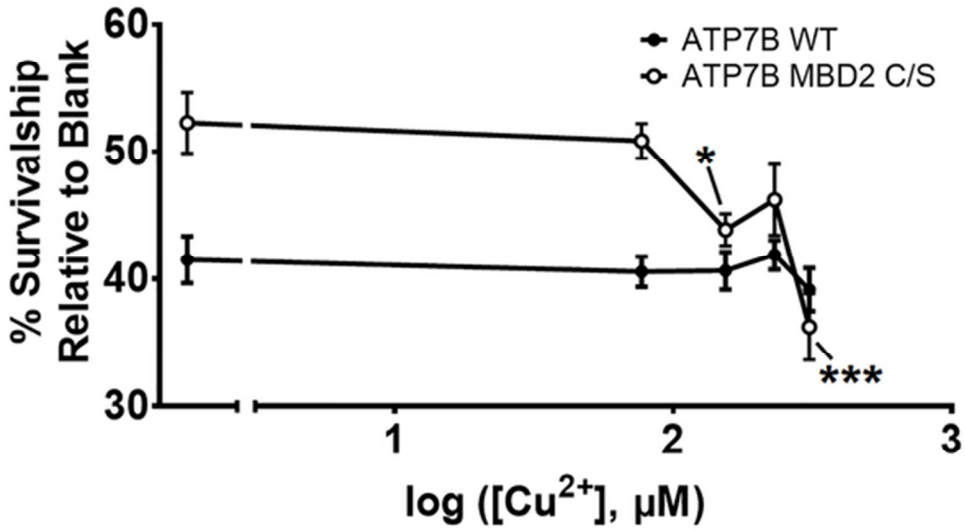

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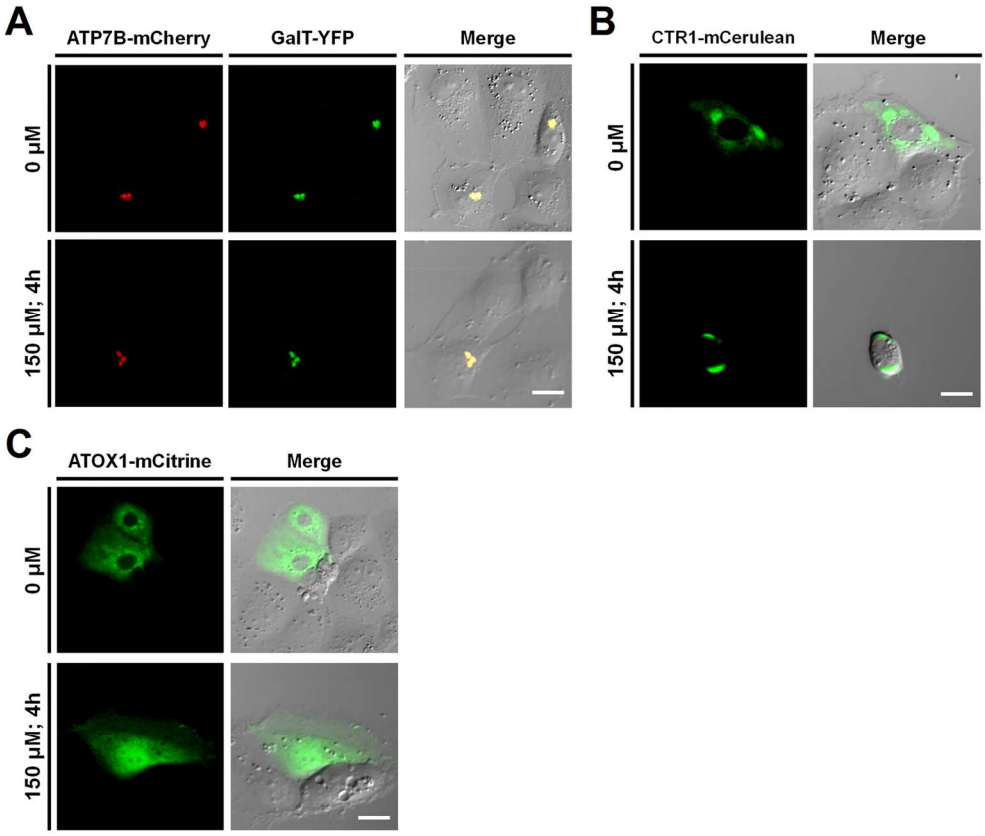

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151x127mm (300 x 300 DPI)