Multiple Cu-ATPase genes are differentially expressed and transcriptionally regulated by Cu exposure in sea bream, *Sparus aurata*

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1. Introduction

The variable valencies of copper (Cu) have been exploited during evolution as a cofactor for many vital enzymes (Kim et al., 2008). On the other hand, these same redox properties enable Cu to promote reactions which lead to the production of reactive oxygen species (ROS) that can have damaging oxidative effects on cellular macromolecules (Halliwell and Gutteridge 1984). In addition, Cu may also manifest toxicity by displacing Zn from functionally essential protein domains (Predki and Sarkar 1992). Thus, specific proteins have evolved for intracellular Cu transport and delivery to avoid damaging non-specific reactions and to enable targeting to cupro-proteins. These cuproproteins include Cu-ATPases which belong to the P_{1B}-subfamily of P-type ATPases present in all eukaryotic phyla (Lutsenko et al., 2007b). Cu-ATPases transfer Cu from the cytosolic carrier, ATOX1 (Hamza et al., 2003), to secretory vesicles (Petris et al., 2000). The essentiality of Cu-ATPases in normal development and pigmentation was reported in *Drosophila* (Norgate et al., 2006), whilst in the zebrafish (*Danio rerio*) ATP7A was shown by mutagenetic analysis and genetic complementation to be essential for notochord formation and pigmentation (Mendelsohn et al., 2006).

Two isoforms of Cu-ATPase (ATP7A and ATP7B) with differing functional roles have been identified in mammals, primarily by investigation of two human genetic disorders, Menkes syndrome and Wilson's disease. Menkes syndrome is caused by mutations of the gene encoding ATP7A (also known as Menkes protein) and is characterized by overall Cu deficiency and accumulation of Cu in intestinal enterocytes and the kidney. Wilson's disease is caused by mutations in ATP7B (also known as Wilsons protein) and is associated with hepatic Cu overload (Mercer 2001). Cu-ATPases have a dual role; they deliver Cu to cuproenzymes within the secretory pathway (e.g. ATP7A delivers Cu to lysyl oxidase and ATP7B delivers Cu to ceruloplasmin) and they are also responsible for excretion of excess intracellular Cu (Lutsenko et al., 2008). Body Cu homeostasis is achieved manly by regulating intestinal absorption (through ATP7A) and biliary excretion by the liver (through ATP7B). Mutated Cu-ATPase phenotypes show that the two mammalian isoforms have distinct homeostatic roles and operate in different tissues, although in some tissues, such as the brain and kidney, there is evidence of functional redundancy (Linz and Lutsenko 2007).

In fish much less is known, but there is evidence that, as in mammals, whilst intestinal absorption is proportional to the concentration of Cu in the diet, regulation is achieved by basolateral membrane extrusion in an ATP-dependent manner and by a Cu/anion symporter (Clearwater et al., 2000; Handy et al., 2000). In the gill the existence of a functional Cu-ATPase (ATP7A-like protein), has been suggested by the inhibition of Cu transport by vanadate, a specific inhibitor of P-type ATPases (Campbell et al., 1999). In addition, Bury et al., (1999) identified an ATP-dependent silver transporter at the basolateral membrane. Thus, since silver is known to mimic Cu, this finding would support a hypothesis of branchial basolateral Cu transport mediated by a fish homologue of the mammalian ATP7A. Recently, Craig et al., (2009) have shown that ATP7A is expressed in the gill, gut and liver of zebrafish and that waterborne Cu increased its intestinal and hepatic mRNA levels. Although these studies provide evidence for a role of ATP7A in Cu transport in fish, there have been no reports of the existence or the expression of an ATP7B homologue in fish. Following our previous study where we demonstrated that a high affinity Cu transporter (CTR1) was mainly expressed in the intestine of the gilthead sea bream (*Sparus aurata*) and that its mRNA levels were differentially regulated by dietary or waterborne Cu (Minghetti et al., 2008), in this study we have investigated the expression of the intracellular Cu chaperone, ATOX1 and Cu-ATPases homologues in this species.

2. Material and methods

2.1 Animals treatments and RNA extraction

The samples analyzed in this study and their processing including RNA extraction and cDNA synthesis are described in Minghetti et al. (2008). Briefly, juvenile gilthead sea bream (*Sparus aurata*), average 40g mass, were obtained from farm stock (Valle Ca' Zuliani, Rovigo, Italy). Experimental tanks were supplied with seawater pumped ashore from the Adriatic Sea. Water temperature was 19.0 ± 0.5 °C and a salinity of 33.6 ± 2.6 ‰. The trial was performed at the Marine Research Centre of Cesenatico, Bologna University, Italy. Fish were fed two experimental diets containing 7.7 ± 0.3 mg Kg^{-1} Cu (low Cu diet) or containing 130 mg Kg^{-1} Cu (high Cu diet), alternatively fish were fed the low Cu diet but were exposed to Cu dissolved in marine water at a concentration of 0.3 mg L^{-1} Cu. There were no mortalities in the tanks. After a period of 15 days (day 15) and 30 days (day 30) five fish chosen randomly from each tank were removed, killed by a blow to the head and tissues immediately sampled for RNA extraction and metal analysis. Total RNA was prepared using TriReagent (Sigma, UK) according to the manufacturer's instructions and RNA quality was checked by spectrophotometry and agarose gel electrophoresis.

2.2 Synthesis of Sea bream ATP7A, ATP7B and ATOX1 cDNAs

Degenerate primers were designed by selecting conserved areas from alignments of protein sequences derived from ATP7 and ATP7-like DNA sequences of human (*Homo sapiens*) (NM_000052), chick (*Gallus gallus*) (XM_420307), zebrafish (*Danio rerio*) (Ensembl transcript ID: ENSDART00000054977) and pufferfish (*Tetraodon negroviridis*) (Ensembl transcript ID: GSTENT00017010001) ATP7A; human (NM_000053), zebrafish (Ensembl transcript ID: ENSDART00000030246), pufferfish (Ensembl transcript ID: GSTENG00020077001) and stickleback (*Gasterosteus aculeatus*) (Ensembl transcript ID: ENSGACG00000014945) ATP7B; and human (NM_004045), rat (*Rattus norvegicus*) (AF177671), chick (XM_001233562) and puffer fish (Ensemble transcript ID: GSTENT00024266001) ATOX1 (Table 1).

Partial ATP7A, ATP7B and ATOX1 cDNAs were generated by PCR using 0.5 µM of primers (ATP7A: 1, Atp7AF2, Atp7AR5 and 2, Atp7AF7, Atp7AR9; ATP7B: Atp7BF1, Atp7B-R5; ATOX1: Atox1F2, Atox1R3), one eighth (2.5 µl) of the cDNA (from sea bream intestine) synthesis reaction, one unit of Taq polymerase with supplied buffer IV (ABgene, UK) and 1 mM $MgCl_2$ in a final volume of 25 µl. The thermocycling conditions were: ATP7A 1: 94˚C for 3 min followed by 30 cycles 94˚C 30 sec - 52˚C 30 sec - 72˚C 2 min followed by 72˚C for 7 min. ATP7A 2: 94˚C for 3 min followed by 30 cycles 94˚C 30 sec - 56˚C 30 sec - 72˚C 1.5 min followed by 72˚C for 7 min. ATP7B: 1 cycle at 95˚C for 2 min, 30 cycles 95˚C 30 sec, 58˚C 30 sec, 72˚C 3 min followed by 72˚C for 7 min. ATOX1: 94˚C for 3 min followed by 30 cycles 94˚C 30sec - 56˚C 30 sec - 72˚C 30 sec followed by 72˚C for 7 min. The PCR products obtained, which were 1500 and 1200 base pairs (bp) for ATP7A-1 and ATP7A-2 respectively, 2270 bp for ATP7B and 200 bp for ATOX1 were cloned into a 2.1 plasmid (Topo TA, Invitrogen, Paisley, UK) and sequenced (CEQ-8800 Beckman Coulter Inc., Fullerton, USA). The probable identities of the cloned PCR products were predicted using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). RACE-PCR (Rapid Amplification of cDNA Ends) was performed using the SMART™ RACE kit (Clontech, USA). RACE cDNA was generated as described in the kit manual from 1µg of intestinal sea bream total RNA. 5' and 3' RACE amplicons were generated by two rounds of PCR using gene specific primers designed from the sequence of the initial cDNAs for ATP7A (5'RACE: 5'Atp7A-R1, 5'Atp7A-R2 and 3`RACE: 3'Atp7A-F1), for ATP7B (5'RACE: 5'Atp7B-R1, 5'Atp7B-R2 and 3'RACE: 3'Atp7B-F2, 3'Atp7B-F1) and for ATOX1 (5'RACE: 5'Atox1-R1, 5'Atox1-R2 and 3'RACE: 3'Atox1-F1, 3'Atox1-F2). The final full-length PCR products were obtained by two round of PCR using primers Atp7AFull-F1, Atp7AFull-R1 and Atp7AFull-F2, Atp7AFull-R2 for ATP7A; Atp7BFull-F1, Atp7BFull-R1 and Atp7BFull-F2, Atp7BFull-R2 for ATP7B or one round of PCR using primers Atox1Full- F, Atox1Full-R for ATOX1, designed from the 5' and 3' RACE products. The annealing temperature for all primers was 60 °C and the extension time was 1 min/Kb of predicted

PCR product, and 3 min were applied for unpredictable RACE PCR products. All primers are listed in Table 1 and were designed using PrimerSelect Ver. 6.1 program (DNASTAR, www.dnastar.com). Sequencing was performed using a Beckman 8800 autosequencer. Lasergene SEQman software (DNASTAR) was used to edit and assemble DNA sequences. Since Cu-ATPases sequence annotation was incomplete in other fish species with a sequenced genome, and in order to predict the full length polypeptide sequences, the sea bream cDNA sequences coding for Cu-ATPase genes were used to search puffer fish (*Tetraodon nigroviridis*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*) and zebrafish genomes (www.Ensembl.org) using TBlastX. Regions of puffer fish, medaka, stickleback and zebrafish chromosomal DNA sequences containing homologous sequences were then processed with GeneWise2 (www.ebi.ac.uk) using the sea bream sequence as a key to generate predicted polypeptide sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of deduced protein sequences. To deduce and bootstrap phylogenetic trees using the neighbour joining method (Saitou and Nei 1987), MEGA version 4 was used (Tamura et al., 2007).

2.3 Quantitative RT-PCR

Complementary DNA synthesis reactions for qRT-PCR contained Superscript RT (Clontech), 3µg of total RNA and 500 ng oligo- dT primer in a volume of 20 µL and were incubated at 42 °C for 1.5 h followed by 70 °C for 15 min. Oligonucleotide primers for target genes, ATP7A (GQ200817), qAtp7A-F and qAtp7A-R, ATP7B (GQ200818), qAtp7B-F and qAtp7B-R, ATOX1 (AJ966735), qAtox1-F and qAtox1-R, and reference genes, β-–actin (X89920), qActinF and qActinR, GAPDH (DQ641630) qGapdhF and qGapdhR and EF1α (AF184170) qEF1 α F and qEF1 α R (Table 1) were used at 0.3 µM 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 at anneal temperatures of 56 °C for β-actin, ATOX1 and ATP7A and 60 °C for GAPDH and EF1α and 62 °C for ATP7B a to give a PCR product of 171, 130, 175, 250, 174 and 157 bp respectively. Each qRT-PCR product was than sequenced to confirm its identity and was found to be 100 % identical to its predicted sequence. Quantification was achieved by a parallel set of reactions containing standards consisting of serial dilution of spectrophotometrically determined, linearised plasmid containing salmon ATP7A, ATP7B, ATOX1, β-actin, GAPDH and EF1 α cDNA sequences. Normalisation of copy number across biological samples was achieved by using a normalization factor (NF), calculated tissue by tissue, based on the geometric mean expression of three reference genes (β-actin, GAPDH or EF1α) determined using geNorm software (Vandesompele et al., 2002). GeNorm was used to select the

two most stable genes for a particular tissue, which were then used to normalize target gene expression level. However, all reference gene expression levels varied widely between tissues and therefore the comparison of expression levels of target genes in different tissues was achieved by normalising the target gene copy number to total input RNA.

2.4 Tissue copper, metallothionein and glutathione reductase mRNA levels

The measurement of total copper and metallothionein (MT) and glutathione reductase (GR) mRNA levels in the tissues of the fish from these experiments have been described previously (Minghetti et al., 2008).

2.5 Statistical analysis

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. Where necessary sample data was transformed to improve normality. Sample data was analysed by full factorial two way analysis of variance (ANOVA). 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. All data are presented as mean ± SD.

Table 1. Primers used for cDNA isolation and qRT-PCR.

ID	*Sequence 5`→3`*	*length*	*Tm*	*GC%*
Atox1F2	gcacgaatttgaggtggccatgac	24	64.4	54.2
Atox1R3	cgtcacttggtgccgatgtactt	23	62.4	52.2
5'Atox1-R1	gacctcctttccacatttctgcagcg	26	66.4	53.8
5'Atox1-R2	gtcacagctcctgaacaaccctcacac	27	68	55.6
3'Atox1-F1	gtgtgagggttgttcaggagctgtgac	27	68	55.6
3'Atox1-F2	cgctgcagaaatgtggaaaggaggtc	26	66.4	53.8
Atox1Full-F	ttcgagtcagccggaggtgaaa	22	62.1	54.5
Atox1Full-R	catctaagaggggaggggtgtca	23	64.2	56.5
Atp7AF2	gargacatgggntttgatgc	20		
Atp7AF7	cgmttygccttccargcctc	20		
Atp7AR5	gctggatrggmgcctttga	19		
Atp7AR9	cccatccarggctgcarmacca	22		
5'Atp7A-R1	catagttttccatgacggaggcggtgaagc	30	69.5	53.3
5'Atp7A-R2	tccgatctggatgtagcatttggagtgc	28	66.6	50
3'Atp7A-F1	agaagcagcagatgtggtgttgat	24	61	45.8
Atp7AFull-F1	gacgtgcctgcttcgctgcttgtaa	25	66.3	56
Atp7AFull-F2	aagacgaagcaatcatgacacagaaagt	28	62.2	39.3
Atp7AFull-R1	cagaggatcacagggtaggccaaagagt	28	68	53.6
Atp7AFull-R2	agttgttgctggaccacgtgaaccttac	28	66.6	50
Atp7B-F1	ttcaartgygtcaacagyctg	21		
Atp7B-R5	cgacgaccgcaggcttctcatttg	24	66.1	58.3
5'Atp7B-R1	aggaagaaggccaggttgaggag	23	64.2	56.5
5`Atp7B-R2	accacctgctcctcgctgatgat	23	64.2	56.5
3'Atp7B-F1	gtsctgatccggaacgayytgct	23		
3'Atp7B-F2	gcatcgagctstcyaaraagac	22		

Atp7BFull-F1	ataagaggccagccggtgtgagag	24	66.1	58.3
Atp7BFull-F2	gtagcttcactttgggttgttagcag	26	63.2	46.2
Atp7BFull-R1	agcatgttttctcctaagtcg	21	55.9	42.9
Atp7BFull-R2	ttcccgccaaagtaagagc	19	56.7	52.6
qAtp7A-F	gatgttgagttggtgcagaggg	22	62.1	54.5
qAtp7A-R	ggttaatggagcctgcgatc	20	59.4	55
qAtp7B-F	cgctggcctcgtgcttcaacc	21	65.7	66.7
qAtp7B-R	cgacgaccgcaggcttctcattt	23	64.2	56.5
qAtox1-F	gtgtgagggttgttcaggagc	21	61.8	57.1
qAtox1-R	gagaacttccacgtctttgtcg	22	60.3	50
qActinF	gaccaactgggatgacatgg	20	59.4	55
qActinR	gcatacagggacagcacagc	20	61.4	60
qGapdhF	tgcccagtacgttgttgagtccac	24	64.4	54.2
qGapdhR	cagaccctcaatgatgccgaagtt	24	62.7	50
qEF1αF	catggttgtggagcccttct	20	59.4	55
qEF1αR	tcctgcacgaccattcatttc	21	57.9	47.6

Tm = 69.3 + 0.41 * GC% - (650/length). Tm temperature and GC % was not calculated for degenerate primers.

3. Results

3.1 Sea bream Cu-ATPase and ATOX1 cDNAs

Copper ATPases and their specific Cu chaperone ATOX1 have not been previously reported in sea bream, it was therefore important to identify their mRNA (cDNA) sequence enabling the measurement of their tissue expression profile in normal and under excess Cu conditions. The full length cDNA of ATP7A obtained from sea bream (saATP7A) was 5474 bp which contained an open reading frame of 4566 bp (1522 amino acids) and 5' end 3' untranslated regions of 294 bp and 614 bp respectively (GenBank accession number GQ200817). The deduced saATP7A protein sequence displayed 63 % identity with human ATP7A and 74 % with zebrafish ATP7A sequences. The full length cDNA of ATP7B from sea bream (saATP7B) of 4294 bp contained an open reading frame of 3981 bp (1327 amino acids) and 5' end 3' untranslated regions of 61 bp and 252 bp respectively (GenBank accession number GQ200818). The deduced protein sequence of saATP7B displayed 59 % identity with the human ATP7B sequence. Sea bream ATOX1 (saATOX1) full length cDNA was 1374 bp, consisting of an open reading frame of 204 bp (68 amino acids) and 5' end 3' untranslated regions of 90 bp and 1080 bp respectively (GenBank accession number AJ966735).

Alignments of the deduced amino acid sequence of Cu-ATPases (ATP7A and ATP7B) proteins derived from genome sequences of other fish species, bird and human and also from Cu-ATPases from insects, worms and yeast show very strong sequence conservation (Figure 1). The proteins' features which characterize mammalian Cu-ATPases such as the 6 MBDs (ATP7A), 8 transmembrane domains (TMDs) including residues in the transmembrane domains believed from modelling and mutational analysis to be involved in Cu-binding, notably the $C^{1000}PC^{1002}$ motif in

TMD6, YN in TMD7 and the MxxS motif in TMD8 were also totally conserved in fish ATP7A (supplementary Figure 1) and with the exception of the $D^{1497}TAL^{1500}$ all the other functionally important motifs (Lutsenko et al., 2007a) were conserved in saATP7A. On the other hand, saATP7B and other fish ATP7Bs including Pufferfish, Fugu, Medaka and Stickleback ATP7Bs possessed only 4 of the 6 metal binding domains (MBDs) present in higher vertebrates, while Zebrafish ATP7B possessed 5 MBDs. The apical membrane targeting domain at the N-terminus of ATP7B (Braiterman et al., 2009) was conserved in all vertebrate sequences analyzed (Figure 2A). Moreover, the di-leucine golgi retention motif (LL) at the C-terminus was conserved in all Cu-ATPases analysed excluding the sea squirt ATP7 and yeast Ccc2 (Figure 2B and supplementary figure 2).

Phylogenetic analysis of Cu-ATPases showed that sea bream and all other vertebrate ATP7A and ATP7B robustly segregated in two separate branches. The fish sequences clustered together in each branch with sea bream showing greatest similarity to the pufferfish sequence (Figure 3).

Sea bream ATOX1 (GeneBank accession number AJ966735) showed 60 % identity with the human sequence. Alignment of the deduced amino acid sequence of saATOX1 with ATOX1 proteins from other fish species, frog, chick, human, insects and yeast showed strong sequence conservation. The MBD (MxCxxC) (Hung et al., 1998; Wernimont et al., 2000) and the recently identified nuclear localization signal (NLS) (xKxGK) (Itoh et al., 2008) are highly conserved from yeast to humans. Moreover, the K^{65} which was demonstrated to be essential for Cu transport by mutational analysis is also conserved in all sequences (Portnoy et al., 2001) (fig.ure 4A). Phylogenetic analysis of available ATOX1 sequences indicated that the fish sequences were monophyletic and that the sea bream ATOX1 was most closely related to the pufferfish protein (figure 4B).

To verify whether fish species contain multiple genes for Cu transporters for which only single mammalian examples exist, the sea bream cDNA sequence was used to search for similar sequences in the zebrafish, pufferfish, medaka and stickleback genomes. In each species a single ATP7A, ATP7B and ATOX1 gene was identified.

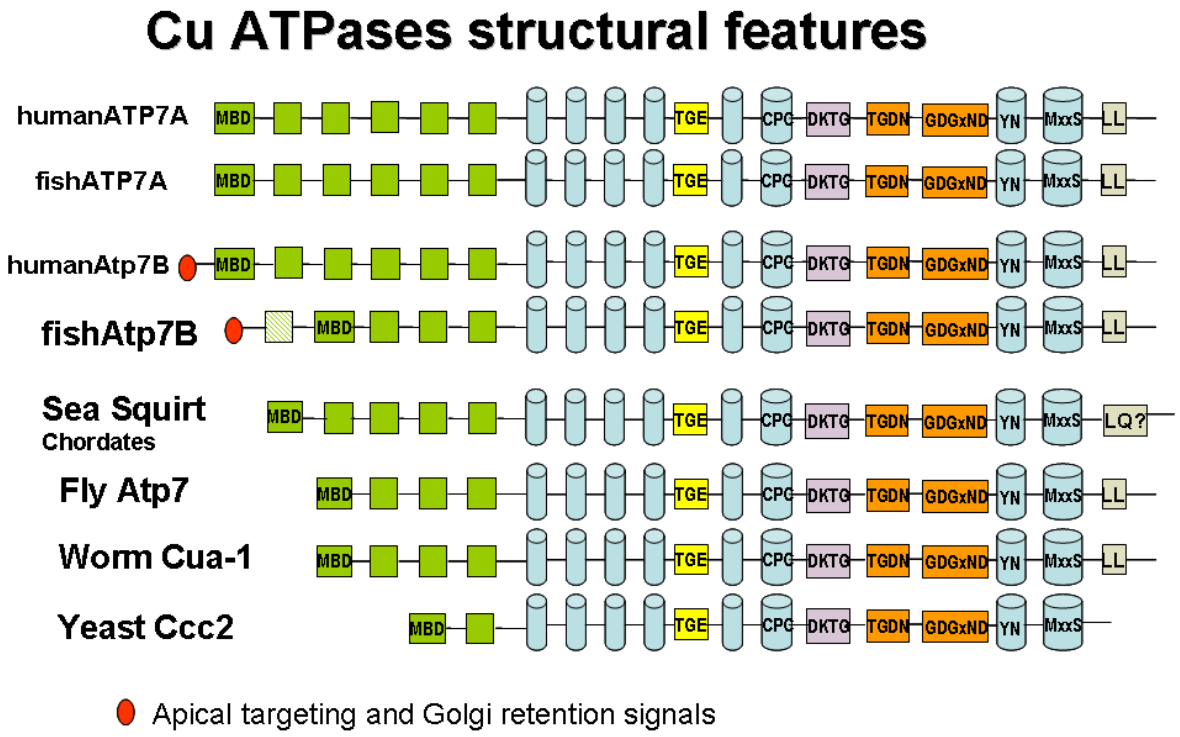

Figure 1: Cu-ATPases structural features. Structural conserved motifs are indicated. The motifs characterizing P-Type ATPases are the motif required for phosphatase activity in yellow, phosphorylation domain in purple, ATP-binding site in orange. The residues predicted to be involved in Cu coordination within the membrane in TMD6, 7 and 8 are indicated (Lutsenko et al., 2007a). Fish ATP7B showed four (sea bream, fugu, medaka and stickleback) or five (zebrafish) MBDs which are shown in green.

A

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Human            MPEQERQITAREGASRKILSKLSLPT--RAWEPAMKKSFAFDNVGYEGGLDGL 51
Chicken          ----------MEPSARMIVVKMYLKTVPKAW---MRQNFAFDNMGYEESFEAM 40
Frog             -------------SARLIVVLWTDSPPVSVWKEAKKPSCAFDNRGYEGSPDDL 40
Sea bream        ICMVECVCEPDCTC-TEHGARHCAARDVKKNG-LDAEKHGLDNLAYE------ 69
Pufferfish       ICMVDCA-----RAAPAPAPGTTVSPGPVLRSAGPDYIQGFDNLAYE------ 66
Zebrafish        LCMKDCKPLCHCDPELC--------ENGSQQEGWIPTKHAFDNFGYE--PDGL 64
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B

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Human-ATP7A      NSLLSDKRSLNSVVTSEPDKHSLLVGDFREDDDTAL---------------- 1500
Chicken-ATP7A    NSLFSDKRSVNSIVLNEPDKHSLLVGDFGEDDDTTL---------------- 1494
Seabream-ATP7A   NSLRSDKHSLNSLVLSEPDKHSLLVGESLCEEEFC----------------- 1526
Human-ATP7B      SLTSDKPSRHSAAADDDGDKWSLLLNGRDEEQYI------------------ 1465
Chicken-ATP7B    SLTSDKLPRHNGFFEEEGDKWSLLMNGGDEEQYI------------------ 1432
Seabream-ATP7B   TPALSGQGLSINSVQEQQDRCSLLDHQTAEDSNV------------------ 1327
Ciona-Atp7       NQTASDQHAINAKSLSEQCRNSLQSVNNRTLKRFEMLK-------------- 1414
Drosophila-ATP7  SRIFMHDYGN-ITSPDAKYGEGLLDPEEQYDGRTKIVRSRFHANDSTELQKL 1219
Nematode-Cua-1   GSLLG--STTSIVSSGSSKKQRLLDNVGSDLEDLIV----------------- 1238
Yeast-Ccc2       GNFWSRLFSTRAIAGEQDIESQAGLMSNEEVL-------------------- 1004
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Figure 2 A: ATP7B N-terminus showing conservation of the apical targeting and Golgi retention signals (Braiterman et al., 2009). **B**: Cu-ATPases C-terminus showing conservation of the LL-motif involved in trafficking signals. Residues identical in all proteins are shaded. Structural motifs are boxed. Full sequence alignments are reported in supplemented figure 1 and 2.

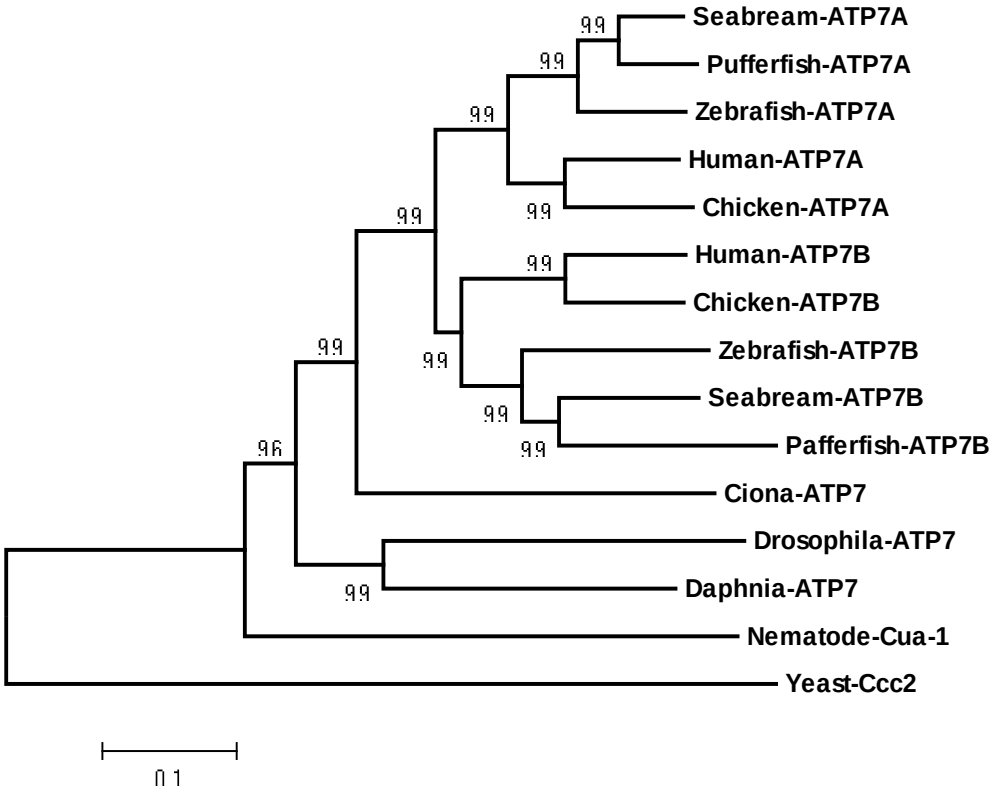

Figure 3: Cu-ATPases phylogeny tree. Human ATP7A (NM_000052) and ATP7B (NM_000053) chick (*Gallus gallus*) ATP7A (XM_420307) and ATP7B (XM_417073), zebrafish ATP7A (*Danio rerio*, Ensemble Chr14 ENSDARG00000003699, NP_001036185) and ATP7B (Ensemble Chr6 ID: ENSDART00000030246) pufferfish ATP7A (*Tetraodon negroviridis*) (Ensemble Chr1 GSTENG00017010001**)** and ATP7B (Ensemble Chr3 GSTENG00020077001), sea bream (*Sparus aurata*) ATP7A (GQ200817) and ATP7B (GQ200818), sea squirt (*Ciona intestinalis*) (Ensembl-Chr14q ENSCING00000007245), fruit fly (*Drosophila melanogaster*) ATP7 (AE014298), Daphnia (*Daphnia pulex*) ATP7 (Daphnia pulex v1.0**,** PASA_GEN_0100302), nematode (*Caenorhabditis elegans*) Cua-1 (NM_067377), and yeast Ccc2 (*Saccharomyces cerevisiae*) (L35270), were used to generate the phylogenetic tree using ClustalW. Numbers (bootstrap values) represent the percentage of times the associated branch topology was returned after 1000 iterations of tree generation.

A

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Seabream        ---MPKHEFEVAMTCEGCSGAVTRVLNKLEG--VTFEIDLPQKLVWIESDKDVEV
Pufferfish      ---MTKHEFEVAMTCEGCSGAVSRILKKLGV--ETFEIDLPKKLVWIETDKDSEV
Human           ---MPKHEFSVDMTCGGCAEAVSRVLNKLGG--VKYDIDLPNKKVCIESEHSMDT
Chicken         ---MPKHEFFVDMTCEGCSNAVTRVLHRLGG--VQFDIDLPNKKVYIESEHNVDT
Frog            ---MSKKEFFVDMTCEGCANAVNRVLSRLEG--VQYEIDLPNKKVVTESDLSVDL
FruitFly        ---MTVHEFKVEMTCGGCASAVERVLGKLGDKVEKVNINLEDRTVSVTSNLSSDE
Yeast           MAEIKHYQFNVVMTCSGCSGAVNKVLTKLEPDVSKIDISLEKQLVDVYTTLPYDF
                            ******
                           MBD

Seabream        LMQTLQKCGKEVKYNGTK- 68
Pufferfish      LMEALKKSGKEVKYNGTK- 68
Human           LLATLKKTGKTVSYLGLE- 68
Chicken         LLETLKKTGKSASYLGEK- 68
Frog            LLETLKKTGKEAKYLGCK- 68
FruitFly        LMEQLRKTGKSTTYVGVKK 71
Yeast           ILEKIKKTGKEVR-SGKQL 73
                     *****
                    NLS
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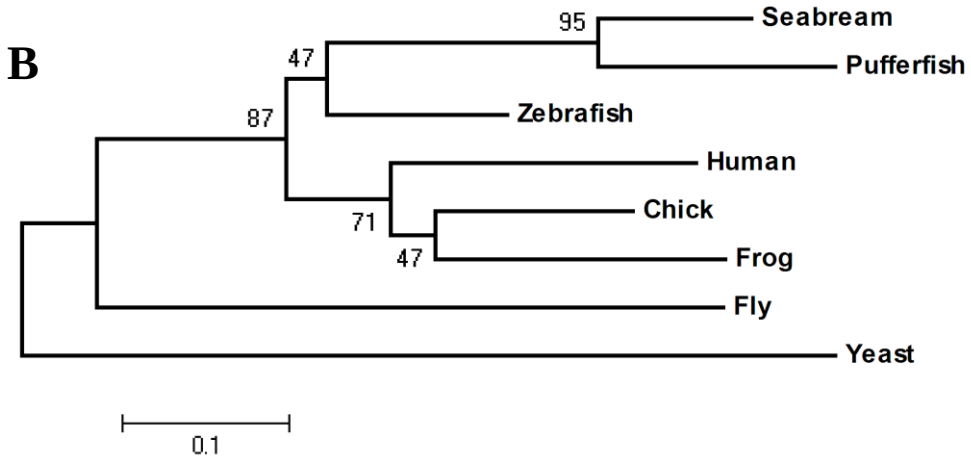

Figure 4. ATOX1 structural features. **A**: ATOX1 sequence alignment. Residues identical in all proteins are shaded. The metal binding domain (MBD) is indicated. The residues shown to be required for the activity of yeast ATX1 (Banci et al., 2007a) are boxed. **B**: Atox1 phylogenetic tree. Human (NM 004045), chick (XM 001233562), frog (*Xenopus tropicalis*, NM 001045773), fruit fly (NM_168932), yeast (L35270), zebrafish (XM 679218), puffer fish (Ensemble-Chr1 scaf14742) and the sea bream ATOX1 (AJ966735) were used to generate the phylogenetic tree using ClustalW. Numbers (bootstrap values) represent the percentage of times the associated branch topology was returned after 1000 iterations of tree generation.

3.2 Tissue distribution of Cu-ATPases and ATOX1

Expression profiles of Cu-ATPases and ATOX1 mRNAs are reported in Figure 5. SaATP7A mRNA was expressed at lower levels in all tissues, compared to ATP7B and ATOX1. For ATP7A, the highest level of expression was found in the brain, whilst kidney, gill, intestine, heart, white and red muscle showed similar levels which were between 2.3 -(kidney) and 4.5- (white muscle) fold lower then brain levels. ATP7A expression in the liver was low with mRNA levels 23.8- fold lower that in the brain and between 10- and 5-fold lower than kidney and white muscle respectively.
Sea bream ATP7B mRNA expression was highest in the intestine and was between 4 -and 200- fold higher than the liver and the heart respectively. Following the intestine, the liver showed the second highest expression, with ATP7B mRNA expression levels between 14- and 54-fold higher than white muscle and heart respectively. ATP7B in the kidney and brain was expressed at similar levels, about one third lower than the liver and between 19- and 5- fold higher than the heart and white muscle respectively.
Sea bream ATOX1 mRNA was expressed at similar levels in the kidney and brain, levels which were between 3- to 13.4- fold higher then those in the gill, intestine, liver, white and red muscle. Moreover ATOX1 expression was 1.8-fold higher in the kidney than in the heart.

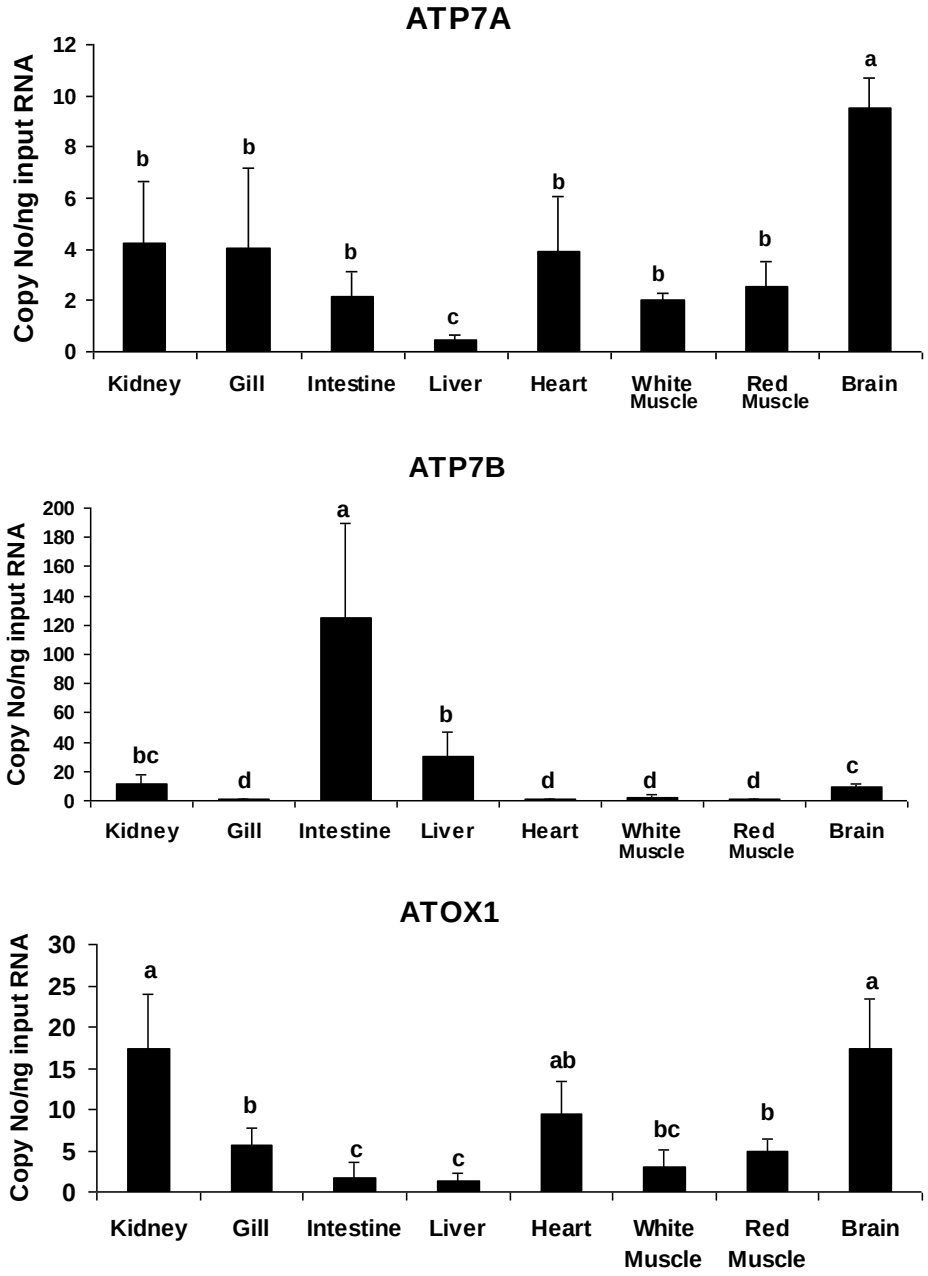

Figure 5. ATP7A, ATP7B and Atox1 tissue expression profile. Messenger RNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different (P<0.05; ANOVA, Tukeys test).

3.3 Effect of excess dietary and waterborne copper on ATP7A, ATP7B and ATOX1 expression

The effects of excess dietary (130 versus 7.7 mg Kg^{-1} Cu) or waterborne (0.3 versus < 0.005 mg L^{-1} Cu) Cu exposure on tissue Cu concentrations and expression of Cu-transporter gene expression after 15 and 30 days exposure in sea bream tissues are shown in Figure 6. Elevated Cu in the diet resulted in a reduction of intestinal (up to 8.5-fold), renal (up to 23 -fold), branchial (up to 5-fold) and hepatic (up to 4.6 -fold) ATP7A at both 15 and 30 d of treatment. Waterborne Cu exposure in fish fed the low Cu diet (7.7 mg Kg^{-1} Cu) resulted in an increase of intestinal (up to 43.5 -fold) and

hepatic (up to 4 -fold) ATP7A compared to unexposed fish. In contrast after waterborne Cu exposure, ATP7A expression decreased in the gill (at 15 d; 5- fold) and kidney (up to 3.4 -fold). ATP7B mRNA expression was affected by Cu exposure only in the liver. In fish fed the high Cu diet hepatic ATP7B was 3.7 -fold higher at 15 d than in low Cu diet fed fish, although there were no differences after 30 days. Waterborne Cu in fish fed the low Cu diet resulted in an increase of hepatic ATP7B mRNA at 15 d and 30 d (up to 5- fold).

ATOX1 expression was reduced by dietary Cu in the liver at 15 d (2.2-fold). Conversely, waterborne Cu reduced ATOX1 expression in the gill (30 d; 2.4 fold), kidney (30 d; 2.6- fold) and liver (up to 3- fold). Moreover, fish fed the low Cu diet showed a decrease in ATOX1 mRNA level in the intestine at 30 d.

3.4 Tissue copper, metallothionein and glutathione reductase mRNA levels

The levels of copper, MT and GR mRNA levels in the tissues of the fish used in this experiment have been reported in detail previously (Minghetti et al., 2008). Briefly, dietary excess copper increased copper levels in intestine and liver, after 30 days but not after 15, and not in gill at either time point. Waterborne excess copper increased copper levels in gill at 15 and 30 days and in liver after 15 days but not after 30, and not in intestine at either time point. Increases in tissue copper are indicated in Figure 6. Markers of toxicity, MT and GR mRNA, were increased in liver and gill after waterborne exposure but not after dietary exposure.

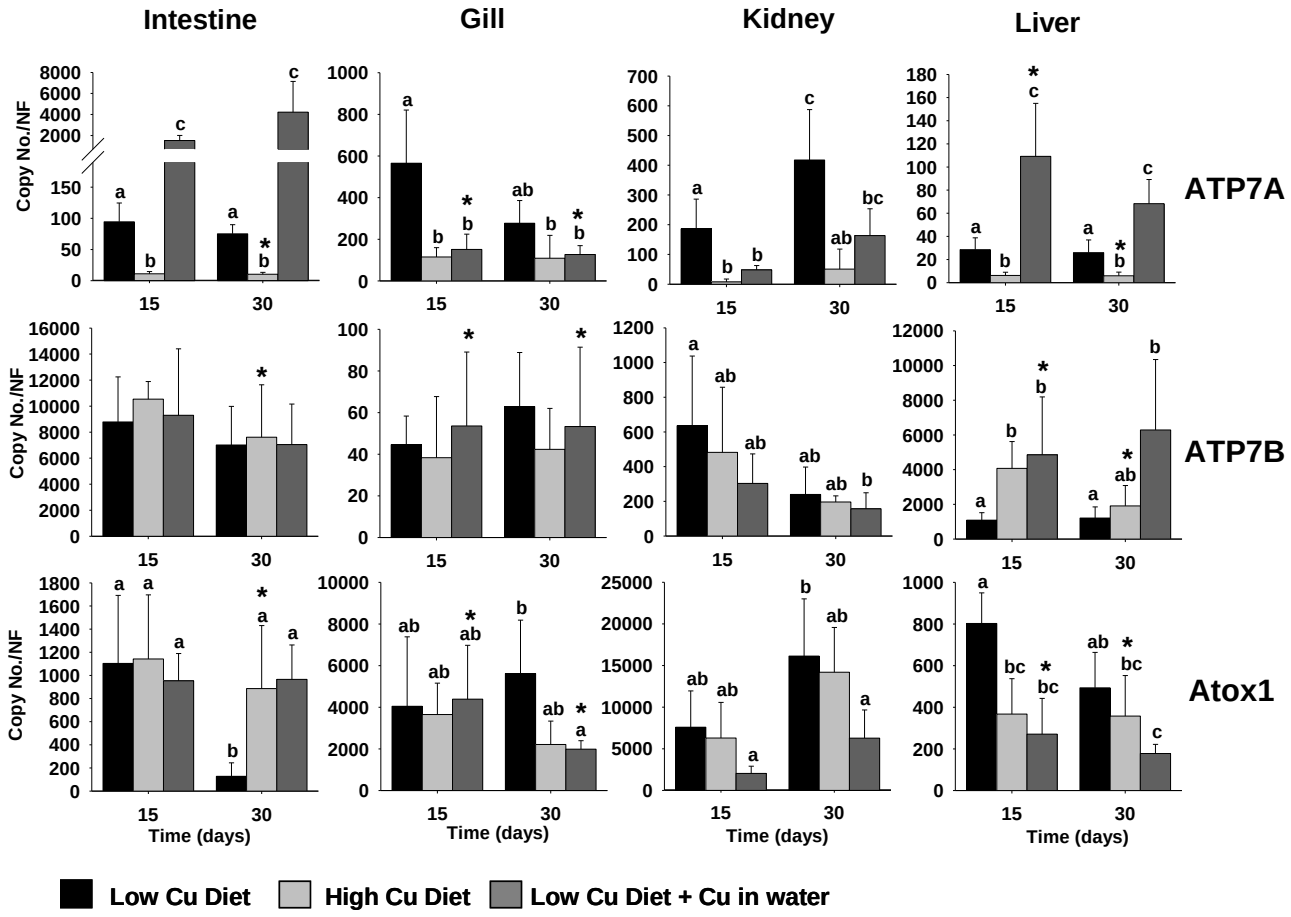

Figure 6. ATP7A, ATP7B and Atox1 expression in sea bream tissues exposed to either dietary (high Cu diet; 130 mg Kg^{-1} Cu) or waterborne Cu (low Cu diet + 0.3 mg L^{-1} Cu). Copy numbers were normalized by geNorm using a normalization factor (NF) based on the geometric mean of β-actin, EF1α or GAPDH reference genes. Values are means ± S.D. N=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test). Stars (*) indicate tissue Cu accumulation (metal concentration not analysed in the kidney) (Minghetti et al., 2008).

4. Discussion

To enable a better understanding of Cu homeostasis in fish we have investigated the expression of genes coding for Cu-transporters in the gilthead sea bream (*Sparus aurata*). We have shown for the first time in a lower vertebrate that homologues of the Cu-ATPases, ATP7A and ATP7B and the chaperone responsible for delivering Cu to these proteins, ATOX1, are expressed. Moreover expression of mRNAs for these proteins is modulated by chronic Cu exposure, in a manner dependent on route of exposure.

4.1 Evolutionary implications

The Cu-ATPases are key proteins in the delivery of Cu to cuproproteins and maintenance of Cu homeostasis, and homologues can be identified in all Phyla from yeast to man (Figure 1). Most notably, monocellular eukaryotes such as yeast (*S. cerevisiae*) and lower animals including arthropods (*D. melanogaster, D. pulex*), nematodes (*C. elegans*) and urochordates (*C. intestinalis*)

have only one isoform of Cu-ATPase whilst tetrapods possess two isoforms of Cu-ATPases, ATP7A and ATP7B. Here we report for the first time ATP7A and ATP7B in teleosts, indicating that their evolutionary origins predate the divergence of lines leading to fish and mammals. In evolutionary history there have been a number of notable whole genome duplications (WGD), and following WGD, duplicated genes can be retained or lost in a lineage dependent manner while a small percentage of the duplicated genes may also undergo sub-functionalization or neo-functionalization to generate new phenotypes on which natural selection can act (Brunet et al., 2006). One of the most significant WGDs occurring in a vertebrate ancestor is hypothesized to have influenced vertebrate evolution and driven their evolutionary success (Dehal and Boore 2005). The Cu-ATPases may provide an interesting example of a functional diversification of genes which were duplicated at the base of the vertebrate lineage. This diversification of function may reflect the dramatic differences in anatomy and physiology of vertebrates compared to invertebrates. Vertebrates have a closed circulatory system, an enterohepatic blood vessel and a hepato-biliary system, which when combined with the tissue specific expression and neo-functionalization of Cu-ATPase genes and their products would enable a finer control of Cu homeostasis. Thus, in *Drosophila*, the single ATP7 has been shown to have a similar role to mammalian ATP7A in intestinal and cellular Cu absorption (Burke et al., 2008). However, *Drosophila* and other arthropods have different mechanisms of Cu excretion to mammals and the role of ATP7, if any is not clear. In fact *Drosophila* and other invertebrates seem to permanently accumulate Cu in insoluble granules and control Cu homeostasis mainly by regulating Cu absorption (Schofield et al., 1997; George and Viarengo 1985). In contrast, vertebrates excrete excess Cu through the hepato-biliary system and this is mediated by the activity of ATP7B; indeed this is known to be the main function of ATP7B in mammals (Lutsenko et al., 2007a). Therefore the duplication and subsequent neo-functionalization of Cu-ATPases genes are an example of an evolutionary advance in Cu homeostasis facilitated by gene duplication as a result of WGD.

4.2 Structure-function comparison

In vertebrates, this neo-functionalisation of the duplicated Cu-ATPase genes accompanying the development of closed circulatory, enterohepatic and hepato-biliary systems is reflected and facilitated by differences in their structures, which enables further control of function by regulation of trafficking of the proteins between different cellular membranes. During Cu transport ATP7A is targeted towards the basolateral membrane of polarized cells (Petris and Mercer 1999). In contrast, ATP7B is targeted to the apical membrane of polarized cells such as enterocytes and hepatocytes. This process is dependent on a motif which is present in ATP7B but not in ATP7A (Guo et al., 2005; Braiterman et al., 2009). These motifs are also conserved in chicken, frog and fish ATP7Bs

(including saATP7B; Figure 1, Figure 2A) but not in other vertebrate ATP7As or in invertebrate Cu-ATPase proteins.

Evolutionary elaboration of Cu-homeostasis may also be evident from other structural distinctions between the Cu-ATPase isoforms, notably by consideration of the number of metal binding domains (MBDs) in various Cu-ATPases enzymes. The role of the MBDs in both ATP7A and ATP7B has been investigated in several mammalian studies by structural (Banci et al., 2004; Achila et al., 2006; DiDonato et al., 2000) and functional (Mercer et al., 2003; Cater et al., 2004) approaches. Not all the MBDs have the same role. MBD5 and/or MBD6 coordinate Cu and deliver it to the CPC (cys-pro-cys) domain, located in the sixth trans-membrane domain, and is required for Cu transport across the membrane (Forbes and Cox 1998). The CPC domain is conserved in all the Cu-ATPases, from yeast, through teleosts to mammals. In contrast MBD 1-4 in mammalian ATP7A and ATP7B seem to have a regulatory auto-inhibitory role as deletion of these domains increases hydrolysis of ATP suggesting an involvement in the regulation of catalytic turnover (Huster and Lutsenko 2003). Guo et al (2005) proposed that these MBDs sense the intracellular Cu concentration and regulate the activity of the protein accordingly. Thus, the number of MBDs in a Cu-ATPase may 'fine-tune' Cu transport. In fish ATP7A has six MBDs, as in mammals, whilst the ATP7B's of the majority of fish including sea bream, Fugu, Medaka , Puffer and Stickleback only have four MBDs. . Thus, it is possible that the numbers of MBDs at the N-terminus of the protein have increased from yeast (2 MBDs) worm and fly (4MBDs), the sea squirt (5 MBDs), fish (6 MBDs in ATP7A and 4-5 in ATP7B) and then to mammals (6 MBDs in both ATP7A and ATP7B) under a selective pressure to acquire finer intracellular tuning of Cu homeostasis.

4.3 Tissue expression profile

In mammals further evidence for functional diversity of ATP7A and ATP7B is apparent from their differential tissue expression patterns. In adult animals very low levels of ATP7A are found in the liver, although it is ubiquitously expressed during development, whereas, ATP7B expression is more delimited and high levels of expression are only found in liver and intestine (Kuo et al., 1997). In sea bream the expression profiles of ATP7A and ATP7B are very similar to those in mammals (Figure 5). The distribution of ATP7A is reflective of its role in delivery of Cu to cuproenzymes eg. peptidyl-α-monooxygenase (El Meskini et al., 2003), tyrosinase (Petris et al., 2000) and lysyl oxidase (Tchaparian et al., 2000). The high level of hepatic ATP7B expression in sea bream is in accord with its known functions in mammals, namely delivery of Cu to ceruloplasmin, a serum ferroxidase which mediates the release of iron from the liver and other tissues (Terada et al., 1998), and its role in the biliary excretion of Cu. Biliary excretion is also the main excretory route for excess Cu in fish, renal excretion being minimal (Grosell et al., 1998). On the other hand, the high

level of expression of ATP7B in the intestine, of both sea bream and mammals has not been satisfactorily explained, since it does not deliver Cu to ceruloplasmin in this tissue. Evidence from Menkes and Wilson diseases indicates that in tissues where ATP7A and ATP7B are co-expressed they have distinct non-overlapping roles since it has been demonstrated that one functionally active ATPase cannot compensate for lack of the other (Kuo et al., 1997; Linz and Lutsenko 2007; Barnes et al., 2005). Thus, even though ATP7B is functionally active in Menkes disease (ATP7A-deficient) patients, Cu still accumulates in the intestine. Possible intestinal roles have been suggested in Cu sequestration (Kaplan and Lutsenko 2009) or in the delivery of Cu to a serum carrier or another cupro-enzyme, such as the Cu-dependent intra-membrane ferroxidase, hephaestin which has a functional role in intestinal iron transport (Wessling-Resnick 2006).

4.4 Effects of copper exposure

In this study, the effect of excess Cu on Cu-ATPase mRNA expression during two alternative routes of exposure, diet and water, was measured. Copper absorption occurs primarily in the small intestine in mammals (Linder 1991) and in the mid posterior region of the gut in fish (Handy et al., 2000). The efficiency of Cu uptake has been demonstrated to be directly related to dietary Cu status; when Cu status is low, Cu absorption is enhanced, whilst when Cu status is high, Cu absorption is repressed (Turnlund 1998; Clearwater et al., 2000; Handy et al., 2000). However, the molecular mechanisms by which these physiological effects are mediated are unknown. Present evidence from mammals suggests that Cu absorption involves both passive diffusion at the intestinal brush border and active transport at the basolateral membrane, with the latter being the limiting step (Arredondo et al., 2000). The protein or mechanism responsible for apical Cu uptake is not clear (Zimnicka et al., 2007), although it has been suggested that low affinity transport of Cu^{1+} is mediated by the non-specific divalent metal ion transporter DMT-1 (Arredondo et al., 2003). Interestingly, when Caco-2 cells are exposed to high Cu levels, DMT1 protein and mRNA expression are markedly decreased (Tennant et al. 2002), similar to the response of ATP7 to excess copper in sea bream intestine shown here. At the basolateral membrane Cu transport from the enterocyte to the blood has been attributed to ATP7A (Voskoboinik and Camakaris 2002) while recent evidence suggest that blood-borne Cu is absorbed by the enterocyte through the high affinity transporter, CTR1 (Zimnicka et al., 2007) (Figure 7). Importantly in fish, there is physiological evidence for ATP-mediated basolateral membrane transport of Cu in both the gill (Bury et al., 1999; Campbell et al., 1999) and intestine (Bury et al., 2003; Handy et al., 2000). Since ATP7A but not ATP7B is expressed in both sea bream intestine and gill, ATP7A is implicated as the probable candidate for basolateral Cu transport in fish.

In mammals, ATP7A function is thought to be regulated mainly at the post-translational level by alterations in membrane trafficking. Under acute conditions of elevated Cu, it has been shown that there is a change in the subcellular localization of the protein from the trans golgi network to the cell periphery (Nyasae et al., 2007). However, in a chronic situation, suckling rats fed a diet containing moderate levels of Cu for 20 days, an increased mRNA level of ATP7A in intestine and ATP7B in the liver was observed (Bauerly et al 2004), inferring transcriptional regulation. In a recent study in zebrafish, Craig et al. (2009) reported an increased expression of intestinal and liver ATP7A mRNA after exposure to 8 μg L^{-1} Cu in the water, a finding confirmed for a seawater fish in the present study. In both intestine and liver of sea bream (Minghetti et al., 2008) and zebrafish (Craig et al., 2009) expression of CTR1 mRNA is also increased by waterborne Cu exposure. In the present study, similar to suckling rats, we also observed induction of hepatic ATP7B expression in sea bream after Cu exposure. Thus, expression of the Cu transporters, CTR1, ATP7A and ATP7B might also be transcriptionally regulated by Cu exposure in fish.

Compared to fish fed the control low Cu diet, in fish fed the high Cu diet there was a reduction of ATP7A mRNA expression in all tissues analyzed after 30 days, which was most marked in intestine (9-fold). This was accompanied by a 50% elevation in intestinal and liver Cu levels. Previous studies have demonstrated accumulation of Cu in intestine, liver and gill after feeding a diet containing 1500-2000 mg Cu Kg^{-1} dry diet in African walking catfish (*Clarias gariepinus*) (Hoyle et al., 2007) and Nile tilapia (*Oreochromis niloticus*) (Shaw and Handy 2006). The accumulation of Cu in enterocytes may therefore be explained by a lack of ATP7A-dependent basolateral efflux of Cu, caused by a decrease in ATP7A expression. In sea bream fed the high Cu diet accumulation of Cu occurred after 30 days in the liver and hepatic ATP7B mRNA expression was elevated by 3-fold after 15 days. Excess Cu is excreted via the bile in fish (Grosell et al., 1998) and biliary excretion is ATP7B-dependent in mammals (Schaefer et al., 1999). Our results together with increase in ATP7B mRNA reported in suckling rats fed elevated Cu (Bauerly et al., 2004) suggest that hepatic ATP7B may also be transcriptionally regulated in a chronic response to dietary Cu-overload. The predicted consequence would be an increase in excreted biliary Cu complex and detoxification as such complexes are less easily reabsorbed in the gut (Linder et al., 1998).

The effects of waterborne Cu exposure on Cu-transporter gene expression were significantly different from those observed under conditions of excess dietary Cu-exposure and are more difficult to interpret since they show tissue dependent differences. It is well documented that fish can also absorb dissolved Cu from the water (Handy et al., 2002; Grosell and Wood 2002) and in trout exposed to 22 μg Cu L^{-1}, Cu appeared in the plasma within 3h of exposure (Kamunde et al., 2002). High levels of waterborne Cu are highly toxic for fish, especially in fresh water (Lauren and McDonald 1985; Li et al., 1998) and although this is not generally a problem in marine fish due to

the insolubility of Cu ions, it can become a problem due to anthropogenic activities (Morillo et al., 2005). ATP7B mRNA was expressed at negligible levels in the gill whilst ATP7A mRNA expression was reduced by 4-fold by waterborne Cu. Cu accumulated in the gills and liver and under these conditions both metallothionein and glutathione reductase are induced, reflective of an oxidative stress response due to Cu toxicity (Minghetti et al., 2008). Thus, any reductions in ATP7A activity resulting from reduced gill mRNA expression were not sufficient to prevent Cu entry to the body.

In intestine after waterborne exposure there was substantial 40–fold induction of ATP7A mRNA expression and there was no accumulation of Cu or an oxidative stress response. In addition, we previously reported a 3-fold induction of CTR1 under these conditions (Minghetti et al., 2008). These results indicate that the effects of Cu were unlikely to have been elicited through uptake of Cu by drinking, since this would involve exposure via the gut and would be expected to produce similar effects to dietary treatment. Induction of ATP7A and CTR1 mRNA's after exposure to waterborne Cu has also been reported in zebrafish intestine (Craig et al., 2009).

Based on the different effects of dietary and waterborne Cu on CTR1 expression in sea bream, we previously hypothesized that the form in which Cu is delivered to the liver and other tissues must differ in some way between intestinal- and gill-derived Cu (Minghetti et al., 2008). Our results show that the liver appears be able to cope with a high level of dietary Cu since Cu arriving from the intestine via the hepatic portal system does not induce biomarkers of toxicity (MT and GR) (Minghetti et al., 2008). Upon exposure to waterborne Cu, the metal is presented to the liver from both hepatic portal and arterial blood and whilst we observed a modest induction of both hepatic ATP7A and ATP7B, toxicity was apparent from the oxidative stress responses of MT and GR. In mammals, Cu uptake is entirely from the diet via the intestine, after which it enters the hepatic portal vein and progresses to the liver where it mixes with arterial blood and is taken up by hepatocytes. The nature of the Cu carrier from the intestine is not clear, the main candidate is transcuprein, an α2-macroglobulin of 190-kDa (Liu et al., 2007). We contend that the difference in effect of Cu from gill uptake may be attributable to the nature of the plasma Cu-complex, most probably as Cu-histidine or Cu-albumin (Lui et al., 2007), rather than a specific enterocyte-derived transporter. Thus the normal mode of entry may be bypassed, resulting in the observed induction of metal and oxidative stress after water borne exposure. This non-specific chelation of Cu after water borne exposure may also explain the unexpected "anti-homeostatic" response (increased CTR1 and ATP7A mRNA levels) we observed in the sea bream intestine (Minghetti et al., 2008) (Figure 6) and also reported in zebrafish (Craig et al., 2009). The increase in intestinal CTR1 and ATP7A mRNA may be induced by exposure to Cu present in the blood stream in a free or non-specifically bound form. Thus, this response could be part of a defense mechanism to increase the rate of

intestinal Cu uptake and detoxification through incorporation into a specific Cu serum carrier, by ATP7A in the same way that dietary Cu is transported to liver. This mechanism would allow the delivery of Cu into the portal vein in a form that subsequently could be metabolized and excreted by the liver. Therefore the gut, like the liver, might be considered an organ of accumulation and detoxification.

The kidney is an organ which has a central role in body Cu homeostasis (Linder et al., 1998). In mammals, kidneys regulate their Cu content more efficiently than many other organs in pathologic conditions of Cu deficiency or excess by the action of ATP7A and ATP7B which are both required for normal function (Linz et al., 2007). In sea bream while renal ATP7A was reduced by waterborne Cu, while under the same conditions CTR1 was increased by waterborne Cu (2.6-6 fold up) (Minghetti et al., 2008) . In fish, as in mammals, the normal role of the kidney is of Cu-reabsorption since urinary excretion is negligible, but significantly Cu accumulates in this tissue after excess to excess (Grosell et al., 1997; Grosell et al., 2003). This might suggest that the kidney could function as an organ of Cu defense through accumulation in conditions of excess Cu.

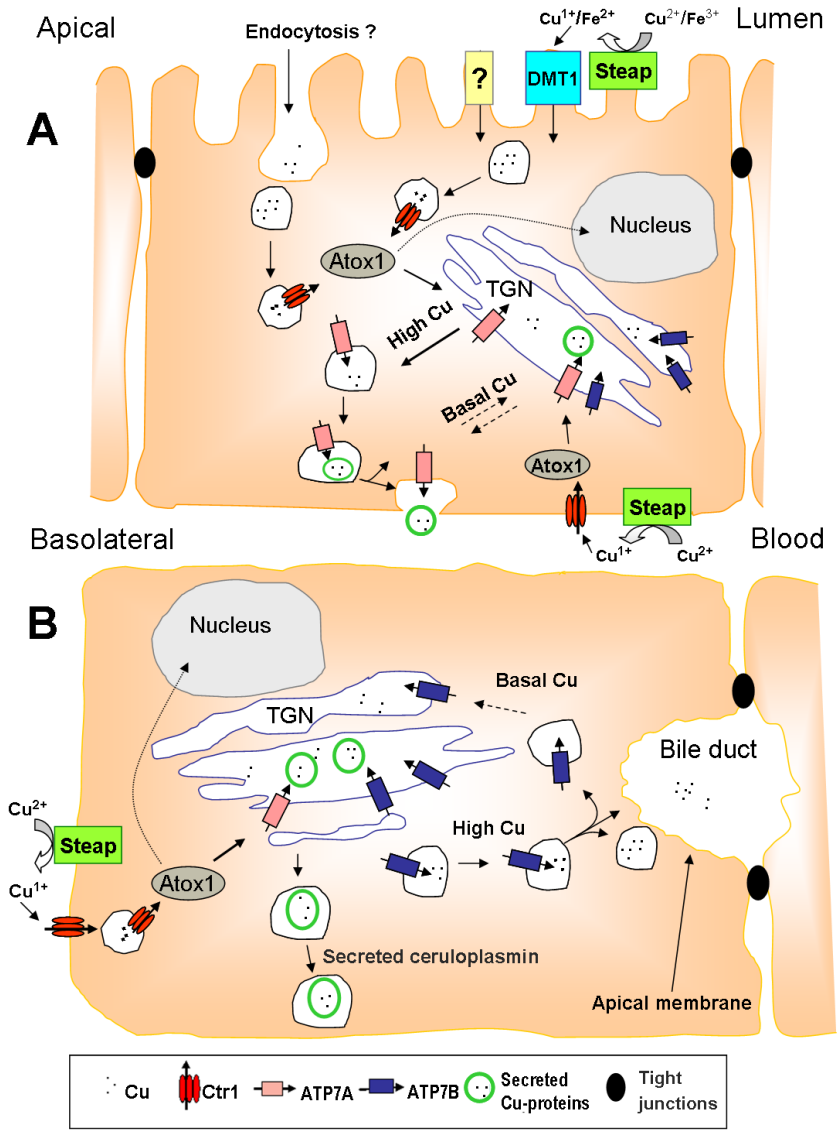

Figure 7. Proposed model of copper transporters trafficking and their intracellular localization in enterocytes (A) and hepatocytes (B). Copper enters via CTR1 at the basolateral membrane in all cells or via DMT1, endocytosis or other unidentified transporters at the apical membrane in enterocytes. Previous entry via CTR1 and DMT1 Cu is reduced by the metalloreductase Steap. After entry Cu is bound to the chaperone ATOX1 which has recently been shown to act as a transcription factor together with its initially identified function to deliver Cu to the Cu-ATPases located at the TGN. ATP7A and ATP7B may then deliver Cu to cupro-enzymes and secretory Cu-proteins. Under basal Cu condition (dotted arrows), ATP7A cycles between the TGN and the basolateral membrane in enterocytes while ATP7B is located at the TGN. When intracellular copper levels increase (solid arrows), in the enterocyte, ATP7A is sorted into vesicles that move toward the basolateral membrane and Cu-loaded vesicles may fuse with the membrane to release copper incorporated into secretory proteins by exocytosis. Conversely with elevated Cu, in the hepatocyte, ATP7B sequester Cu into vesicles that move toward the apical membrane (or canalicular membrane) where vesicles may fuse with this membrane and release Cu, empty vesicles containing ATP7B would then be recycled trafficking back to the TGN (Adapted from La Fontaine and Mercer, (2007)).

4.5 Conclusions

In summary, in this study we report for the first time that fish possess two isoforms of Cu-ATPases which are homologues of the human ATP7A and ATP7B, and structural consideration together with basal tissue expression levels suggest similar functional roles in teleosts and tetrapods. Phylogenetic analysis of diverse Cu-ATPases suggest that the two vertebrate genes arose and neo-functionalized from a single ancestral gene at a similar time to the evolutionary appearance of a closed blood circulation, an enterohepatic blood vessel and a hepato-biliary system. The expression of sea bream Cu-ATPase (saATP7A and saATP7B) mRNAs are consistent with the available physiological evidence from various fish species for the involvement of ATP dependent (ATP7A-B-like) Cu transporters under conditions of both normal and excess Cu exposure. The alterations in expression of these genes, when fish are exposed chronically to excesses of dietary or waterborne Cu, show that they may under Cu-dependent transcriptional regulation and that the route of exposure is critical to maintenance of Cu homeostasis and the manifestation of toxic effects.

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