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Copper induces Cu-ATPase ATP7A mRNA in a fish cell line, SAF1.

Matteo Minghetti[1*], Michael J. Leaver[1], John B. Taggart[1], Elisa Casadei[1], Meirav Auslander[2], Moshe Tom[2] and Stephen G. George[1]

1: Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK.

2: Israel Oceanographic and Limnological Research, P.O.B. 8030 Haifa 31080, Israel.

*To whom correspondence should be addressed:

Matteo Minghetti

Institute of Aquaculture

University of Stirling

FK9 4LA

Tel: +44 (0) 1786 467929

Fax: +44 (0) 1786 472133

E-mail address: matteo.minghetti@stir.ac.uk

Abstract

Copper transporting ATPase, ATP7A, is an ATP dependent copper pump present in all vertebrates, critical for the maintenance of intracellular and whole body copper homeostasis. Effects of copper treatment on *ATP7A* gene expression in fibroblast cells (SAF1) of the sea bream (*Sparus aurata*) were investigated by qRT-PCR and by a medium density microarray from a closely related species, striped sea bream (*Lithognathus mormyrus*). To discriminate between the effects of Cu and other metals, SAF1 cells were exposed to sub-toxic levels of Cu, Zn and Cd. Expression of Cu homeostasis genes copper transporter 1 (*CTR1*), Cu ATPase (*ATP7A*), Cu chaperone (*ATOX1*) and metallothionein (*MT*) together with the oxidative stress markers glutathione reductase (*GR*) and Cu/Zn superoxide dismutase (*CuZn/SOD*) were measured 0, 4 and 24 hours post-exposure by qRT-PCR. Microarray was conducted on samples from 4 hours post Cu exposure. Cu, Zn and Cd increased *MT* and *GR* mRNA levels, while only Cu increased *ATP7A* mRNA levels. Microarray results confirmed the effects of Cu on *ATP7A* and *MT* and in addition showed changes in the expression of genes involved in protein transport and secretion. Results suggest that *ATP7A* may be regulated at the transcriptional level directly by Cu and by a mechanism that is different from that exerteted by metals on *MT* genes.

Keywords: Copper; metals; *ATP7A*; *MT*; microarray, qRT-PCR, fish, *Sparus aurata*.

1. Introduction

Cu is present as an essential catalytic and structural cofactor in many enzymes involved in diverse vital biological processes including respiration, protection from reactive oxygen species (ROS), development, neurological functions and iron acquisition (Kim et al., 2008). However, free Cu can generate ROS, which cause severe damage to cellular macromolecules (Halliwell and Gutteridge 1984) culminating in apoptotic or necrotic cell death (Krumschnabel et al., 2005). Thus, living organisms have evolved homeostatic systems, in the form of a heirarchical network of Cu-binding proteins, which control Cu cellular uptake, storage, trafficking and excretion. Whilst some proteins involved in these pathways are known in some detail, there are still major gaps in our knowledge of how cellular Cu homeostasis is achieved at the molecular level (Turski and Thiele 2009). Known pivotal proteins in the regulation of cellular Cu homeostasis include the high affinity Cu transporter CTR1, responsible for cellular Cu uptake (Puig and Thiele 2002), and the Cu-ATPases, ATP7A and ATP7B, responsible for delivery of Cu to cupro-enzymes and for excretion of Cu in condition of excess Cu (Lutsenko et al., 2008). Much of the effort investigating molecular mechanisms of Cu homeostasis has been directed toward mammals. In terms of Cu exposure and uptake, fish differ from mammals in one crucial regard; they can absorb Cu by two routes, *via* the gut as in mammals, and also, unlike mammals, *via* the gill. Most research effort in fish has concentrated on the toxicokinetics of Cu, whilst the molecular mechanisms of piscine Cu-homeostasis have so far received much less attention.

Recently we reported the characterization of *CTR1*, *ATP7A* and *ATP7B* from the marine fish, Sea bream (*Sparus aurata*) (Minghetti et al., 2008; Minghetti et al., 2010). *In vivo* experiments showed that the mRNA levels of these transporters were increased or decreased by Cu excess depending upon the route of metal exposure and on the tissue in which they were measured. Thus, after waterborne exposure *ATP7A* and *ATP7B* and markers of metal

toxicity and oxidative stress, *MT* and *GR* respectively, were highly induced in the liver. In contrast, after dietary exposure hepatic *ATP7A* mRNA was reduced (Minghetti et al., 2008; Minghetti et al., 2010). These results suggest that Cu is presented to the liver in a different complex during waterborne and dietary exposure routes in fish, and also indicates potentially complex intracellular sensing and response mechanisms.

In the present study we report the transcriptional responses to sub-lethal Cu exposure in a fibroblast cell line from *Sparus aurata* fibroblast (SAF1) (Bejar et al., 1997), in order to assess its suitability as an *in vitro* system for studying Cu homeostasis and to gain insight into the mechanisms specifically involved in Cu-response in sea bream. We compared the responses of specific Cu-homeostasis and oxidative stress genes after exposure to different metals by quantitative RT-PCR and also carried out an analysis of broader effects by use of a medium density cDNA microarray from a closely related fish species, the striped sea bream (*Lithognathus marmoratus*).

2. Materials and Methods

2.1 SAF1 cell culture

The established cell line SAF1 (Bejar et al., 1997) was routinely propagated and maintained at 25˚C in Leibovitz's L-15 with GlutaMAX™-l supplemented with kanamycin (100 µg/ml), penicillin G (50 U/ml), streptomycin (50 µg/ml) and 10% of fetal bovine serum (FBS). All components were purchased from Gibco®/Invitrogen, UK. Prior to experimental seeding procedures, viable cells were counted using trypan blue exclusion dye (0.4%;Sigma, UK) using a standard Neubauer haemocytometer (0.1 mm).

2.2 Cytotoxicity assays

To determine the dose-response of Cu in SAF1 cells, measures of cell death and cell viability were performed in 96 well culture micro-plates. Cell death was estimated by determining the total protein per well using the kenacid blue (KB) dye binding assay (Clothier et al., 2006). Cell viability was determined colorimetrically using the neutral red (NR) assay (Borenfreund and Puerner 1985).

SAF1 cells were plated in sealed 96-well plates at 1×10^4 cells/well which gave satisfactory absorbance values in the cytotoxicity assays and avoided overgrowth of cells. $CuSO_4.5H_2O$ (Sigma, UK) and $CdCl_2.2H_2O$ (Sigma, UK) were dissolved in purified water, filtered with 0.45 µm filter units (MILLEX®-HV, MILLIPORE, UK) and added to medium at one tenth volume to give final concentrations ranging form 0.01 mM to 2.5 mM. Growth medium was replaced with metal-containing growth medium 24 hours (h) after seeding and assays were conducted after 24 h exposure. The dose response curve was plotted and the effective concentration causing a 50% (EC50) decline in viability was determined using Sigma plot software version 11.

2.3 Metal exposure

SAF1 cells were plated in sealed 6-well plates at a density of 0.5×10^6 cells/well resulting in 80-90% confluency after 24 h. 24 h post-seeding cells were exposed to 25 µM Cu, 10 µM Cd or 100 µM Zn and samples were taken at 0, 4 and 24 h. Each time point/metal exposure was represented in four biological replicates (different wells) for qRT-PCR. Samples for microarray analysis (6 wells) were taken at 4 h from controls and from Cu-treated cells (3 replicate were obtained combining 2 wells from the 6 wells control and treated plate). Based on cytotoxity curves and previous reports, metal treatment concentrations were selected to minimise the possibility of acute cellular toxicity, whist retaining the probability of eliciting

physiological responses. . Thus, SAF1 exposed to 25 µM Cu after 24 h showed 95 % viability by NR assay. SAF1 exposed to 10 µM Cd for 24 h showed 73 % cell viability. This compares to a sub-toxic Cu level of 20 µM for trout hepatocytes (Denizeau and Marion 1989) and a Cd level which has previously been shown to induce MT (Kling and Olsson 2000). Zinc cytotoxicity was not performed on SAF1 cells, however, previous studies on a variety of other fish cell lines grown on the same medium used in this experiment (Lebovitz supplemented with 10% FBS) showed an extremely high EC50 (~ 1500 µM $ZnCl_2$) (Muylle et al., 2006). The level of 100 µM Zn was chosen based on previous results showing this amount caused maximal MT promoter induction in rainbow trout cells (Mayer et al., 2003).

2.4 Striped sea bream liver cDNA microarray

The 4608 cDNAs which were used to construct the array were isolated from libraries constructed from the livers of striped sea bream (*Lithognathus marmoratus*) exposed to a variety of toxic compounds, and represent 1886 distinct contigs (GEO NCBI, GPL5351; (Auslander et al., 2008). Contigs were annotated by comparison to Genbank using TBLASTX, BLASTX and BLASTN as implemented by BLAST2GO.

The relative efficiency of cross-species hybridization between striped sea bream and gilthead sea bream was previously shown to be 80.5% and demonstrated the validity of using this array as a tool to measure gene expression in *Sparus aurata* (Cohen et al., 2007).

2.5 Total RNA extraction and cDNA synthesis

After exposure, cells were washed twice with 1 ml of PBS per well. Then, the cell monolayer was scraped from each well in 0.5 ml of PBS, transferred into a sterile Eppendorf tube and centrifuged at 3000 g for 10 min. PBS was then aspirated off and 0.5 ml of TRI Reagent® RNA extraction buffer (Sigma, UK) was added. Total RNA was extracted following the manufacturer's instructions. Four biological replicates (different wells) were taken for

qRT-PCR. For microarray analysis RNA was extracted from 6 wells and 3 RNA samples, each containing material from two wells was generated. RNA quantitation and quality checks (28S rRNA and 18S rRNA integrity, and absence of significant quantities of contaminating DNA) were determined with a ND-1000 Nanodrop spectrophotometer (Labtech Int., UK) and by gel electrophoresis in 1% agarose respectively. cDNA for quantitative PCR was synthesised using 1 µg of total RNA, 2 µM of anchored oligo dT_{20}, 500 µM dNTPs, 200 units of SuperScript™ II RT reverse transcriptase (Invitrogen, UK) in a final volume of 20 µl. Reactions were incubated for 60 min at 42 °C followed by 70 °C for 15 min.

2.5.1 RNA amplification for microarray analysis and labelling

Five hundred ng of total RNA from each of the three vehicle and three Cu-treatments was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, UK). The resulting aRNA was purified from unincorporated dNTPs, salts, enzymes and inorganic phosphate labelled using gel filtration micro-spin columns (as provided in the Ambion kit). Each test RNA (vehicle and Cu-treated) was labelled with Cy3 and a pool of equal amounts of aRNA from all samples was labelled with Cy5. Dye solutions were prepared fresh, by re-suspending a tube of dye from a Cy3 or Cy5 Monoreactive Dye Kit (product code PA23001 or PA25001 respectively, GE Healthcare; UK) in 70 µl of ultrapure dimethyl sulphoxide (DMSO; Sigma UK). A total of 1.2 µg aRNA was resuspended in 5 µl of 2× coupling buffer (0.1 M NaHCO3, pH 9.0; Sigma, UK) and added to an equal volume (5 µl) of resuspended dye. Following gentle mixing the solution was incubated for one hour in the dark at 25°C. To remove unincorporated dye, labelled aRNA samples (10 µl total volume) were purified using spin columns (illustra AutoSeq™ G-50, GE Healthcare, UK). Dye incorporation and labelled aRNA quality was assessed by separating 0.5 µl of the purified samples on a 1% agarose gel and visualising fluorescent products on a Typhoon scanner (GE Healthcare, UK). Labelled

aRNA samples were simultaneously quantified for both aRNA and for dye incorporation by nanodrop spectrophotometry. Samples were stored at -70°C until required.

2.6 Quantitative RT- PCR

Primers for qRT-PCR were designed using PrimerSelect 6.1 (DNASTAR, USA), and all target sequences and database accession numbers for sequences from which the primers were designed are provided in Table 1. All primers were optimised to anneal at 60 ˚C. Target sequences were selected for qRT-PCR after identifying *S. aurata* database entries with >95% identity to the *L. mormyrus* features (ESTs) of interest (GEO ncbi database, GPL5351; Auslander et al. 2008) as determined by BLASTN. This was considered to a minimum requirement for the selection of PCR primers which were specific for *S. aurata* homolgues of *L mormyrus* ESTs.

Table 1. Primers used for qRT-PCR.

Gene name	*Forward primer 5`→3`*	*Reverse primer 5`→3`*	*GeneBank ID**
CTR1	cgggtctgctcatcaacaccc	tgtgcgtctccatcagcaccg	AJ630205
ATOX1	gtgtgagggttgttcaggagc	gagaacttccacgtctttgtcg	AJ966735
ATP7A	gatgttgagttggtgcagaggg	ggttaatggagcctgcgatc	GQ200817
ATP7B	cgctggcctcgtgcttcaacc	cgacgaccgcaggcttctcattt	GQ200818
MT	gctcctgcacctcctgcaag	gggtcacacacaggcgccat	U58774
CuZn/SOD	ccatggtaagaatcatggcgg	cgtggatcaccatggttctg	AJ937872
GR	caaagcgcagtgtgattgtgg	ccactccggagttttgcatttc	AJ937873
β-Actin	gaccaactgggatgacatgg	gcatacagggacagcacagc	X89920
GAPDH	tgcccagtacgttgttgagtccac	cagaccctcaatgatgccgaagtt	DQ641630
EF1α	catggttgtggagcccttct	tcctgcacgaccattcatttc	AF184170
saCoxI	ttcgctattatggctggctttgtc	agggtgtatgcatcggggtagtc	DQ248312
lmC1q	cacgcaattgagggagcaggtttc	gcggcgacagaggcagagatttat	DQ849914
sa-Trap-α	aattgctgctgcttttcctg	atcggcgtcatcttcgtctcct	FM150285
sa-Trap-γ	gaggcgacgatgacgagaac	cccgcaaggagaaagacgag	AM964008

For qRT-PCR reaction all primers were used at 300 nM with one fortieth of the cDNA synthesis reaction (5 µl of a 1:10 dilution) and 10 µl of SYBR-green qRT-PCR mix (ABgene, UK) in a total volume of 20 µl. Reactions were run in a Techne Quantica thermocycler.

A number of qRT-PCR quality and efficiency checks were performed. The amplification efficiency of each primer set was measured by testing against a serial dilution of standard cDNA template. Primers with <90% efficiency were rejected. Random selection of qRT-PCR products were visualized by agarose gel electrophoresis to check that a single product was amplified, and these PCR products were sequenced to confirm identity.

Quantification was achieved, where possible, by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing sea bream CTR1, ATP7A, ATP7B, ATOX1, MT, GR, CuZn-SOD, β-actin, GAPDH and EF1α cDNA sequences. Normalisation of copy number across biological samples was achieved by using the geometric mean expression of two reference genes (β-actin and EF1α) as determined by geNorm software (Vandesompele et al., 2002). Statistical analysis was performed using the Minitab v.15.1 statistical software package (Minitab Inc., USA). Data was first assessed for normality with the Kolmogorov-Smirnov test and for homogeneity of variances by Bartlett's test and examination of residual plots. Sample data were natural-log transformed to improve normality. However row data figures are presented as mean ± SD. Sample data were analysed by full factorial two way analysis of variance (ANOVA) where time and treatments were the fixed factors. Post hoc multiple comparisons were applied using Tukey's test (Zar 1999). A significance of $p<0.05$ was applied to all statistical tests performed. In addition, to enable comparison with fold expression changes estimated by microarray analysis, qRT-PCR expression levels relative to untreated controls were calculated using the relative expression method described by Pfaffl et al., (2002). Thus, statistically significant differences between control samples and treated samples were evaluated in group means by randomization test using REST software. Differences were considered to be significant at $P \leq 0.05$. The changes in expression levels of *ATP7A* and *MT*

mRNAs across treatments were similar after both relative and absolute quantification methods were applied.

2.7 Hybridization protocol and analysis

All 6 microarray hybridisations were carried out at the same time. Pre-hybridisation consisted of washing slides in purified Milli-Q water three times for 30 seconds, then transferring into pre-hybridization solution (5 × SSC, 0.2% SDS and 1.5% BSA, Sigma, UK) and incubating at 50˚C for 2 hours. Following pre-hybridisation slides were immediately washed in warm filtered water for 30 seconds (× 3). Then slides were dried by centrifugation (500 × g for 5 min) and loaded into a Lucidea Semi-automatic hybridisation system (GE Healthcare, UK).

Appropriate Cy3 and Cy5 labelled samples (7-9 µl, 150-250 ng cDNA, 16-30 pmol) were combined in a PCR tube and water was added to a final volume of 25 µl. These samples were then denatured at 95˚C for 3 min and immediately added to hybridisation buffer equilibrated at 60˚C. The hybridization buffer consisted of 170 µl of 50% UltraHyb solution (Ambion, UK), 2× SSC, pH 7.0, 1 mg mL^{-1} poly(A) (Sigma, UK) and 0.5 mg mL^{-1} herring sperm DNA (Sigma, UK). Arrays were hybridised for 18 h at 45˚C, with a programmed mixing step every 15 min. Slides were then automatically washed with 2 × SSC, 0.5% SDS for 10 min at 60˚C. After hybridisation slides were washed manually as follows: two washes in 0.3 × SSC, 0.2% SDS for 5 min each at 42˚C; followed by three washes in 0.2 × SSC for 3 min each at 42˚C. The slides were finally dried by centrifugation, before being scanned.

Hybridised slides were scanned at 10 µm resolution using a Perkin Elmer ScanArray Express HT scanner. The detected fluorescence was adjusted for each slide by altering both laser power (80-90%) and photo-multiplier tube (PMT) (80-90%) to ensure that maximum number of features was within the linear range of detection, and that the intensity ratio of the Cy3 and Cy5 signals was close to one. BlueFuse software (BlueGnome) was then used to

identify features and quantify the fluorescent signal from scanned images. Abnormal hybridization signals were flagged and omitted from subsequent analyses. Similarly all positive and negative control features were removed prior to implementation of transformation and normalisation procedures. Linear intensity values from duplicate features were combined ('fused'; BlueFuse proprietary algorithm). The fused data were then imported into Genespring GX version 7.3.1 (Agilent Technologies). Within Genespring all linear intensity values less than 0.01 were set to 0.01, to remove any potentially confounding negative values. Then a 'per spot per chip' intensity dependent (Lowess) normalisation was undertaken (40% of data used for smoothing). Data were subsequently filtered using a BlueFuse spot confidence value > 0.1 in four or more slides and BlueFuse spot quality of > 0.4 in four or more slides. Array features which formed contigs were treated as within-chip replicates. This gave a final list of 1625 features which were available for statistical analysis. ANOVA (t-test) was used to compare controls and Cu exposed groups and statistical significance was set to $p < 0.05$.

3. Results

3.1 Cytotoxicity assays

The cytotoxicity assays results are shown in Figure 1. The NR assay which is a functional assay was more sensitive than the KB clonal assay which determined total cell protein (hence cell numbers). SAF1 cells exhibited toxic effects after 24 h of exposure at 0.1 mM Cu, cell viability dropped to 80% (NR) (EC50 0.556 mM Cu) and total protein to 89 % (KB) (EC50 1.309 mM Cu). Thus 24 h exposure to 0.25 mM Cu was used subsequently as a sub toxic level of exposure since 95% of the cells were viable (NR) and 100% of total cell protein was stained (KB). SAF1 cells showed higher sensitivity to Cd than to Cu. With 0.01 mM Cd (the lowest Cd concentration tested) after 24 h of exposure cell viability was reduced to 73% (NR

assay) with an EC50 of 0.03 mM, the EC50 for cytotoxicity determined by the KB clonal assay was 0.335 mM.

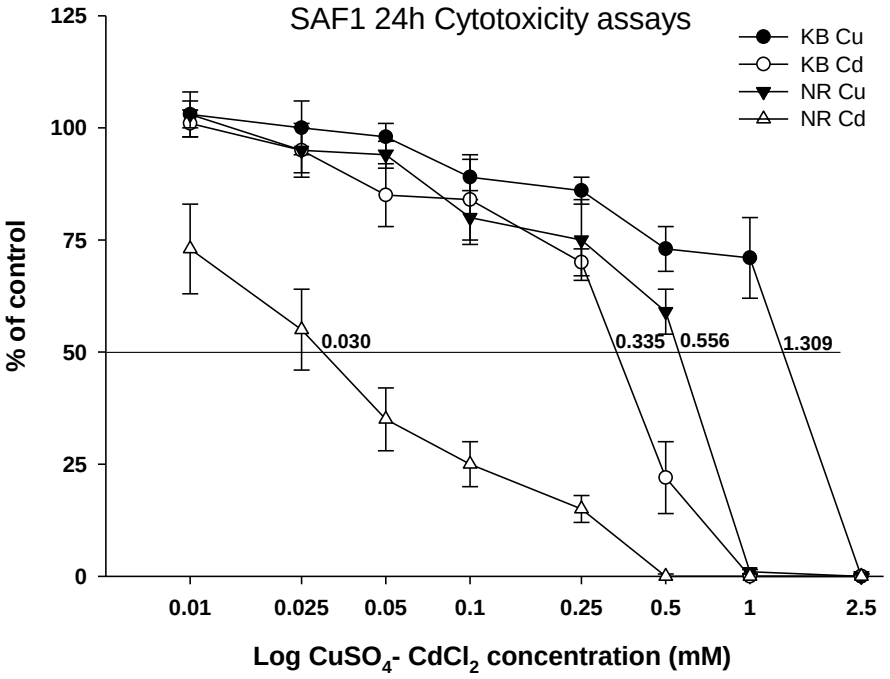

Figure 1. Cytotoxicity of $CuSO_4$ and $CdCl_2$ in L15 medium. SAF1 cells were exposed for 24 h to a range of $CuSO_4$ and $CdCl_2$ concentrations (0.01-2.5 mM). Viability was assessed by NR uptake and total protein by KB staining. Y-axis shows % absorbance at 540 nm for KB and 492 nm for NR compared to controls (0 mM condition). EC50 values are represented in mM. Each data point represents the mean ± S.D. (n= 4).

3.2 Gene Expression

Following statistical analysis, performed in Genespring (ANOVA t-test, $p<0.05$) the expression of 103 genes (contigs) were scored as changed by Cu exposure (Supplementary table). The application of a false discovery rate test did not return any genes, and we therefore applied a filter to return genes that were only 1.2 fold up- or down-regulated (16 genes; Table 2), and then validated the expression results for selected gens by qRT-PCR. The array was not large enough or sufficiently well annotated to undertake a reliable statistical GO analysis with Genespring. Analysis of the list of genes whose expression was changed by Cu showed that

two genes involved in copper homeostasis (*MT* and *ATP7A*) were significantly altered as was ferritin, which is involved in iron homeostasis. Interestingly, expression of three genes involved in protein translocation, secretion and trafficking, *Sec61*, *TRAP-α* and *TRAP-γ* were also altered. Selected genes which were changed according to microarray analysis were also measured by qRT-PCR (Table 2). All genes measured by qRT-PCR showed similar expression changes to those measured by microarray with the exception of cytochrome c oxidase, which showed a reduction in expression level on the array but an increase by qRT-PCR.

Table 2. Genes whose expression was changed in SAF1 cells exposed to 25 μM Cu for 4 hours.

	Microarray		**qRT-PCR**			
Process	**p-value**	**Fold Change**	**p-value**	**Fold Change**	**Uniprot ID and Blast-Hit**	**GenBank**
Copper homeostasis	0.0463	2.285	0.0001	7.4	(AAC32738) metallothionein	U58774
	0.0108	1.802	0.041	2.9	(ACX37119) ATP7A	GQ200817
Iron homeostasis	0.0115	1.546	-	-	(FJ788424) ferritin M subunit	DQ850994
Protein translocation	0.0065	1.252	0.028	2.1	(P45433) TRAP-α	DQ850702
	0.0156	1.215	0.036	2.1	(Q7ZUR5) TRAP-γ	DQ850967
	0.0177	0.747	-	-	(Q8AY34) Sec61 alpha subunit	DQ849773
Prot processing/membrane targeting	0.0422	0.816	-	-	(Q5VZH5) PDZ and LIM domain 1 (Elfin)	DQ850471
	0.0468	1.328	-	-	(Q4VBR7) N-myristoyltransferase 1	DQ850006
Immune response	0.0418	1.231	-	-	(O97375) Scavenger receptor cysteine-rich protein type 12	DQ850955
	0.0041	0.763	-	-	(Q9XS13) MHC class I antigen (Fragment)	DQ850397
Immune/Blood coagulation	0.0182	0.812	-	-	(Q6NYE1) Fibrinogen, B beta polypeptide	DQ850270
Proteome	0.0213	0.816	-	-	(Q6AZC1) 26S protease regulatory subunit 8	DQ849657
Oxidative Phosphorylation	0.0402	0.806	0.031	5.4	(Q94TF4) Cytochrome c oxidase subunit I	DQ850258
Lipid Metabolism	0.0215	0.792	-	-	(O42364) Apolipoprotein Eb precursor (Apo-Eb)	DQ849686
Unknown	0.019	0.807	0.054	0.22	(Q8JI26) C1q-like adipose specific protein	DQ849868
	0.0316	0.463	-	-	(Q6PS61) Ovary-specific C1q-like factor	DQ850938

Genes whose expression was smaller than 1.2 fold up or down were omitted. ANOVA (t-test) was performed between controls and Cu exposed groups and statistical significance was set to $p < 0.05$. qRT-PCR fold change values and p-values ($n = 4$; $p<0.05$) were calculated by REST software. Microarray and qRT-PCR validation results are relative to 4h exposure. Further 16 features passing filters and statistcal testing, but which could not be identified due to low BLAST scores are omitted. The full list is reported in supplementary Table.

To distinguish the effects of Cu from the effects of other related metals, a subset of genes of interest were also measured in Zn- and Cd-treated SAF1cells from a simultaneous, parallel series of treatments (Figure 2). Significant effects were observed for both treatment and time

after 2-way ANOVA. After 4 hours of exposure to metals the mRNA levels of the Cu transporters, *CTR1*, *ATP7A* or *ATOX1* were not changed when compared to controls in any of the metal-treated SAF1 cells, whilst MT mRNA levels were increased 5.1 fold and 23 fold by Cu- and Cd- exposure respectively. In addition, SAF1 cells exposed to Zn and Cd for 4 h showed increases in levels of GR of 2.6 and 5.5 fold respectively. Following 24 h exposure, Cu induced *ATP7A* mRNA level by 3.1 fold whilst *CTR1* and *ATOX1* mRNA levels were unaffected by metal exposures. All three metals induced *MT* mRNA between 15 and 300 fold, with Cd-exposed cells showing the highest level of *MT* induction and Cu the lowest. In Cu- and Cd-exposed cells there was an induction of the oxidative stress marker *GR*. *CuZn/SOD* mRNA levels were unaffected by these levels of Cu, Zn or Cd in SAF1 cells.

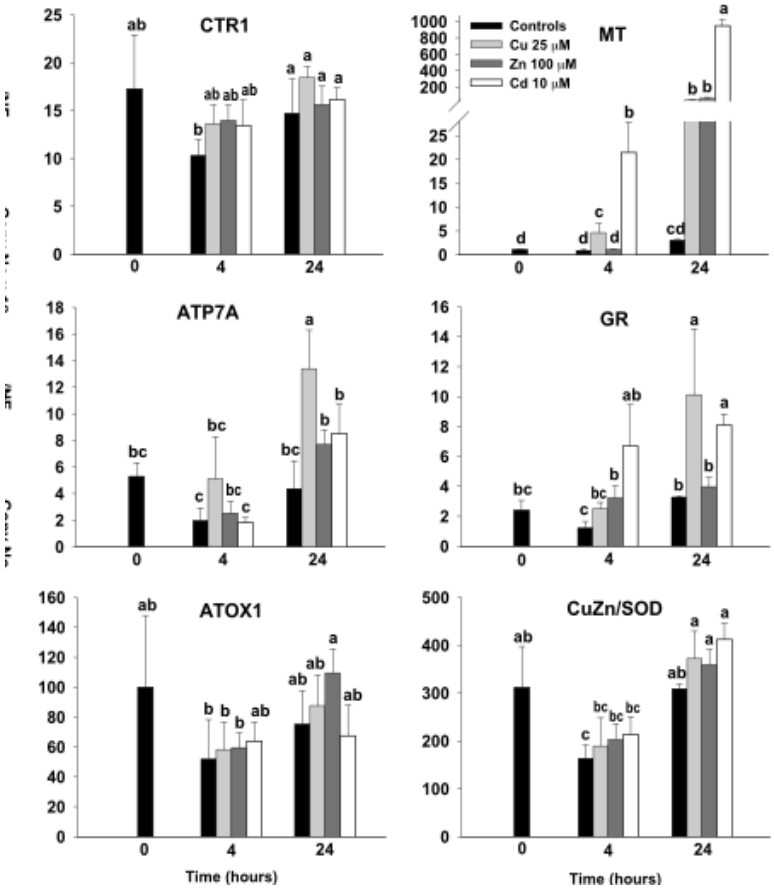

Figure 2. SAF1 transcriptional response to 25 µM Cu, 100 µM Zn and 10 µM Cd. Gene expression measured by qRT-PCR of Cu transporter genes *CTR1*, *ATP7A* and *ATOX1* and *MT* and oxidative stress genes *GR* and *CuZn/SOD*. *CTR1*, *ATP7A*, *ATP7B*, *MT*, *GR* and *CuZn-SOD* copy

numbers were normalized by geNorm using a normalization factor (NF) based on the geometric mean of *β-actin*, *EF1α* reference genes. Values are means ± S.D. n = 4. Lettering indicates significant differences following ANOVA and post-hoc Tukeys test ($p<0.05$).

4. Discussion

Sea bream SAF1 cells demonstrated a clear response to Cu which included increases in mRNAs for *ATP7A* and *MT*, as well as for components involved in protein translocation such as *Sec61*, *TRAP-α* and *TRAP-γ*. This response of *ATP7A* expression was specific for Cu exposure since it was not elicited by Cd or Zn treatment. The inductive effects on *MT* mRNA and *GR* mRNA were common to all three metal treatments indicating both a stimulation in metal detoxification ability and an oxidative stress response. These responses were in general agreement with previous *in vivo* results for sea bream where there were changes in tissue *ATP7A* mRNA levels after exposure to excess waterborne Cu (Minghetti et al., 2010). This was particularly apparent in the liver where waterborne Cu resulted in the induction of *ATP7A* as well as *MT* and *GR* mRNA levels. Additionally, in the liver, *ATP7B* mRNA levels were also induced. In SAF1 cells *ATP7B* mRNA was expressed at negligible levels under all treatments tested. This might be explained by the fact that SAF1 is a fibroblast-like cell line and the low level of *ATP7B* expression in SAF1 cells is in line with the characteristics of this cellular type (Kuo et al., 1997).

MT induction is known to be transcriptionally regulated by different stimuli including metals, glucocorticoids and oxidative stress (Coyle et al., 2002). The zinc finger metal transcription factor-1 (MTF1) modulates *MT* induction by binding to specific *cis*-acting promoter elements (Andrews 2000; Bury et al., 2008). The mechanism by which MTF1 senses intracellular variation in metals is not completely understood. It is hypothesised that MTF1 activation occurs through mobilization of free pools of Zn from other proteins by

metals such as Cd or oxidative stress (Li et al., 2006). However, there is also evidence for induction of *MT* by silver and arsenic through mechanisms other then Zn mobilization (Mayer et al., 2003; He and Ma 2009). Importantly, since neither Cd nor Zn resulted in changes in *ATP7A* mRNA, it is likely that the increase in *ATP7A* expression caused by Cu treatment is not by an MTF1-dependent mechanism.

One of the most sensitive and consistent responses to oxidative stress is an increase in glutathione reductase activity both in mammals where it is regulated by the oxidative stress-activated transcription factor Nrf2 (Harvey et al., 2009) and in fish, where it is used as a biomarker (Sturve et al., 2008). In the present study, in SAF-1 cells *GR* mRNA levels were induced both by Cd and Cu indicating that these exposures elicited oxidative stress which is in accord with previous *in vivo* results (Minghetti et al., 2008; Hansen et al., 2006). In SAF1 exposure to 100 µM Zn induced a 2.6 fold induction of GR mRNA after 4 hours, but returning to control levels after 24 h exposure suggesting that SAF1 cells have efficient homeostatic mechanisms which regulate Zn intracellular concentration. Murine GR is regulated by the oxidative stress-activated transcription factor Nrf2 (Harvey et al, 2009), and although similar oxidative mechanisms may also play a role in MT induction (Ohtsuji et al., 2008), the lack of change in *ATP7A* mRNA after Cd treatment suggests that Cu is not affecting ATP7A mRNA expression *via* a mechanism dependent on oxidative stress. Recently Singleton et al. (2010) have provided evidence that the reversible glutaredoxin1-catalysed glutathionylation of the Cu-binding Cys residues of ATP7A is required for normal copper transport. Since depletion of glutathione inhibits the Cu-transporting activity of ATP7A, the induction of GR shown in our study may be part of the cellular response to excess Cu, working to maintain glutathione levels in order to facilitate Cu excretion by ATP7A.

In contrast to *GR, CuZn/SOD* mRNA level were unaltered by the conditions applied in this experiment. This could suggest that *CuZn/SOD* is not as sensitive as *GR* at the mRNA level in response to metal toxicity.

Microarray analysis also highlighted an increase in ferritin mRNA levels. A similar response, attributed to metal-induced oxidative stress has been reported in mammals (Torti and Torti 2002) and a microarray analysis of copper exposed fish, *Solea senegalensis*, also identified an increase in ferritin mRNA as a response (Prieto-Alamo et al., 2009). The response of the ferritin gene further highlights the inter-relationships between iron and copper metabolism and in the ferroxidases responsible for their uptake (Sharp 2004).

Overall the specific induction of *ATP7A* by Cu compared to the response of *MT* to all metals tested, suggest that there may be a specific intracellular Cu sensor which mediates *ATP7A* transcription. Although the transcriptomic analysis reported here was limited by the relatively small number of available and annotated features on the array, SAF1 cells exposed to excess Cu (25 μM for 4 hours) indicated that, in addition to *ATP7A* and *MT* induction, the pathways most significantly affected were protein transport, trafficking and secretion (Table 2), principally represented by three genes of the translocon (*TRAPα*, *TRAPγ* and *Sec61*), these proteins are responsible for the translocation of proteins from the cytosol to the luminal space of the ER (Menetret et al., 2008). The connection between Cu homeostasis andthe translocon pathway is currently unknown. However, interestingly, changes in Sec61 gene expression have been previously reported in fish exposed to Cu (Osuna-Jimenez et al., 2009). suggesting that the relation between these pathways should be further investigated.

Previously, most similar studies have focused on mammalian ATP7A regulation at the post-translational level and several of these studies have shown that the translocation of ATP7A from a compartment localized within the trans-Golgi network to a compartment near the plasma membrane is required for regulation of intracellular Cu levels (Nyasae et al., 2007;

Lutsenko et al., 2008). Transcriptional regulation of *ATP7A* levels in liver and intestine of zebrafish and sea bream exposed to waterborne Cu (Craig et al., 2009; Minghetti et al., 2010) and in rat intestine after Cu-exposure (Bauerly et al., 2005) indicate an additional mechanism of regulation in Cu efflux. Thus the transcriptional regulation of *ATP7A* and of cytosol/ER protein translocation imply that both elevated Cu efflux effected by *ATP7A* and an increase in metal-detoxification capacity by *MT* induction observed in the present study are all important responses to conditions of excess Cu.

In conclusion we have shown that Cu specifically induces *ATP7A* mRNA in a fish cell line by a mechanism which appears to be distinct from either MTF1 regulation or from oxidative-stress mediated gene regulation. This effect of Cu on *ATP7A* is accompanied by changes in expression of mRNAs for components of the protein translocation pathway, consistent with known mechanisms of intracellular Cu regulation by *ATP7A*. Finally, the SAF1 cell line represents a useful system in which to study Cu homeostasis in fish, and may enable Cu-dependent mechanisms of *ATP7A* gene regulation to be elucidated in future.

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