CHARACTERISATION AND EXPRESSION OF COPPER HOMEOSTASIS GENES IN SEA BREAM (*Sparus aurata*)

A THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

by

MATTEO MINGHETTI

INSTITUTE OF AQUACULTURE, UNIVERSITY OF STIRLING, STIRLING, SCOTLAND

JANUARY 2009

to Frances

and

to my parents

The Myth of Sisyphus

… At that subtle moment when man glances backward over his life, Sisyphus returning toward his rock, in that slight pivoting he contemplates that series of unrelated actions which become his fate, created by him, combined under his memory's eye and soon sealed by his death. Thus, convinced of the wholly human origin of all that is human, a blind man eager to see who knows that the night has no end, he is still on the go. The rock is still rolling.

I leave Sisyphus at the foot of the mountain! One always finds one's burden again. But Sisyphus teaches the higher fidelity that negates the gods and raises rocks. He too concludes that all is well. This universe henceforth without a master seems to him neither sterile nor futile. Each atom of that stone, each mineral flake of that night-filled mountain, in itself forms a world. The struggle itself toward the heights is enough to fill a man's heart. One must imagine Sisyphus happy.

Albert Camus

Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged the work described in this thesis is the result of my own investigations.

Signed

Matteo Minghetti

Date

Acknowledgements

If I am here now, writing this page, then there are many people I need to thank!

I would like to thank my supervisors Stephen George and Micheal Leaver for their steady guidance and also for their sometimes harsh criticisms which have helped shape my attitudes towards research and made this PhD what it is.

Many thanks to Emilio Carpenè for his support, without which, this PhD would not have been possible. Thanks also to Giulia Andreani, Marta Monari, Michaela Fabbri and Roberta Gatta for their kind help during the sampling procedure and to Giovanni Vitali for his assistance in setting up the aquarium system. A big thank you is also due to Elisa Casadei for her help with sampling, cytotoxicity assay of cell cultures and for her support throughout this PhD.

Thanks to Amer Diab for his generous help in introducing me to the laboratory and for his good counsel. Thanks to John Taggart for his expert help setting up the microarray experiment and to James Bron for his help during the bioinformatic analysis. Thanks to Ann Gillmor and Catheryn Dixon for always being available to help solve any technical or practical issues. Thanks to Matthew Sprague for his help with data analysis and for his support. I would like to thank all of my fellow PhD colleagues here at the Institute of Aquaculture. I may not have space to name them all, but their sense of co-operation and collaboration undoubtedly makes the success of this institute possible.

Many thanks to my fiancée, Frances for taking the time to proof-read this thesis and above all for her encouragement, understanding and ability to look forward.

Finally, my gratitude goes to my parents, Daniela and Tiziano. Their constant encouragement, financial support and belief in me and the path I have chosen, even if it has lead away from home and family, is a constant source of strength to me and I thank them for it.

Abstract

The redox properties of Copper (Cu) make it both an ideal cofactor for many enzymes, and, in its free form, a highly toxic molecule capable of stimulating production of reactive oxygen species or binding to protein thiol groups. Therefore, living organisms have evolved homeostatic systems to "handle" Cu avoiding dangerous and wasteful aspecific interactions. These systems comprise uptake, carrier, storage and excretion proteins. The importance of Cu-homeostatic systems was initially discovered in humans where alterations of Cu-excretory proteins were shown to be responsible for two lethal genetic disorders; the Wilson and Menkes diseases. The levels of bioavailable Cu in the aquatic environment is important because concentrations in oceanic waters tend to be minute, whilst in some fresh and coastal waters, particularly around areas of mineral extraction, viniculture and farming operations, concentrations can be excessive. In contrast to terrestrial vertebrates, fish are not only exposed to dietary sources of copper but are also exposed to dissolved ionic copper that may enter via the skin and gills. Indeed, the latter route is important in fish and it has been demonstrated in physiological studies that under conditions of dietary deficiency, fish can satisfy their own body requirements by uptake from water. Therefore, fish must have systems relating to both gill and gut to enable maintenance of body homeostasis of this essential, yet toxic, metal.

In an attempt to understand the mechanisms of Cu homeostasis in fish, whether under conditions of deficiency, adequacy or excess, it is essential to consider the expression of known Cu-homeostasis proteins. Thus, cDNAs for sea bream (*Sparus aurata*) homologues of copper transporter 1 (Ctr1), antioxidant protein 1 (Atox1), Menkes protein (ATP7A), Wilson protein (ATP7B), and metallothionein (MT), which are responsible for the uptake, delivery to the secretory pathway and scavenging of

intracellular Cu, were cloned and their mRNA tissue expression levels measured. To investigate the molecular basis of the different homeostatic and toxic responses to waterborne or dietary Cu, sea bream were exposed to sub-toxic levels of Cu in the diet (130 mg/Kg of dry diet) or water (0.3 mg/L) and tissue mRNA and Cu levels were measured. Moreover, to discriminate between the effect of different metals on the transcriptional regulation of Cu homeostasis genes in fish, *Sparus aurata* fibroblast (SAF1) cells were exposed to sub-toxic levels of Cu (25 μM), Zn (100 μM) and Cd (10 μM). In addition, a microarray was used to gain a broader overview of the transcriptional response of SAF1 cells to Cu (25 μM).

Waterborne or dietary Cu resulted in distinct expression profiles of Cu-homeostasis genes and markers of oxidative stress. After dietary exposure, Cu increased in intestine and liver, whilst after waterborne exposure Cu increased in gill and liver. Exposure to dietary Cu resulted in decreases in Ctr1 and ATP7A mRNA in both liver and intestine. Renal Ctr1 levels remained unchanged, whilst ATP7A mRNA decreased. In contrast, waterborne Cu exposure increased intestinal Ctr1 and ATP7A mRNA, and increased renal Ctr1 and decreased renal ATP7A mRNA. Both dietary and waterborne Cu increased ATP7B mRNA in liver. Metallothionein (MT) mRNA increased in liver and gill after waterborne Cu. Glutathione reductase (GR), a marker of oxidative stress, increased expression in liver and gill after waterborne Cu exposure, but decreased in intestine.

Thus, exposure to Cu via water or diet has different, often opposite effects on Cu-homeostasis genes. The decrease in expression of both Cu-transport genes in intestine after dietary exposure may indicate a defensive mechanism to limit uptake of Cu. The opposite effects in intestine after waterborne exposure are more difficult to explain, but again may reflect a defence mechanism against excess bloodborne Cu coming from the

gill. Since both dietary and waterborne Cu increased Cu levels in liver and increased hepatic ATP7B it is likely that well-characterised mammalian route of Cu excretion to bile is active in sea bream. However, only hepatic Cu derived from gill increased the expression of the stress markers MT and GR. This suggests that Cu is delivered to liver in a different form from gill as that from intestine, the intestinally derived pool being less toxic. Thus the increase in copper transport gene expression in intestine after gill exposure might be a mechanism to enable incorporation of excess bloodborne Cu into the intestinal pathway of Cu delivery to liver, thus minimizing toxicity.

The *in vitro* exposure of SAF1 cells to Cu showed a similar response to liver of fish exposed to waterborne Cu indicating similar Cu availability and complexation. ATP7A mRNA levels were induced by Cu but not by Zn or Cd suggesting Cu-specific regulation. Conversely, MT and GR were induced by all metals tested. The transcriptomic analysis highlighted that the biological processes most significantly affected by Cu were secretion, protein trafficking and stress.

Overall, these results show that in fish copper has distinct effects on tissue Cu transporter genes and oxidative stress depending on whether it is taken up via the gill or gut and that intestinal absorption may be required for normal uptake and metabolism of Cu, regardless of the route of uptake. Moreover, changes in mRNA levels indicate that Cu homeostasis genes, at least in fish, may be regulated at the transcriptional level. Although more work needs to be done to identify genes that are robust predictors of Cu toxicity, the microarray results presented here show a clear transcriptional fingerprint which may characterize Cu toxicity in fish.

Keywords: Copper homeostasis, Fish, *Sparus aurata*, Ctr1, ATP7A, ATP7B, MT, Microarray.

Abbreviations and Acronyms

This list contains the most frequently used abbreviated terms. All other abbreviated terms are indicated in the text.

ARE (Antioxidant Responsive Element)

Atox1 (Antioxidant protein 1)

ATP (Adenosine-5'-triphosphate)

ATP7A (Menkes protein)

ATP7B (Wilson protein)

BLAST (Basic Local Alignment Search Tool)

BSA (Bovine Serum Albumin)

CCS (Copper Chaperone for Superoxide dismutase)

COX (Cytochrome *c* Oxidase)

Cox17 (Copper Chaperone for cytochrome c oxidase)

CP (Ceruloplasmin)

CT (Copper toxicosis)

Ctr1 (High affinity copper transporter 1)

CuZn-SOD (Copper Zinc Superoxide Dismutase)

COMMD1/Murr1 (Copper metabolism domain containing 1)

DAVID (Database for Annotation, Visualization and Integrated Discovery)

DMT1 (Divalent Metal Transporter 1)

dNTP (Deoxynucleotide triphosphate)

DOM (Dissolved Organic Matter)

EF1 α (Elongation factor 1 alpha)

ENaC (Epithelial sodium channels)

EST (Expressed Sequence Tags)

FW (Fresh water)

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)

GO (Gene ontology)

GR (Glutathione reductase)

GSH (Tripeptide glutathione)

GTPase (Guanosine triphosphate hydrolyze)

MBD (Metal Binding Domain)

MD (Menkes disease)

MRE (Metal Responsive Element)

MT (Metallothionein)

MTF-1 (Metal Transcription Factor)

PBS (Phosphate-buffered saline)

PCR (Polymerase Chain Reaction)

PM (Plasma membrane)

QPCR (Quantitative reverse transcription PCR)

RACE (Rapid Amplification of cDNA Ends)

ROS (Reactive Oxygen Species)

SAF1 (S*parus aurata* Fibroblast)

SW (Sea water)

TGN (Trans Golgi Network)

TMD (Trans-membrane domains)

USF (Upstream Stimulating Factor)

UTR (Untranslated regions)

WD (Wilson's disease)

β-actin (Beta actin)

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Chapter 1. General introduction

1.1 Introduction

Copper (Cu), atomic number 29, is a transition metal which has two common valencies Cu (I) and Cu (II), known as cuprous and cupric respectively, enabling it to easily undergo changes in its oxidation state and to donate or accept electrons. This variable valency feature gives copper great biological importance making it an ideal cofactor for enzymes that require redox activity. Therefore copper is an essential trace metal required by all organisms, acting as a bound cofactor in a variety of enzymes and proteins which perform specific metabolic functions crucial for life (Table 1-1) (Linder 1991; Kim et al., 2008). On the other hand, when copper is present as a free ion, its redox property also allows copper to take part in radical reactions with oxygen that lead to the formation of highly damaging reactive oxygen species (ROS) and other reactive free radicals (eg thiyl radicals). These radicals can cause catastrophic damage to lipids, proteins and DNA (Halliwell and Gutteridge 1984) (see 1.4). Consequently, to avoid cytotoxicity, highly specific proteins for uptake, transport, storage and excretion of copper have evolved to prevent uncontrolled reactivity of the free ion. Therefore, in normal conditions, “free” copper ions are not present in the cytoplasm or in the extracellular fluids (Rae et al., 1999; Tapiero et al., 2003).

Table 1-1: Cuproenzymes and Copper-binding proteins of biological and pathological importance.

Common Name	**Biological Function**
Cytochrome *c* oxidase	Respiration; it is the last protein in the electron transport chain.
CuZn-SOD	Free radical detoxification.
Lysyl oxidase	Crosslinking of collagen and elastin.
Ceruloplasmin	Ferroxidase activity, extracellular free-radical scavenger and serum copper transport.
Tyrosinase	Melanin biosynthesis.
Dopamine β-hydroxylase	Neurological function; catecholamine production.
Angiogenin	Induction of blood vessel formation.
Clotting Factors, V, VIII	Blood clotting.
Plastocyanin	Photosynthesis; electron transfer.
Hemocyanin	Oxygen blood transport in some molluscs and arthropods.
Peptidylglycine monooxygenase	Bioactivation of peptide hormones.
Metallothionein	Bind heavy metal including copper; free radicals scavenger ?
β-amyloid precursor protein	Neuronal development?; miss-functioning in Alzheimer's disease.
Hephaestin	Intestinal iron efflux.
Prion protein	Function unknown; Copper binding properties suggests potential role in copper uptake.

The informations summarized in this table have been extracted from (Linder 1991; Kim et al., 2008).

1.2 Copper abundance in the environment

Although Cu is a relatively rare element, it has been estimated that the Earth's crust may contain a total of 15 x 10^{17} kg, of which only a small proportion is present in tillable soil and water (6.7 x 10^{12} and 5 x 10^{12} kg, respectively), and an even more minute proportion in living organisms (2.9 x 10^{10} kg in plants and 2.4 x 10^{5} kg in animals) (Linder 1991). Copper is widely distributed in nature, especially in sulphide, arsenide, chloride and carbonate deposits. Because of its properties, copper has been used by human beings

since the beginning of civilization. Copper is extracted as ore which is then processed to be commercialized as pure metal (www.copper.org). Copper becomes a pollutant where human activity, primarily in extraction procedures, releases "free" soluble Cu ions which can cause environmental damage (Nriagu et al., 1998). As a result of mining and its widespread industrial and agricultural use, Cu is one of the most common environmental pollutants (Sadiq 1992). In unpolluted soil and sediments total Cu concentration is generally found to be below 10 μg kg^{-1} (Sadiq 1992).

1.2.1 Copper in fresh and marine waters

All bodies of water that sustain life (oceans, tidal pools, lakes, rivers, and ponds) contain Cu as a vital, naturally occurring element. Levels of total dissolved Cu in fresh and marine water are generally low, but sufficient enough to sustain biological growth; in fact Cu deficiency in aquatic organisms has not been reported (Sadiq 1992). Whilst reported Cu levels in unpolluted rivers range from 0.15 μg l^{-1} [ppb] in the Manuherikia River, New Zealand to 1.4 μg l^{-1} in the Amazon River, Brazil, a world average Cu concentration in river water of 10 μg l^{-1} has been reported with higher concentrations where there are effluent discharges from industrialized cities (Kennish 2001). Similarly, the average Cu concentration in marine waters of 0.1 μg l^{-1} ranges between 0.008 μg l^{-1} (in the Framvaren Fjord, Norway), to as high as 29 μg l^{-1} (in Shark Bay, Western Australia) (Sadiq 1992). Even though the concentration of Cu in different locations varies widely, it is possible to conclude that the highest levels of dissolved Cu are found in estuarine waters and coastal sea waters near big cities or industrial sites showing that anthropogenic inputs contribute significantly to Cu contamination of the marine environment (Sadiq 1992). The Huelva estuary, in southern Spain, is a prime example of metal pollution caused by mining

activities and between 2001 and 2002 it was one of the most polluted estuaries in Europe with an average level of 97 μg l^{-1} of Cu (Morillo et al., 2005).

Once it enters the marine or fresh water system, Cu may remain dissolved in ionic form or it may bind to other inorganic or organic molecules. Furthermore it may be adsorbed to particulates which may remain in suspension or sink to the sediments. (Bjorklund and Morrison 1997; Guthrie et al., 2005). The major fraction (80-99.9 %) of total dissolved Cu is complexed to dissolved organic matter (DOM) in both fresh and marine water (Guthrie et al., 2005; Buck et al., 2007). Sediments play an important role in Cu water chemistry as ionic Cu is primarily adsorbed to calcite, clays, organic matter, and oxides and hydroxides of Fe and Mn and can be released when water Cu concentrations are low (for detailed review on sediments see Sadiq 1992).

The total dissolved Cu concentration in marine waters increases with depth, (about 100 μg l^{-1} more per 1000 m) (Boyle and Huested 1981). This phenomenon is thought to be biological. Marine plankton directly take up Cu from sea water through their cell walls thus depleting Cu in the surface layers, as organisms die they fall as detritus thus concentrations in deeper water are greater (Sadiq 1992). However, whilst the free Cu^{2+} concentration does rise below 500 m, 99 % of Cu is complexed to organic ligands up to 3000 m (Moffett and Dupont 2007).

Most Cu is complexed in the environment, however, it is the free Cu^{2+} ion which is cytotoxic and also the species which is often transported across cell membranes, therefore when assessing the potential biological risk of Cu pollution in the aquatic environment, Cu speciation should be evaluated (Bowles et al., 2006; Guthrie et al., 2005). Since ionic Cu concentrations found in the aquatic environment are generally below analytical detection limits, computer models have been developed to derive free Cu concentrations. These are

of questionable practical use due to the complexity of ”real world” situations (Guthrie et al., 2005; Buck et al., 2007) and therefore attention has been diverted to the use of sentinel species which may accumulate Cu (Morillo et al., 2005) and molecular biomarkers that could show animal exposure to Cu (George and Olsson 1994; Knapen et al., 2007).

1.3 Dietary copper requirements

The average daily intake of Cu by human adults, varies from 0.6 to 1.6 mg/d (Linder 1991; Scott and Turnlund 1994) and the main sources are seeds, grains, nuts, beans, shellfish and liver. Drinking water does not normally contribute significantly to intake. The Recommended Dietary Allowance (RDA) of Cu which prevents deficiency in human adults is 0.9 mg of Cu daily and the tolerable upper intake level (UL) which prevents overt signs of Cu toxicity is < 10 mg daily (Trumbo et al., 2001). Inadequate or excessive intake of Cu can be pathogenic and life threatening (Harris 2003). Good models for Cu deficiency and toxicity in humans are Menkes disease (MD) and Wilson’s disease (WD) respectively (see 1.5.4.1).

Copper is required for the normal growth and development of fish. Rainbow trout (*Oncorhynchus mykiss)* fed with a low-Cu diet (0.8 μg Cu g^{-1}) and kept in low-Cu water (0.37 μg Cu l^{-1}) showed a marked reduction in growth over a 50-day experimental period (Kamunde et al., 2002b) and Cu deficiency profoundly altered embryo development of zebrafish (*Danio rerio*) (Mendelsohn et al., 2006). The requirement for Cu in fish depends mainly on the physiological state of the animal, the species, the Cu content of the water and dietary level of zinc, iron, cadmium and molybdenum, which can all be metabolic antagonists of Cu (Watanabe et al., 1997; Clearwater et al., 2002; Kamunde et al., 2002b; Kamunde et al., 2002b; Sharp 2004). The optimal level of Cu in the diet, as determined for several fish, ranges from 3 to 5 mg Cu Kg^{-1} dry diet (Watanabe et al., 1997). For example,

dietary requirements for common carp (*Cyprinus carpio)* and rainbow trout are at 3 mg Cu Kg^{-1} dry diet, for channel catfish 5 mg Cu Kg^{-1} dry diet and 5 –10 mg Cu Kg^{-1} dry diet for Atlantic salmon (*Salmo salar)* parr. However in rapidly growing Atlantic salmon fry, a significantly higher requirement of 35 mg Cu Kg^{-1} dry diet has been claimed (Clearwater et al., 2002).

1.4 Copper toxicity

The great advantages of the aerobic "lifestyle" gave primordial living organisms the opportunity to benefit from more energy, since 36 molecules of adenosine-5'-triphosphate (ATP) are produced by aerobic organisms through cellular respiration vs 2 ATP produced by anaerobic organisms through fermentation. However, at the same time as reaping benefits cells had to evolve systems to cope with atmospheric oxygen toxicity as damaging **R**eactive **O**xygen **S**pecies (ROS) (O_2^-, H_2O_2, $OH^\bullet$) are the unavoidable by-products of aerobic cellular metabolism.

Oxidative stress: Oxidative stress occurs when the antioxidant enzyme detoxification and ROS production is not balanced (Ahmad 1995). To detoxify environmental and metabolic ROS the cell has evolved a series of specific proteins including Cu/Zn superoxide dismutase, catalase, glutathione peroxidase, transferase and reductase and metallothionein.

The redox activity ($Cu^{1+} \rightarrow Cu^{2+}$) of free Cu ions promotes the formation of reactive oxygen species (Halliwell and Gutteridge 1984), such as superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2):

$$Cu^{2+} + O_2^- \rightarrow Cu^+ + O_2$$

$Cu^{+} + O_2^{-} \rightarrow Cu^{2+} + O_2^{2-}$

$O_2^{-} + 2H^{+} \rightarrow H_2O_2$

Net reaction: $O_2^{-} + O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$

Copper (I) can also react with H_2O_2 to make hydroxyl radicals:

$Cu^{+} + H_2O_2 \rightarrow OH^{\bullet} + OH^{-}$

ROS have the capacity to disrupt membrane systems and become foci for peroxidation reaction affecting phospholipids of cell membranes. Due to their high reactivity with thiol groups (R-SH), they directly oxidise proteins. In addition ROS can cleave DNA and RNA. Thiol groups oxidized in the presence of Cu can generate a wide range of radicals including $OH^{\bullet}$, O_2^{-} and the thiyl radical ($RS^{\bullet}$) (Halliwell and Gutteridge 1984):

$RSH + Cu^{2+} \rightarrow RS^{\bullet} + Cu^{+} + H^{+}$

In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands (according to the Irving Williams series), for example, the replacement of Zn (II) by Cu (II) in the zinc-finger DNA binding domain of the human estrogen receptor renders this protein defective, altering its role in hormone-dependent signal transduction *in vivo* (Predki and Sarkar 1992).

Therefore, although Cu is essential, it is also highly toxic. This places a special importance on systems that transport Cu. To be safe, these systems should operate with a very low Cu level and have a high specificity for Cu. Free Cu concentration in normal physiologic conditions is virtually zero with the concentration of Cu ions estimated to be

in the order of 10^{-18} to 10^{-13} M both in yeast cells and in human blood plasma, respectively (Tapiero et al., 2003).

Fish living in coastal waters are exposed to a mixture of inorganic and organic pollutants including heavy metals, arising from human activities. Indeed global Cu emission tripled between 1950 and 1980 (Segner et al., 2001) and this alarming increase in marine ecosystems has led Cu to be classified as one of the more hazardous metals.

1.4.1 Implications of Dietary and Environmental Copper in Aquaculture

Understanding the mechanisms of both essential and excess Cu uptake in fish is also important in aquaculture since alarmingly high sediment Cu levels have been detected close to both Mediterranean (sea bream) and Atlantic (salmon) farming operations (Mendiguchia et al., 2006; Dean et al., 2007; Chou et al., 2004). This accumulation is due to the deposition of Cu present in uneaten food and unabsorbed dietary metal in faeces. In order to resolve these issues it is necessary to both understand the mechanisms and regulation of essential dietary uptake by fish as well as the toxicological consequences of environmental Cu contamination. Therefore, it is important to prepare diets containing the optimal levels of Cu in order to avoid deficiency while at the same time reducing Cu output in the marine environment.

1.4.2 Dietary copper toxicity

In mammals, excess dietary Cu is primarily accumulated in the liver and secondarily in the brain and kidney (Linder 1991). When large amounts of Cu accumulate in the hepatocyte they can be destructive when the ability of the cells to store excess Cu has been exceeded and ROS are formed by free Cu ions catalysis. This excessive Cu

accumulation in the liver, especially when associated with defects in biliary excretion as in WD patients, results in the formation of cirrhotic nodules which in chronic Cu conditions is accompanied by dramatic morphological changes and eventually ends in organ failure (Linder 2001; Buiakova et al., 1999) (see 1.5.4.1).

Dietary Cu toxicity in fish depends on the species, the life stage (early life stages are more vulnerable), daily dose and probably also diet type (purified, practical or live diet), and composition (levels of Zn, ascorbic acid and nitrilotriacetic acid) (Clearwater et al., 2002). Moreover, Clearwater et al. (2002) have defined toxicity thresholds as daily doses of > 1 mg Cu Kg^{-1} body weight per day for channel catfish, 1-15 mg Cu Kg^{-1} body weight per day for Atlantic salmon, and 35-45 mg Cu Kg^{-1} body weight per day for rainbow trout, depending on the life stage. The dietary bioavailability of Cu is lower compared to waterborne Cu (Grosell et al., 2004b; Grosell et al., 2004a) (see 1.4.4 and 1.5.5) and the dietary toxic effect of Cu exposure occurs only at high concentration and results in tissue accumulation, primarily in the liver but also in gill, gut, kidney and carcass. In more sensitive species or at high doses decreased growth and survival rate are found (Clearwater et al., 2002; Kamunde et al., 2001). Moreover, trout and catfish exposed to sublethal dietary Cu (5-16 μmol Cu g^{-1} fed and 1500 mg Cu Kg^{-1} dry weight feed respectively), have shown Cu tissue accumulation in the liver, intestine and gill. On the other hand there were no effects on growth rates or Na^+/K^+-ATPase activity, suggesting a high level of tolerance to dietborne Cu (Hoyle et al., 2007; Kamunde and Wood 2003). Even so in catfish increased lipid peroxidation level in gill and intestine and increases in total glutathione show that dietary Cu exposure results in tissue oxidative stress probably related to Cu tissue accumulation (Hoyle et al., 2007). Glutathione constitutes an important primary mechanisms of defence against ROS in the cell (Ferreira et al., 1993) (see 1.5.3.6).

In marine fish there is considerably less data, however Bielmyer et al., (2005) have shown that hybrid striped bass have a high level of tolerance to dietborne Cu. Fish fed 1013 mg Cu Kg^{-1} dry diet for 42 days accumulated significant liver and intestinal Cu but showed no significant change in growth rates. Baker et al., (1998) showed that juvenile grey mullet, fed 2400 mg Cu Kg^{-1} dry diet for 70 days exhibited increased hepatic lipid peroxidation and decreased food intake and growth. Whilst the latter could be due to the energetic cost of Cu detoxification it is more likely that it is due to a reduction in food intake due to decreased palatability (Smith et al., 2001; Baker et al., 1998; Lanno et al., 1985) (see 1.5.5).

1.4.3 Waterborne copper toxicity

Since it is freely soluble, Cu can pose a serious threat to aquatic animals. Waterborne Cu toxicity is influenced by biotic factors such as fish species and life stage. Embryos and larvae of the common carp exposed to Cu (50 μg Cu l^{-1}) showed spinal cord deformations and increased incidence of larval mortality (Flik et al., 2002). Moreover, since the size of the fish is strongly correlated with the Na turnover rates in fresh water (FW) fish (Grosell et al., 2002) this might explain the higher sensitivity of smaller fish to Cu toxicity due to higher sensitivity to perturbation of sodium osmoregulation. In addition abiotic factors such as pH, water hardness, DOM concentration can influence Cu toxicity at different levels (Taylor et al., 2002). Abiotic factors which alter Cu speciation (eg. hardness and pH) alter Cu toxicity (Lauren and McDonald 1985; Matsuo et al., 2004) whilst [Na^+] may influence transporters which are sodium sensitive (Handy et al., 2002). DOM also alters metal ion concentrations, therefore influencing uptake, similarly, excreted mucoproteins at the gill surface will also affect Cu availability.

In aquatic animals the toxicity of most waterborne heavy metals including Cu increases with decreasing salinity (Hall and Anderson 1995). However, a recent study comparing acute Cu toxicities across the full range of salinity in fish and invertebrates has shown the highest tolerance at intermediate salinity and the highest sensitivity at the two extreme salinities (Grosell et al., 2007). Cu speciation fails to explain this result because ionic Cu^{2+} and $CuOH^{+}$, which are considered to be the most toxic forms of Cu are most abundant at intermediate salinities (Paquin et al., 2002). Therefore, Grosell et al., (2007) proposed that the mechanism of toxicity of waterborne Cu in aquatic animals is strictly related to their osmoregulatory physiology.

Comparing Cu toxicity between FW and sea water (SW) fish requires some consideration since their osmoregulation differs. All FW fish maintain internal Na^{+} concentrations higher than the surrounding environment and SW fish lower than surrounding seawater (Grosell and Wood 2002; Kamunde et al., 2002b). In freshwater, osmotic influx of water occurs across the gill. The gills lose electrolytes from their concentrated extracellular fluids by diffusion to the dilute freshwater environment (Powers 1989; Grosell et al., 2002). Moreover, FW fish excrete osmotic water loads through the kidney via production of diluted urine (Beyenbach 2004). The key transporters for the compensation of lost electrolytes are the epithelial sodium channels (ENaC), which is located at the apical membrane of polarized epithelial cells including the gill and facilitates passive uptake of Na^{+} from the dilute environment and Na^{+}/K^{+}-ATPase that is located at the basolateral membrane of epithelial cells including the gill cells (Bury 2005). Na^{+}/K^{+}-ATPase actively pumps Na^{+} into the blood and requires Mg^{2+} as a cofactor (Dang et al., 2000; Grosell et al., 2002). Conversely, all SW teleost fish maintain extracellular ionic concentrations below that of SW (Grosell 2006) and must drink to replace fluid lost by diffusion (mainly through the gill) to the high-salt external environment. Intestinal

water absorption is driven by active absorption of Na^+ and Cl^-. The NaCl gained by this process and by diffusion across other surface areas is extruded by active transport across the gill (Talbot et al., 1992). In addition, differently form FW fish, the urine of SW fish is generally isosmotic to the plasma (Beyenbach 2004) and in conditions of hypersalinity the kidney reabsorbs Na^+ to conserve water excreting concentrated Mg^{2+}, Cl^- and $SO4^{2-}$ in the urine (McDonald and Grosell 2006). Both intestinal Na^+ uptake and branchial Na^+ excretion are carried out by Na^+/K^+-ATPases (Blanchard and Grosell 2006).

The primary acute toxic effect of water borne Cu exposure of FW fish is exhibited by the gill where at relatively low concentrations it inhibits the Na^+/K^+-ATPase. Reduced sodium (and chloride) uptake results in imbalanced osmoregulation leading to mortality (Lauren and McDonald 1985; Li et al., 1998a). Li et al. (1996) have shown that Cu^{2+} non-competitively inhibits Na^+/K^+-ATPase by binding to –SH groups, and also by binding to the Na^+/K^+-ATPase's Mg^{2+} co-factor binding site required for its normal function. Furthermore at higher concentrations waterborne Cu stimulates mucus production causing respiratory distress through blockage of water circulation and thus reduced gas exchange (Laurén et al. 1985). Chronic waterborne Cu exposure in FW results in tissue Cu accumulation especially in the liver and reduced growth rates (McGreer et al., 2000a; McGreer et al., 2000b; De Boeck et al., 1997). The locations at which Cu and Na are most likely to be competing for entry are in the initial absorption onto the epithelia across secreted mucoproteins and at the apical/mucosal pathways used for Na and Cu entry into the epithelial cells. There is evidence for both Cu-specific channel (Ctr1) and Cu leak through ENaC but the latter may be more significant in conditions of low Na^+ such as in the FW gill (Handy et al., 2002).

The effects of waterborne Cu exposure have been poorly studied in SW fish. Marine gulf toadfish (*Opsanus beta*) exposed to 761 μg l^{-1} of Cu for both 96 h and 30 days showed Cu toxicity through failure of Na^+ and Cl^- regulatory systems resulting in increased plasma sodium, chloride and osmolality followed by fluid loss in muscle tissue as well as disturbance of chloride absorption in the intestine (Grosell et al., 2004a). Furthermore, gulf toadfish exposed to 761 or 3508 μg l^{-1} of Cu for 30 days rapidly accumulated Cu in the gill, and had a net increase in drinking rate resulting in intestinal and liver Cu accumulation (Grosell et al., 2004b). The mechanism of chronic Cu accumulation in SW and FW fish therefore appears to be similar. The increases of plasma sodium observed during acute waterborne Cu exposure in SW (Grosell et al., 2004a) could be due to either the inhibition of water transport, facilitated by uptake of Na^+ in the intestine or inhibition of the Na^+ excretion in the gill. Even though both these scenarios could be explained by an inhibition of the Na^+/K^+-ATPases in the intestine and gills, no inhibition of these enzymes has been demonstrated, however this may be an assay artefact. The ion-regulatory disturbance could be due to a change in gill permeability (Grosell et al., 2004a). One known consistent effect of Cu exposure in SW fish is an increase in plasma ammonia (Grosell et al., 2004b; Wilson and Taylor 1993). This has been considered a secondary effect of Cu toxicity, but how Cu affects nitrogen metabolism is still unknown (Lauren and McDonald 1985; Blanchard and Grosell 2006). As mentioned above, killifish (*Fundulus heteroclitus*) exposed to Cu across the full salinity range showed the highest sensitivity in FW (96h LC50: 18 μg l^{-1}) followed by SW (96h LC50: 294 μg l^{-1}) with fish at intermediate salinities being the most tolerant (96h LC50 > 963 μg l^{-1}) at 10 ppt (Grosell et al., 2007). In conclusion, even though FW was the condition where Cu had the highest toxicity this result suggests that Cu toxicity is directly related to the Na^+ gradient for osmoregulating organisms. Even though the toxic symptoms

following waterborne Cu exposure in FW and SW fish are similar the mechanism of toxicity in the latter is still not understood and further studies are therefore required.

1.4.4 Dietborne *vs* waterborne copper toxicity

The higher sensitivity to waterborne Cu compared to dietary Cu, especially in FW fish, could be due to lower Cu complexation of waterborne Cu compare to dietary Cu, the more permeable architecture of the gill tissue compared to the intestinal tissue and also that in the gill of FW fish more Cu may enter through leakage of the Na channel (Handy et al., 2002). Whilst Miller et al. (1993) suggested that fish tolerate dietary Cu more than waterborne Cu because dietary Cu uptake is better regulated, Clearwater et al. (2002) argued that Cu uptake from water and diet may be the same but the different toxicity effect could be due to faster passage of water across the face of the gill cell than food passage across the face of the intestinal cell resulting in a higher tissue accumulation compared to dietary Cu.

Understanding the different mechanism of toxicity and the homeostatic response to either waterborne or dietborne Cu in fish is one of the main objectives of this thesis, which approaches these issues by trying to understand Cu homeostasis by investigating the tissue expression profiles of specific Cu transporters.

1.5 Copper homeostasis

The oldest microfossil ever discovered was found on a volcanic rock in deep sea and was estimated to be 3.2 billion years old (Rasmussen 2000). This finding contributed to the hypothesis that todays hydrothermal vents are the sites most resembling the environment in which the first living organisms on earth evolved. The hot, acidic output of

the hydrothermal vents release metals ions like iron, manganese, Zn and Cu and reduced sulphur (H_2S) from volcanic rocks (Zierenberg et al., 2000). Therefore the evolution of resistance to those metal ions could have been an evolutionary prerequisite for the first life forms.

The identification of Cu homeostasis proteins comes from studies in model systems such as bacteria and yeast (see 1.5.2). The study of these proteins show what Pena et al., (1999) call "modular function domains" that are functional domains of proteins which are conserved throughout evolution and are able to bind and deliver Cu with high specificity and efficiency (see 2.1.4). These modular functional domains are the "signature" of the family of Cu proteins and they may be the key to identify more components of this complex network.

1.5.1 Copper homeostasis in prokaryotes

The gram-positive bacterium *Enterococcus hirae* has been utilised as a model system for studying Cu homeostasis in prokaryotes (Solioz and Stoyanov 2003). In *E. hirae* the intracellular Cu concentration is transcriptionally regulated by the *cop* operon that regulates uptake, availability and export. The *cop* operon contains a promoter and four genes that encode: a repressor, CopY, a Cu chaperone, CopZ, and two CPx-type Cu ATPases (see 1.5.4), CopA and CopB responsible for Cu uptake and export respectively (Solioz and Stoyanov 2003) (Figure 1-1). The *cop* operon of *E. hirae* is regulated by the Cu-responsive repressor CopY (Odermatt and Solioz 1995). At physiological or low levels of Cu, CopY binds to the *cop* promoter as a Zn^{2+} complex and the transcription of the *cop* operons is down-regulated. If the intracellular Cu level is high, then Cu^{+}CopZ chaperone donates Cu to the Zn^{2+}CopY repressor, thus displacing Zn. The resulting Cu^{+}CopY complex dissociates from the promoter allowing the transcription of CopZ, CopA and

CopB (Cobine et al., 1999). Paradoxically, both uptake (CopA) and efflux (CopB) proteins are regulated together by the *cop* operon so that in conditions of Cu excess both the uptake protein CopA and the efflux protein CopB are expressed. This is hypothesised to be a safety mechanism to enable the efflux of excess Cu in conditions where sudden environmental Cu increases cause deleterious levels of intracellular Cu through uptake by CopA (Solioz and Stoyanov 2003). Copper is delivered to its target enzymes by maintaining Cu^{+}CopZ levels within strict limits with excess Cu^{+}CopZ being degraded by a Cu-activated serine-type protease (Cobine et al., 1999). The Cu released by this process is then utilised as a cofactor for various cuproenzymes (Solioz and Stoyanov 2003). There is evidence that Cu^{+} is the species taken up by uptake proteins in *E.coli* (Rapisarda et al., 2002), *S. cerevisiae* (Hassett and Kosman 1995) and mammals (Frieden 1980). The most common speciation of environmental Cu in Cu^{2+}, Cu^{+} ions are insoluble in free form at physiological pH and in standard bacteriological media. On this basis Solioz et al. (2003) suggest that any Cu uptake system must have an extracellular Cu reductase (CorA), even though it is still not characterized in *E. hireae*.

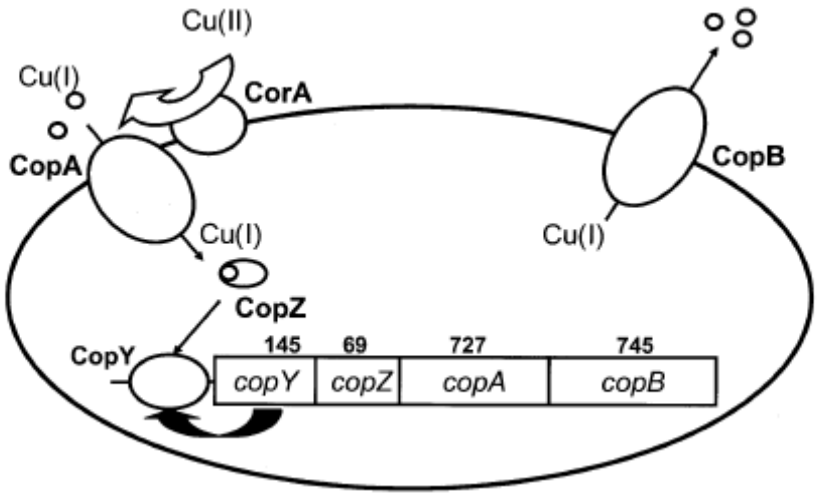

Figure 1-1 Model of the *cop* operon and copper homeostasis in *E.hirae.* (from Solioz et al. 2003).

1.5.2 Copper uptake in eukaryotes

The organism in which Cu homeostasis and the associated proteins are most well known is baker's yeast (*Saccharomyces cerevisiae*). The high degree of conservation of the components of the Cu homeostatic pathways, combined with the ease with which genetics can be used to isolate and study Cu transport mutants, and the fact that the entire genome of this yeast has been sequenced, makes *S. cerevisiae* an excellent model organism to study Cu metabolism (Pena et al., 1999). The current understanding of Cu uptake, distribution, storage and detoxification in yeast is summarized in Figure 1-2. Knowledge of Cu homeostasis genes has been achieved through the generation and identification of yeast mutants lacking genes responsible for specific cellular functions such as Cu-uptake, transport and excretion. These mutants can then be used to screen genes for their ability to resore a normal Cu phenotype (Dancis et al., 1994b; Lin et al., 1997). Subsequently, the identification of Cu homeostatic genes in mammals has been achieved by similar functional complementation methods (known as the yeast two hybrid system) using yeast Cu mutants (Zhou and Gitschier 1997). Sequence comparisons with characterized yeast genes and screening of sequence databases have been also used to identify Cu homeostatic candidate genes in mammals and other species (Puig and Thiele 2002). Most of the yeast genes responsible for uptake, intracellular transport and excretion have now been identified in mammals showing that considerable levels of conservation exist between the Cu homeostatic system from yeast to vertebrates (Pena et al., 1999; O'Halloran and Culotta 2000; Prohaska and Gybina 2004). This high level of conservation infers their essentiality to life.

1.5.2.1 Copper uptake in yeast

In the environment the most stable and therefore most common atomic species of Cu is Cu^{2+} (Krot et al., 2005; Buck et al., 2007). However, the Cu-trafficking proteins operate with a trigonal planar coordination chemistry, which binds Cu^{+} (Finney and O'Halloran 2003) (see 1.5.3). Since Cu^{+} is the species required by uptake systems and chaperones, to be able to be specifically taken up, Cu has to be reduced from Cu^{2+} to Cu^{+} by one or more cell-surface reductases e.g. the yeast Fe^{3+}/Cu^{2+} reductases, Fre1 and Fre2 (Hassett and Kosman 1995; Dancis 1998). Cu^{+} can then be taken up through the high affinity Cu transporting membrane receptors ScCtr1 and ScCtr3 (Dancis et al., 1994a; Knight et al., 1996). High affinity uptake by ScCtr1 and ScCtr3 is specific for Cu^{+} and is saturable, with a Km of 1-4 μmol l^{-1} as determined by ^{64}Cu uptake studies in whole yeast cells (Lin and Kosman 1990). The chromosomal disruption of ScCtr1 results in phenotypes associated with Cu starvation, such as aerobic growth arrest due to a defect in Cu-Zn superoxide dismutase (CuZn-SOD) activity, and respiratory deficiency due to the inability to provide cytochrome c oxidase with Cu, showing that ScCtr1 is a gene essential for Cu uptake, normal mitochondrial respiration and CuZn-SOD activity (Dancis et al., 1994a). ScCtr3 was identified as a suppressor of the Cu starvation phenotypes associated with strains lacking ScCtr1. Moreover yeast which possess both transporters have a distinctive growth advantage under Cu deficiency (Knight et al., 1996).

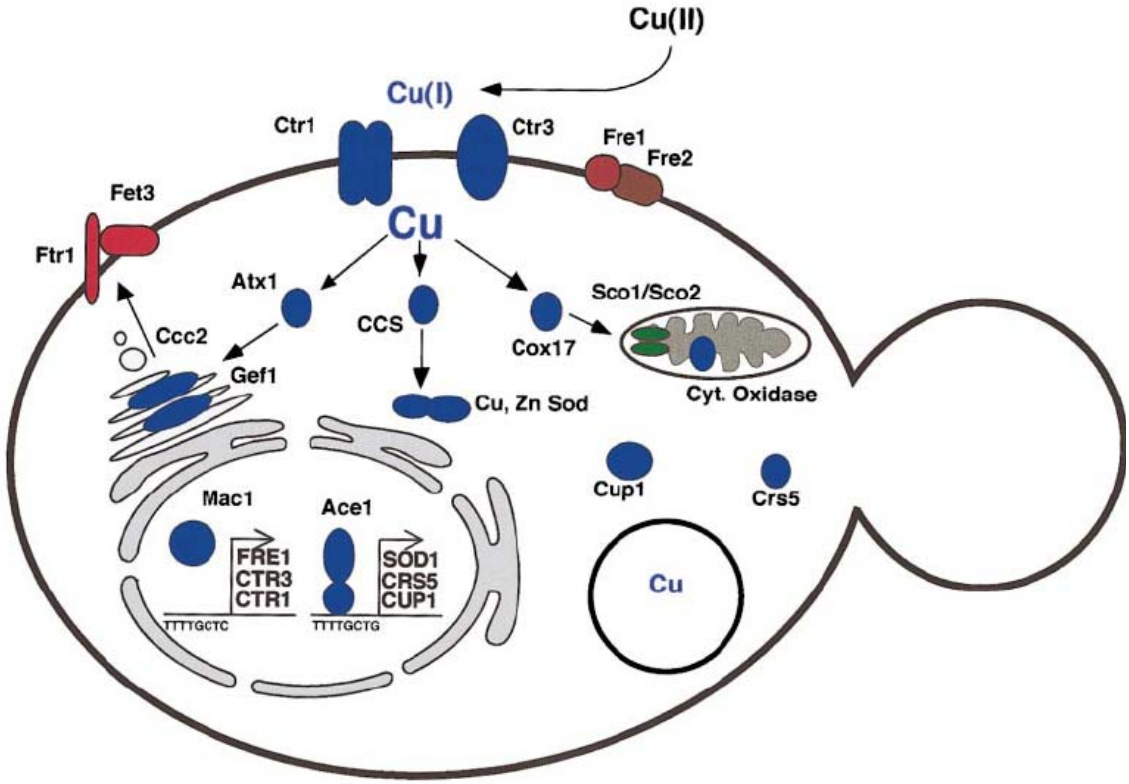

Figure 1-2. Copper transport and distribution in *Saccharomyces cerevisiae* (from Pena et al. 1999).

Yeast adjusts its internal Cu concentration predominantly through two Cu sensing transcription factors, Mac1 and Ace1, which are sensitive to Cu deficiency and excess respectively, although other transcription factors have been discovered which may also have a role in Cu homeostasis (Rutherford and Bird 2004; van Bakel et al., 2005). Excess Cu directly binds Mac1 which represses the expression of the genes involved in Cu uptake, Fre1, ScCtr1 and ScCtr3, through interaction with the *cis*-acting DNA response elements (CuREs) located in their gene promoters. Conversely, low levels of Cu up-regulates Cu uptake genes by inhibiting Mac1 repression (Winge 1998; Pena et al., 1998). In addition to the repression of Cu uptake, Cu excess results in the expression of three genes involved in Cu storage and detoxification, the metallothioneins Cup1 and Crs5, which can bind free Cu in the cytoplasm, and CuZn-SOD, which is involved in free

radical scavenging (Gralla et al., 1991; Jensen et al., 1996). The expression of these three genes is regulated by the transcription factor Ace1 (or Cup2) which, upon binding Cu, changes its conformation enabling interaction with Metal Responsive Elements (MREs) (Winge 1998), located on metallothionein and CuZn-SOD gene promoters (Searle et al., 1985). Yeast can also regulate Cu uptake through post-translational mechanisms. Levels of Cu that exceed the Km (~10 μM Cu) of Ctr1 trigger its endocytosis and delivery to the vacuole for degradation (Ooi et al., 1996). Moreover, the post-translational degradation of ScCtr1 under excess Cu requires Mac1 and it has been postulated that either an uncharacterized Mac1 target gene encodes a protein that is essential for this degradation, or that Mac1 itself functions as a protease or protease-recruiting factor (Yonkovich et al., 2002).

1.5.2.2 Cu uptake in mammals

Aligning the protein sequences of known Ctr1 family members from yeast, plants and mammals shows a very high level of conservation in the protein structure (Figure 1-3). This level of sequence conservation in such a broad group of species also suggests functional conservation (Puig and Thiele 2002).

Zhou et al. (1997) isolated the human high-affinity Cu transporter 1 (hCtr1) by functional complementation of the respiratory defect in yeast cells defective in Cu transport due to inactivation of both ScCtr1 and ScCtr3. The hCtr1 amino acid sequence was determined to be 29 % identical to ScCtr1. Ctr1 mRNA is ubiquitously expressed in mammals with the highest levels found in the liver and kidney, important organs for metabolism and reabsorption, respectively, and lower levels detected in spleen and brain (Zhou and Gitschier 1997; Lee et al., 2000). Even though hCtr1 is significantly smaller in size (190 amino acids compared to 406 ScCtr1 and 241 ScCtr3), it has conserved the same

structural/functional domains that characterize the Ctr1 family of proteins (Figure 1-3) and suggests that the mammalian Ctr1 and ScCtr1 and ScCtr3 share a common ancestral gene.

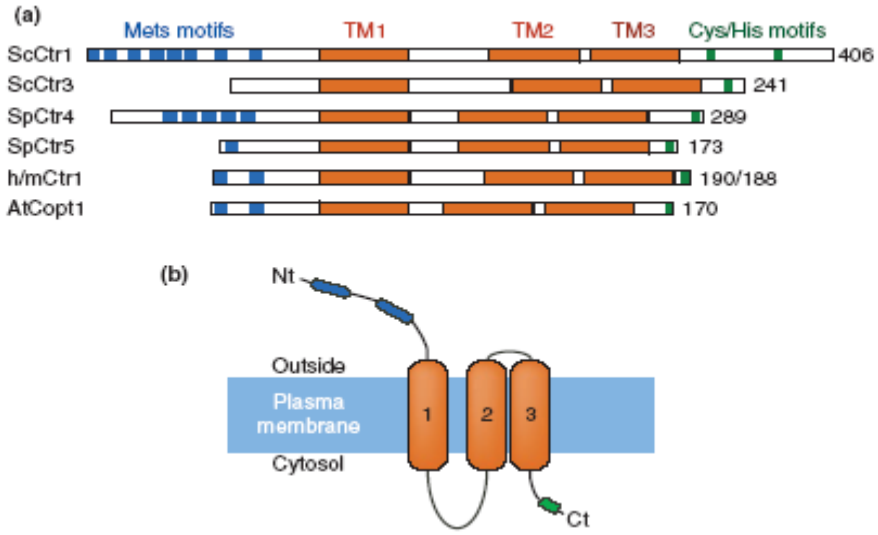

Figure 1-3 Model for the structure of Ctr family high-affinity copper transport proteins. (a) Copper transport proteins from *S. cerevisiae* (ScCtr1, ScCtr3), *S.pome* (SpCtr4, Sp Ctr5), human and mouse (h/mCtr1) and *A.thaliana* (AtCopt1) were aligned, consensus sequences are represented from Nt (left) to Ct (right). (b) Proposed topological structure of Ctr1 family of high-affinity copper transporters (from Puig S. and D.J. Thiele, 2002).

The function of mammalian Ctr1 has been established showing that both mouse and human Ctr1 expression can rescue the lost uptake activity of ScCtr1 yeast mutants (*Ctr1*Δ). *Ctr1*Δ are associated with phenotypes attributable to lack of Cu by specific enzymes such as cytochrome c oxidase (COX) which result in mitochondrial respiratory deficiency, CuZn-SOD which result in oxidative stress sensitivity, and multi-Cu ferroxidase (Fet3) which result in inability to transport iron (Lee et al., 2000). Moreover transfection experiments have shown that both mouse and human Ctr1 proteins are able to stimulate saturable uptake of Cu with high affinity and preference for Cu^{+} in human and mouse cells (Lee et al., 2001; Eisses and Kaplan 2005). Lee et al. (2002) have shown that by over expressing Ctr1 in the human cell line Hek239, Ctr1 Cu uptake is energy

independent and is stimulated by acidic extracellular pH and high K^+ concentrations, in a process that is time dependent and saturable. The stimulation of Cu uptake by K^+ combined with the observation in yeast that Cu uptake is coupled with K^+ efflux may suggest that Cu uptake is mediated by a $Cu^+/2K^+$ antiport mechanism (De Rome and Gadd 1987; Lee et al., 2002a; Lee et al., 2002b).

Recently, a family of metalloreductase, homologues to Fre1-2 in yeast, has been identified in humans (STEAP1-4), suggesting the possibility that hCtr1, like ScCtr1/ScCtr3, transports Cu^+ reduced by these metalloreductases (Ohgami et al., 2006). In addition, another metalloreductase has been localized at the apical membrane of enterocytes which may also function in Ctr1-mediated Cu^+ transport (McKie et al., 2001).

Targeted deletion of the Ctr1 gene (Ctr1 knockout) has revealed an important role for Ctr1 in embryonic development. Mice with a complete deficiency of Ctr1 die *in utero,* approximately at mid-gestation, following growth and developmental defects whilst partially deficient heterozygous mice survive but exhibit tissue specific defects in Cu accumulation. A 50% reduction in total brain and spleen Cu level as compared with wild-type mice was observed, although no differences were apparent in liver and kidney. Furthermore a reduction in the activities of the cupro-enzyme CuZn-SOD and COX were found in the brain (Lee et al., 2001; Kuo et al., 2001). In agreement with the tissue expression profile, tissues with low Ctr1 expression, such as brain, are more vulnerable to Cu imbalances. Liver tissue may recycle Cu more efficiently as liver is the main tissue for Cu storage (Linder 1991), or alternatively there may be other Cu transporters (low affinity) which are more active in some tissues than others (Puig and Thiele 2002). Moreover, Ctr1 has a crucial role in intestinal Cu absorption, as specifically knocking out Ctr1 from the intestinal epithelium in mice results in severe Cu deficiency phenotypes

such as low Cu in peripheral tissues, hepatic Fe overload due to lack of holo-ceruloplasmin, growth defects and death at 8-10 days after birth. These phenotypes can be rescued bypassing the intestinal uptake by a singular intraperitoneal injection of Cu at 5 days after birth. This further evidence confirms the role of Ctr1 in intestinal uptake of Cu and its essential involvement in Cu distribution to peripheral tissues (Nose et al., 2006a).

The mechanism by which mammals regulate Cu uptake through Ctr1 has not yet been clearly established. Lee et al. (2000) have reported that Ctr1 mRNA levels, detected by Northern blot, were not changed in response to Cu availability in brain, liver or intestine whilst Bauerley et al. (2005) reported that Ctr1 mRNA, detected by QPCR, and protein levels, were higher in the liver of rat pups exposed to moderately high levels of Cu (25 μg/ day) in the milk but no differences were found in the intestine. However, there is also evidence from *in vitro* studies for a post-translational mechanism of regulation of Ctr1 in response to high levels of Cu (Petris et al., 2003). In basal growth medium hCtr1 was localized at the cell membrane in growing cells (human embryonic kidney, HEK293), whilst in the presence of a high concentration of Cu (100μM), hCtr1 displayed a cytoplasmic vesicular distribution and levels in the plasma membrane were reduced. Degradation of Ctr1 was also shown to be more rapid when cells were cultured in medium containing high concentrations of Cu. Taken together these results indicate that intracellular Ctr1 distribution plays a role in the regulation of Cu levels in mammals and that this may reflect a defensive mechanism in response of Cu excess (Petris et al., 2003). Thus in polarised cells such as absorptive epithelia, identification of Ctr1 localization and trafficking to specific intracellular locations is going to be crucial to understand its role in the regulation of Cu homeostasis and its other cellular biochemical functions. To date there is disagreement on the functional location of hCtr1 in polarized cells. Kuo et al. (2006) showed by immunostochemical analysis of mice duodenal tissue that Ctr1 protein

is predominately distributed at the apical membrane in newborn mice whereas in adults it is predominantly localized in intracellular vesicles. Conversely, Zimnicka et al. (2007) have shown, in polarized enterocytes (human colonic adenocarcinoma cells, Caco2), that basolateral Cu uptake substantially exceeds the apical uptake. Moreover, surface specific biotinylation and immunohistochemistry experiments have confirmed Ctr1 location at the basolateral but not the apical membrane. Furthermore, immunofluorescence studies have localized hCtr1 to a perinuclear compartment, cytoplasmic vesicles and cell surface in non polarized cell lines (cervical cancer cell line, HeLa, human placenta choriocarcinoma, BeWo and Caco2) (Lee et al., 2002b; Klomp et al., 2002). Klomp et al. (2002) suggest that hCtr1 location is related to specific cell type; in some cell lines, hCtr1 is located predominantly in an intracellular vesicular perinuclear compartment (HeLa cells) and in others, hCtr1 was located also at the plasma membrane (BeWo and Caco2). The results in this study suggested a cell-specific control of Cu uptake, which involves subcellular localization of the hCtr1 protein. Taken together these results suggest that, Ctr1 functions at the plasma membrane in conditions of low/normal environmental Cu and in conditions of high environmental Cu Ctr1 function is impeded by endocytosis of Ctr1, by reducing Ctr1 expression and by increasing degradation of the Ctr1 protein. However, further studies are required to clarify Ctr1 functional location in polarized cells to better understand its homeostatic role at cellular and systemic level.

1.5.2.3 Cu uptake in plant, insects and other vertebrates

Ctr1 has also been reported and studied in plants *Arabidopsis thaliana*, insects *Drosophila megalogaster,* reptiles *Podarcis sicula* and fish *Danio rerio.* The protein structure that characterizes the Ctr1 protein family is conserved in all these species (Sancenon et al., 2003; Zhou et al., 2004; Riggio et al., 2002; Mackenzie et al., 2004). As

in mammals (Lee et al., 2001), in plants, insects and fish Ctr1 has been shown to be essential for development, functional disruptions presenting a range of phenotypes related to Cu deficiency, possibly due to imbalances in the biosynthesis of cuproenzymes. Ctr1 gene knock-out is lethal at early larval stages in fish (Sancenon et al., 2004; Sancenon et al., 2003; Zhou et al., 2004; Mackenzie et al., 2004). It has been proposed that in lizards Ctr1 functions in Cu acquisition in growing oocytes and eggs (Riggio et al., 2002). In fish and lizards the highest level of Ctr1 mRNA is found in intestine, highlighting the role of this tissue in Cu absorption (Riggio et al., 2002; Mackenzie et al., 2004; Bury et al., 2003). Interestingly, in plants and insects Ctr1 is transcriptionally regulated in response to Cu exposure (Ruzsa and Scandalios 2003; Zhou et al., 2004). Since the transcriptional regulation of Ctr1 by Cu is so widespread, similar mechanisms may exist in vertebrates which, if true, would be important in understanding Cu homeostatic processes.

1.5.2.4 Low affinity Cu uptake

In *S. cerevisiae* besides the high affinity Cu transporters ScCtr1 and ScCtr3 (see 1.5.2.1) other transporters may also uptake Cu ions. Ferrus transporter (Fet4) (Hassett et al., 2000) and the suppressor of mitochondria import function (Smf1) (Liu and Culotta 1999) have been shown to transport Cu with low affinity (Hassett et al., 2000; Cohen et al., 2000). In addition, intracellularly, ScCtr2 has been demonstrated to mobilize Cu pools stored in vacuoles, although with low affinity for Cu (Rees et al., 2004). Identification of the human gene for hCtr2 was based on sequence homology with hCtr1 (Zhou and Gitschier 1997). This has a low affinity for Cu attributable to a lack of well defined Cu binding motifs and a lower abundance of histidines and methionine residues. The tissue expression profile of these two hCtr genes is also significantly different, hCtr2 is highly expressed in placenta and has a low level of expression in liver (Zhou and Gitschier 1997).

There is evidence that the function and localization of hCtr2 is analogous to ScCtr2, ie mobilization of Cu from endosomal and lysosomal Cu pools maintaining Cu homeostasis (Rees et al., 2004; van den Berghe et al., 2007). Recent studies reported by Bertinato et al. (2008) have shown that Ctr2 can be also localized at the plasma membrane in African green monkey kidney cells (COS-7) and over-expression of Ctr2 results in accumulation of Cu. Ctr1 homozygous knock-out mice still accumulate Cu in some tissues (Lee et al., 2001) and Ctr1 knock out mouse embryonic cells show ~30% residual Cu transport activity (Lee et al., 2002b), indicating the presence of Ctr1-independent mechanisms for Cu acquisition by cells. Considering that hCtr2 cannot complement phenotypes of yeast deficient in high affinity Cu uptake, Bertinato and L'Abbe (2004) considered it unlikely that this low affinity Cu transporter could compensate for reduced uptake of Cu due to the absence of hCtr1 although later this group have proposed that hCtr2 may be involved in tissue specific Cu uptake in conditions of Cu starvation (Bertinato et al., 2008). Further studies are required to clarify hCtr2 function.

The divalent metal transporter 1 (DMT1) is a member of the natural-resistance-associated macrophage protein (Nramp2) family, of which Smf1 in yeast is also a member. DMT1 has been demonstrated to mediate proton coupled transport of a broad group of divalent ions, primarily iron (Tandy et al., 2000), but also Cu with lower efficiency (Gunshin et al., 1997). In conditions of dietary Cu overload, iron absorption is reduced indicating competition between the two metals (Tennant et al., 2002). Treatment of Caco-2 cells, an intestinal model, with a DMT1 antisense oligonucleotide resulted in 80 and 48% inhibition of iron and Cu uptake, respectively, indicating that DMT1 may also function in intestinal Cu absorption (Arredondo et al., 2003). DMT1 is not essential for Cu uptake since DMT1 deficient mice do not show Cu deficiency (Conrad et al., 2000). Furthermore, even though the name of this transporter suggest transport of Cu^{2+}, Cu^{+} was

shown to be the species transported by DMT1 (Arredondo et al., 2003; Tennant and Sharp 2004).

The nature of Cu uptake pathways, in mammals, is still open to debate but up to now the evidence is that Ctr1 is able to uptake Cu with high affinity in Hek293 (Human embryonic kidney) cell line (Km 1-5 μM) and is essential for intestinal Cu absorption (Nose et al., 2006a). However, Nose et al (2006) have also shown that mice with an intestinal specific Ctr1 knock out, despite showing a peripheral Cu deficiency phenotype accumulate Cu in the intestine indicating that apical Cu uptake is Ctr1 independent. The apical mechanism of Cu uptake is unknown and further studies are therefore required.

1.5.3 Intracellular copper distribution

Copper is required by a number of essential enzymes (Table 1-1). However, in order to distribute this crucial cofactor and at the same time avoid toxicity, the eukaryotic cell has evolved a special family of proteins called Cu chaperones (O'Halloran and Culotta 2000). Copper chaperones can acquire Cu under conditions where the metalloenzymes cannot and they function to traffic Cu to specific cellular targets. In essence, these molecules act to escort Cu ions and protect them from Cu-scavenging detoxification mechanisms (Field et al., 2002). In fact, in spite of the micromolar quantities of Cu that accumulate in a yeast cell, there is, virtually, not a single free atom available in the cytoplasm (Rae et al., 1999).

Cu trafficking proteins must bind their cargo tightly to prevent non-specific reactions, but must also allow for transfer of Cu to the target enzyme. The metal binding domain (MBD) that characterizes metal trafficking proteins –GMXCXXC- (Arnesano et al., 2002) makes possible a Cu coordination chemistry comprising two cysteine residues in

a low coordination number environment that allows for very tight binding but at the same time can allow the entrance of a third binding residue or ligand from outside the protein, or domain itself. This coordination chemistry is typical of Cu trafficking proteins whereas enzymes that ultimately incorporate Cu^{+} as a cofactor exhibit a coordination chemistry of four (Finney and O'Halloran 2003). These low-coordination-number environments are thus thought to create the conditions for the observed metal transfer chemistry.

Up to now, there are three well known Cu chaperone pathways, even though other putative Cu chaperones have been identified (Bertinato and L`Abbe 2004) (see Figure 1-2 for yeast and Figure 1-4 for mammals): (i) Cu delivery to the secretory pathway for activation of enzymes destined for the cell surface or extracellular milieu by Atx1 in yeast and Atox1/Hah1 in mammals (Lin and Culotta 1995; Lin et al., 1997; Klomp et al., 1997); (ii) Cu trafficking to CuZn-SOD in the cytoplasm carried out by CCS (Culotta et al., 1997); and (iii) delivery of Cu to mitochondria for activation of cytochrome oxidase carried out by Cox17 (Glerum et al., 1996).

Consistent with the function of this family of proteins is the presence in all Cu chaperones and Cu ATPases of typical Cu binding domain (MBD). Because of their functional importance these domains are very highly conserved from bacteria to mammals (Arnesano et al., 2002).

1.5.3.1 Copper delivery to the secretory pathway (Atx1/Atox1)

The Cu chaperone Atx1 was initially discovered by Lin and Culotta (1995) who found this protein capable of protecting the yeast cell against oxidative stress. In fact, the oxygen toxicity of yeast mutants lacking CuZn-SOD can be suppressed by the expression of Atx1, which was subsequently shown to be a Cu chaperone (Lin et al., 1997). The mammalian orthologue of Atx1, called Atox1 or HAH1 was discovered by the same

research group shortly after (Klomp et al., 1997). The human Atox1 is a small protein of 68 amino acids that shows a 47% amino acid identity to Atx1 including conservation of the MTCXGC Cu-binding domain at the N terminus (Klomp et al., 1997). Through genetic and protein-protein interaction studies, Atx1 first (Pufahl et al., 1997) and Atox1 later (Hamza et al., 1999; Walker et al., 2004; Hamza et al., 2003) were shown to be responsible for carrying Cu to the Cu transporting ATPases, namely the yeast Ccc2 and the mammalian ATP7A (Menkes protein) and ATP7B (Wilson protein). Consistent with the proposed role for Atox1 in Cu delivery to the secretory pathway, a marked increase in intracellular Cu content secondary to impaired Cu efflux was observed in Atox1-deficient cells (Hamza et al., 2003). Moreover Atox1 has been shown to be essential in perinatal Cu homeostasis, as mice with Atox1 gene knockdown die after birth and display other defects including growth retardation, skin laxity, hypopigmentation and seizures - all defects referable to specific cuproenzyme impairment (Hamza et al., 2001).

1.5.3.2 Copper trafficking to Cu/ZnSOD (CCS)

The second Cu chaperone to be identified was CCS (**C**opper **C**haperone for **S**uperoxide dismutase) (Culotta et al., 1997). The copper zinc superoxide dismutase is a cytosolic enzyme which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide ($O_2\cdot^- + O_2\cdot^- + 2H^+ \rightarrow O_2 + H_2O_2$) (McCord and Fridovich 1969). Mutations in the CuZn-SOD gene resulting in a distupted free radical detoxification have been associated with the amyotropic lateral sclerosis (ALS) (Andersen 2001). CCS is a homodimer with a 35 kDa subunit and 3 functional domains (Field et al., 2002). At the amino terminus, domain I contains the Cu binding site MXCXXC and crystallographic studies have shown structural conservation of this domain to the corresponding domain of Atox1 (Lamb et al., 1999). Domain I is essential in conditions of Cu deficiency but is not

the primary donor of Cu to CuZn-SOD (Schmidt et al., 1999a). The central and main part of CCS constitute domain II. Domain II sequence is very homologus to CuZn-SOD sequence conserving all the Zn binding residues and 3 of the 4 Cu binding histidines, but CCS does not have SOD activity (Schmidt et al., 1999a; Schmidt et al., 1999b). Domain III is the smallest of CCS domains comprising only 30 amino acids in yeast, however it is the most conserved region across diverse species and contains an essential CXC motif capable of binding Cu. Deletion of this motif prevents Cu transport from CCS to CuZn-SOD (Schmidt et al., 1999a). Mice with CCS deletion show dramatic reduction in SOD activity as a result of impaired Cu incorporation into CuZn-SOD (Wong et al., 2000). Furthermore this CCS deletion does not alter other Cu chaperone pathways, such as ceruloplasmin activation (Wong et al., 2000). CCS and SOD have been localized in the mitochondria as well, which makes sense considering that mitochondria are highly exposed to oxidative stress (Sturtz et al., 2001). Interestingly, elevated protein levels of CCS have been reported in tissues of mice and rats in conditions of Cu starvation (Bertinato et al., 2003; Prohaska et al., 2003). This effect was not due to transcriptional regulation but to slower degradation by the 26S proteasome complex (Prohaska et al., 2003; Bertinato et al., 2003; Bertinato and L'Abbe 2003). Higher levels of CCS in Cu deficient conditions may increase or prioritize Cu delivery to CuZn-SOD (Bertinato and L`Abbe 2004). In contrast to CCS, Atox1 levels are not affected by Cu deficiency (Hamza et al., 1999). Copper chaperones may therefore play a role in determining the hierarchy of Cu utilization in Cu-deficient cells (Bertinato and L`Abbe 2004).

1.5.3.3 Delivery of copper to cytochrome c oxidase (Cox17)

The Cu chaperone pathway to the mitochondria was determined by genetic studies on yeast, describing the isolation of yeast mutants incapable of assembling the

multisubunit complex cytochrome c oxidase (COX). Yeast lacking the Cu chaperone Cox17 are respiratory-deficient due to a complete lack of COX activity (Glerum et al., 1996). COX or complex IV is a large transmembrane protein (13 subunits) found in the mitochondrial inner membrane and it is the last protein in the electron transport chain (Hamza and Gitlin 2002). COX subunit I and II (Cox1 and Cox2) contain Cu centers CuB and CuA, respectively (Hamza and Gitlin 2002). The mitochondrial proteins Sco1 and Sco2 are required to incorporate Cu to COX (Carr and Winge 2003). The Cu chaperone for COX, Cox17, is found both in cytoplasm and in the mitochondrial intermembrane space. Cox17 is the main donor of Cu to Sco1 and Cox11, involved in formation of the mature COX complex. Recently, mutations in Sco1 and Soc2 in humans have been associated with pathologic COX deficiency (Sue et al., 2000; Valnot et al., 2000). Moreover, other Cu chaperones (Cox19 and Cox23) which show structural similarity to Cox17 may be involved in the assembly of COX in yeast. However their specific function is not clear (Cobine et al., 2006).

1.5.3.4 Other putative Cu Chaperones

Reddy et al. (2000) have reported a transcript variant of the ATP7A, the Cu-ATPase associated with Menkes disease named NML45. This small protein of 103 amino acids contains the first metal binding domain of ATP7A and it has been observed to localize to the nucleus of three human cell lines. This evidence may suggest that NML45 is a specific nuclear Cu chaperone in mammals (Reddy et al., 2000).

Another putative chaperone, Murr1 (or COMMD1), has been recently identified in inbred Bedlington terriers that develop Cu toxicosis (van de Sluis et al., 2002). Copper toxicosis (CT) in Bedlington terriers is an autosomal recessive disorder which presents an abnormal hepatic Cu metabolism (Ludwig et al., 1980; Watson et al., 1983) with a severe

impairment in biliary Cu excretion (Hultgren et al., 1986). Murr1 is a small cytoplasmatic protein of 188 amino acids, which has been related to the biliary excretory pathway mediated by ATP7B (Tao et al., 2003). Even though the Cu metabolic function of Murr1 is still not clear it has been proposed that it may play a role in vesicular Cu movement and excretion at the canalicular membrane of hepatocytes (van de Sluis et al., 2002). The loss of Murr1 generates Cu overload in the liver and embryos died *in utero* between 9.5 and 10.5 days *postcoitum* (van de Sluis et al., 2007). Recently it has been shown that Murr1 functions to regulate not only Cu homeostasis but also sodium transport regulating the amiloride-sensitive epithelial sodium channel (ENaC), responsible for sodium reabsorption (Biasio et al., 2004) and also the nuclear factor-kappa-B (NF-kB) (Burstein et al., 2004; Ganesh et al., 2003) that plays an important role in the transcriptional regulation of a wide array of genes involved in immune and stress responses (Pahl 1999). The involvement of Murr1 in multiple cellular processes is consistent with its ubiquitous expression pattern (de Bie et al., 2005). Although the exact molecular functions of Murr1 are unknown, they probably involve protein-protein interactions rather than direct Cu binding and more studies are therefore required in this direction (de Bie et al., 2005).

An important outstanding question is: where are the chaperones taking the Cu from? The C-terminus of ScCtr1 can interact with Atx1 and Cu is transferred between these two proteins (Xiao and Wedd 2002; Xiao et al., 2004). However, previously, a series of studies in yeast failed to demonstrate protein-protein interaction between the chaperones Atx1 and CCS and membrane transporters Ctr1, Ctr3, Ctr2 and FET4 (Pena et al., 2000; Portnoy et al., 2001). Nevertheless, the fact that all of these transporters have been demonstrated to be able to deliver Cu to the chaperones indiscriminately (Portnoy et al., 2001) suggested that another intermediate chaperone(s) may exist to ferry Cu between the membrane transporters and the Cu chaperones (Field et al., 2002).

1.5.3.5 Metallothionein

The most studied protein in the metabolism of heavy metals so far, is metallothionein (MT) (Margoshes and Vallee 1957; Kagi et al., 1961), although a single primary role has not been defined and further functions continue to be discovered. Metallothioneins are intracellular, low molecular weight, cysteine-rich proteins. Ubiquitous in eukaryotes, MTs have unique structural characteristics to give potent metal-binding and redox capabilities (Coyle et al., 2002).

Several biological roles have been proposed for MT:

- Detoxification of non essential metals such as cadmium and mercuric ions (Kaegi et al., 1981);
- Detoxification/storage of excessive essential metals such as Cu and Zn (Templeton and Cherian 1991);
- Protection against ROS "scavenger" (Thornalley and Vasak 1985);
- Recruitment of Cu in a Cu-deficient environment by sequestering Cu from degraded Cu-enzymes and delivering it to Cu chaperones (Suzuki et al., 2002).

In mammals the highest concentration of MT in the body is found in the liver, kidney, intestine and pancreas - all organs involved in absorption, metabolism and excretion of heavy metals (Hamer 1986). Hepatic MT synthesis is induced by a number of metals, cytokines and stress hormones as well as a wide range of chemicals, many of which act indirectly via a stress or inflammatory response (Hamer 1986; Bremner 1987a). Even though Zn, Cu, Cd, Hg, Au and Bi all induce MT, Zn is the primary physiological inducer since the other metals regulate MT expression indirectly and through toxic response mechanisms (Coyle et al., 2002). Zinc directly interacts with the metal transcription factor (MTF-1), which is a zinc finger protein, promoting the binding of MTF-1 to the metal

responsive elements (MREs) in the promoter region which, in turn, initiates MT-gene transcription. MT can also be induced by Cd and H_2O_2 through indirect activation of MTF-1 and also by direct activation of the upstream stimulating factor (USF) which promotes the binding to the cis-acting antioxidant responsive element (ARE) on MT promoter (Andrews 2000). How MTF-1 is indirectly activated is not yet understood completely, however Cd and Cu in excess displace Zn from Zn-protein, creating a free pool of Zn which is then suggested to be sensed by MTF-1 promoting MT synthesis (Zhang et al., 2001; Andrews 2001). Interestingly, sub-toxic levels of Cu do not induce MT, although Cu-MT complex can be detected probably because of displacement of Zn from existing Zn-MT (Bremner 1987b; Oestreicher and Cousins 1985). It may be that Cu induces MT only when present at toxic levels, when the excretory homeostatic system is saturated and free Cu begins to generate ROS, which then cause up-regulation of MT through different mechanisms from MTF1 activation. Interestingly, rats kept in conditions of Cu deficiency show initially a cessation of biliary excretion and limitation of the Cu supply to ceruloplasmin and then an increase in Cu-MT with maintenance of the Cu concentration in the liver. These results suggest that MT functions to limit loss of Cu in a Cu-deficient environment by sequestering Cu from degraded Cu-enzymes and delivering it to Cu chaperones (Suzuki et al., 2002).

By exposing several species of fish (sea bream and flounder) to Cu, it has been demonstrated hepatic MT mRNA increases and MT level is related to Cu accumulation. In addition, in fish MT is induced by several heavy metals such as Cd, Hg, Cu, Zn and Pb. Therefore, MT induction has been used as a biomarker for the presence of heavy metals in aquatic environments (George and Olsson 1994). Other tissues can accumulate heavy metals bound to MT, including Cu, such as kidney, gill and intestine. However, this is not seen in all tissues (eg. brain), indicating a tissue specific response (Isani et al., 2003;

George et al., 1996b). In the presence of dietary Cu excess, Cu is prevented from entering the body by retention in the gut tissue bound to MT (Olson 1996). Potentially, this MT-bound Cu may then be excreted into the faeces *via* sloughing of the epithelial membrane (Handy 1996; Clearwater et al., 2000). Similarly in the gill tissue MT expression can limit the absorption of excess essential and non essential heavy metals (Dang et al., 1999; George et al., 1996b).

1.5.3.6 Glutathione

Another molecule that plays an important role in metal detoxification is the tripeptide glutathione (GSH) which has been shown to bind Cu^{+} and to play a role in the biliary excretion of Cu (Houwen et al., 1990). In addition, *in vitro* studies show that GSH can transfer Cu to cuproproteins including metallothioneins (Ferreira et al., 1993; Freedman and Peisach 1989). GSH may also have a role in Cu absorption considering that Cu-deficient rats have shown increased levels of GSH (Chao and Allen 1992) and rats injected with Cu show decreased levels of GSH (Kawata and Suzuki 1983). Moreover cells with decreased GSH levels have a reduced rate of Cu uptake from the medium and have lower steady-state Cu concentrations (Tong and McArddle 1995).

Taken together this evidence suggests a role for GSH and metallothionein in intracellular Cu metabolism that extends beyond their role as metal detoxification proteins (Bertinato and L`Abbe 2004).

1.5.4 Copper transporting ATPases

Copper transporting ATPases are crucial components of the secretory pathway as they are responsible for delivering Cu to cuproenzymes and for the excretion of excess Cu (Camakaris et al., 1999). Cu-ATPases use the energy of ATP-hydrolysis to transfer Cu

from the cytosol into the lumen of the secretory pathway compartments (Voskoboinik et al., 1998). Copper transporting ATPases are a sub class of P-type ATPases (Solioz and Vulpe 1996a). Copper and cadmium (Cd-ATPases are known only in bacteria) P-type ATPases are characterized by: (1) putative heavy metal-binding sites in the polar amino-terminal region; (2) a conserved intramembrane CPC, CPH or CPS motif (cysteine-proline motif; CPx); (3) conserved histidine-proline dipeptide (HP locus) 34 or 43 amino acids carboxy-terminal to the CPx motif; and (4) a distinctive number and topology of membrane-spanning domains (Lutsenko and Kaplan 1995). Vulpe and Solioz, (1996) propose the designation of CPx-type ATPases for this group of P-type ATPases, based on the conserved CPx motif.

In yeast, the Cu-ATPase Ccc2 accepts Cu from Atx1 (Banci et al., 2007b), then pumps Cu into the late secretory component, where Cu incorporation into the cuproenzyme, Fet3 multi-Cu ferroxidase occurs with the aid of Gef1 (Greene et al., 1993). Gef1 is an anion channel protein that provides Cl^{-1}, which serves an allosteric role to facilitate Cu loading onto Fet3 within the lumen of a secretory compartment (Davis-Kaplan et al., 1998). Fet3 forms a complex with the iron permease Ftr1 and both proteins are responsible for high affinity iron uptake at the plasma membrane, (Askwith et al., 1994) (Figure 1-2).

In mammals like in yeast, the delivery of Cu to cuproenzymes occurs in the secretory pathway and depend on the Cu-ATPases, ATP7A and ATP7B (homologues of Ccc2), which receive Cu from Atox1 (Larin et al., 1999; Strausak et al., 2003) and pump Cu^{+} to the Golgi lumen for incorporation into cuproenzymes (Lutsenko et al., 2007a). Moreover both ATP7A and ATP7B can restore Fet3 ferroxidase activity in yeast lacking Ccc2 (Yuan et al., 1995). How Cu is incorporated into cuproenzymes is not clear.

However the incorporation of Cu into ceruloplasmin by ATP7B has been reported (Hellman et al., 2002) and this pathway may be relevant to other cuproenzymes. Incorporation of six atoms of Cu into newly synthesised apo-ceruloplasmin results in protein conformational changes that activate the ferroxidase activity of ceruloplasmin. The mutation of one of the Cu binding sites of ceruloplasmin which impede the binding of one of the six Cu atoms results in an inactive protein. Furthermore, this study shows a precise and sensitive mechanism for the formation of holo-ceruloplasmin under limiting conditions of Cu availability which may be applicable to the biosynthesis of other cuproproteins (Hellman et al., 2002). Additionally, ATP7A has been shown to deliver Cu to other enzymes such as tyrosinase, lysyl oxidase and peptidylglycine α-amidating monooxygenase (PAM) (Lutsenko et al., 2007a).

Structure characteristics of Cu-ATPases are described in (2.1.4.3). ATP7A is expressed in all mammal tissues except the liver where its expression is very low (Paynter et al., 1994; Paynter et al., 1994). In contrast, ATP7B is highly expressed in liver, intestine and kidney but is very low in all other tissues (Yamaguchi et al., 1993). In basal or low Cu conditions ATP7A and ATP7B are localized in the trans-Golgi network (TGN) where they function to deliver Cu to cuproenzymes (Voskoboinik and Camakaris 2002). In conditions of high levels of Cu the cellular location of these ATPases is altered. In enterocyte, ATP7A migrates from the TGN to near the basolateral membrane and the excess Cu is pumped into a vesicle which is then exocytosed in the portal vein. ATP7A may also have a crucial role in the transport of Cu to the brain through the blood brain epithelium and to the foetus across the placenta (Monty et al., 2005; Mercer and Llanos 2003). Similarly in hepatocytes, in conditions of Cu overload ATP7B moves from the TGN to a cytoplasmic vesicular compartment near the canalicular membrane where, interacting/assisted by Murr1, it excretes Cu into the bile (Schaefer et al., 1999; Tao et al., 2003). ATP7A and

ATP7B are therefore crucial not only for intracellular Cu homeostasis but also for systemic Cu homeostasis. Interestingly, in human hepatocytes two ATP7B proteins appear to be present, one of 160-kDa and one of 140-kDa. Immunofluorescent staining of HepG2 cells has shown that the 160-kDa protein is present in the trans-Golgi network whilst the 140-kDa protein is located in the mitochondrion. It has been postulated that the lower molecular weight form is a product of proteolytic cleavage within the metal binding repeats at the amino terminus and is targeted to the mitochondria where it is suggested to play a role in mitochondrial Cu ion homeostasis (Lutsenko and Cooper 1998).

Another alternative form of ATP7B, generated by alternative splicing of the ATP7B gene, encodes a pineal gland, night-specific ATPase (PINA) which has been identified by subtractive hybridization studies (Borjigin et al., 1999). PINA was found to be expressed in the pinealocytes and a subset of photoreceptors in adult rats, and transiently in the retinal pigment epithelium and ciliary body during retinal development. Furthermore, PINA expression exhibits a dramatic diurnal variation in both pineal gland and retina with 100-fold greater expression at night than in the day. In mammals, nocturnal pineal expression of PINA is under the control of the suprachiasmatic nucleus, a region of the brain, located in the hypothalamus, which is responsible for controlling endogenous circadian rhythms and innerves the pineal gland (Foulkes et al., 1997). Interestingly, in some non mammalian species, including fish there is a temporal inversion of expression of melatonin (Besseau et al., 2006; Iigo et al., 1997), and this could be related to the fact that melatonin is a potent antioxidant and in tissue such as retina exposed to UV radiation, a system of antioxidant protection could be required (Beyer et al., 1998). Previous studies have shown that the gene expression and protein activity of the rate-limiting enzyme of melatonin synthesis, serotonin N-acetyltransferase (NAT), increases dramatically with the onset of darkness (Borjigin et al., 1995). NAT has been

demonstrated to respond to cAMP which seems to be an essential signal for temporal regulation of melatonin synthesis (Foulkes et al., 1997). Consistent with the role of clock and cAMP signalling in temporal regulation of pineal rhythms, PINA transcription is under clock control and inducible *in vivo* and *in vitro* using agents activating the β-adrenergic receptor and cAMP signalling pathway (Borjigin et al., 1999). Moreover the presence of a number of cis-acting elements that are recognized by a novel pineal/retina-specific nuclear factor, pineal responsive elements (PIRE), in the promoter regions of PINA and NAT could explain the similarity in temporal expression patterns and tissue distributions (Li et al., 1998b). Sequence studies show that, the alternative spliced protein PINA, results in a protein devoid of the amino-terminus, including all the Cu-binding motifs and the first two transmembrane domains. Despite these deletions, PINA is able to restore Cu transport in Ccc2Δ strains (strains lacking Ccc2 gene) of *Saccharomyces cerevisie,* suggesting a novel role for rhythmic light-regulated Cu homeostasis in pineal and retinal circadian functions (Borjigin et al., 1999).

In yeast, many Cu homeostasis genes are transcriptionally regulated through Cu-sensing transcription factors (Winge 1998; Pena et al., 1999), whereas in mammalian cells Cu homeostasis seems to be controlled by post-transcriptional mechanisms (Huffman and O'Halloran 2001). The transcriptional regulation of both ATP7A and ATP7B is still unclear in vertebrates. Promoter studies on the human ATP7A and ATP7B genes show the presence of putative metal responsive elements (MREs) in both genes, which are cis-acting elements typically present in metallothionein genes (Harris et al., 2003; Oh et al., 1999). This evidence might suggest that ATP7A and ATP7B can be transcriptionally regulated by metals under some conditions. There is some support for this view. Bauerly et al. (2005) showed that when rat pups were exposed to Cu in milk the mRNA levels of Cu-homeostasis genes were different in exposed than control groups. In the small intestine

MT and ATP7A and in the liver Ctr1, MT and ATP7B mRNA levels were increased after Cu exposure. Furthermore Ctr1 and ATP7A protein levels were higher in the intestine of Cu-exposed rats compared to controls. Bauerly and colleagues (2005) hypothesized that higher expressions of hepatic Ctr1 and intestinal ATP7A increases Cu transport to the liver and may facilitate the biliary excretion although this is not consistent with the previous theory of regulation of Cu status by basolateral membrane extrusion (in intestine) which would involve a reduction in intestinal expression or activity of Ctr1 and or ATP7A (Linder 1991; Turnlund 1998).

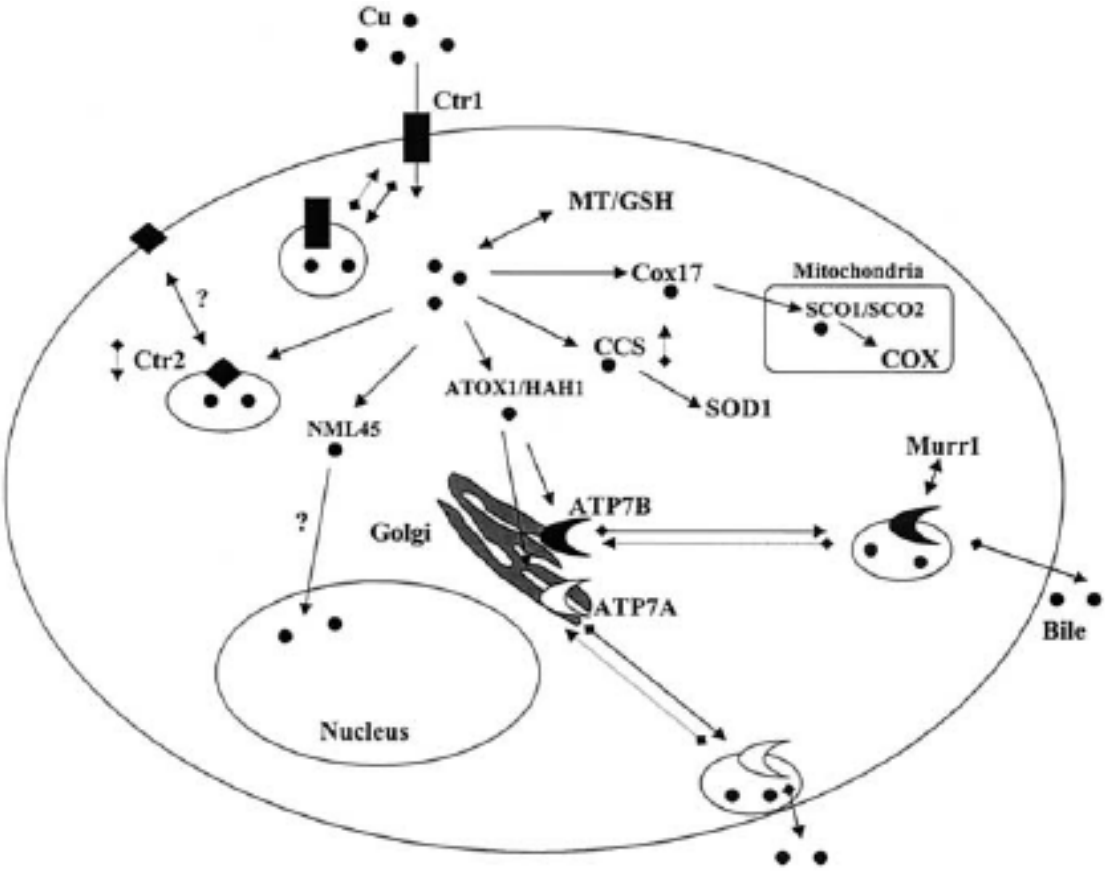

Figure 1-4 Copper transport and distribution in Mammals. (from Bertinato and L'Abbé, 2004).

1.5.4.1 Genetic disorders related to Cu-ATPases

Both ATP7A and ATP7B are well characterised in humans and rodents because they are responsible for Cu deficiency and overload disorders, Menkes Syndrome and Wilson's Disease, respectively (Mercer 2001).

Menkes Disease (MD) is an X-linked Cu deficiency syndrome with a frequency of about 1 in 200000, first described in 1962 by John Menkes. Affected boys usually die in early childhood and have multiple abnormalities that can be related to deficiencies in cuproenzymes (Danks 1995). MD is caused by mutations in the gene encoding the Menkes protein and is characterized by overall Cu deficiency and accumulation of Cu in intestinal enterocytes and the kidney. The molecular explanation of MD is a number of mutations on the ATP7A gene that generate a non-functional protein. This results in nutritionally inadequate amounts of Cu available to the system (Mercer 2001). Milder forms of Menkes Syndrome are characterized by a missense mutation in the seventh transmembrane domain that impedes migration of the ATP7A protein to the plasma membrane in response to Cu (Ambrosini and Mercer 1999). The explanation of a milder course of the disease (mild neurological effect and mild connective tissue disease) could be because of the localization of a partially active protein in the trans Golgi network (TGN), which is only able to deliver trace amounts of Cu to lysyl oxidase (Mercer 2001). Defects in the Menkes gene have demonstrated the importance of adequate supplies of Cu during development, in humans, mice (Cecchi et al., 1997) and zebra fish (Mendelsohn et al., 2006) and demonstrate that Cu is an essential nutrient for normal development. The effect of Cu deficiency either from a lack of Cu in the diet or from genetic disorders results in an impaired function of cuproenzymes. Copper deficiency in early developmental stages results in embryo resorption and in later stages in severe

neurological impairment and multiple defects in organogenesis. These abnormalities are indicative of connective tissue weakness, probably attributable to lysyl oxidase deficiency (Hamza et al., 2001; Keen et al., 1998; Mercer and Llanos 2003). In a recent study, a fish model was chosen to investigate the effect of Cu deficiency during embryonic development. The optical clarity and rapid external development of zebrafish embryos enabled the characterization of deficient phenotypes from the moment of fertilization. The effect of Cu deficiency during embryonic development was experimentally recreated by Cu deficiency using molecules with Cu chelating activity (neocuproine) that impede normal Cu uptake or by using a genetically modified mutant defective in the ATP7A gene (*calamity*). In either case a Cu deficient condition resulted in abnormal embryonic development showing that ATP7A is essential for the notochord formation (Mendelsohn et al., 2006). Moreover, the severity of the phenotype was related to the gene dosage and Cu availability. While neocuproine did not affect melanin pigmentation, which is the result of the cuproenzyme tyrosinase, in wild-type zebrafish embryos, the same dose of neocuproine prevents melanin pigmentation in *calamity* heterozygotes and worsened the notochord formation in calamity homozygotes. The cuproenzyme lysyl oxidase in addition to its fundamental role in connective tissue formation may be important also for the notochord formation as high levels of expression of lysyl oxidase were detected within the developing notochord in *Xenopus laevis* (Geach and Dale 2005).

Wilson's Disease (WD) is an autosomal recessive Cu toxicosis condition and while MD affects young boys WD is an adult-onset disorder with an incidence of one in 35000-100000. WD is caused by a slow build-up of Cu first in liver (because the biliary excretion is defective) and subsequently in kidney, brain, cornea and mammary gland (Gitlin 2003). The build up of Cu causes death of the hepatocytes, release of Cu and accumulation in extra-hepatic tissues including the central nervous system; Cu deposits can sometimes be

seen in the cornea of the eyes, known as Kayser-Flescher rings (Gitlin 2003). WD patients often have a low level of plasma holo-ceruloplasmin but the apo-ceruloplasmin protein (which is inactive) concentration may be close to normal (Danks 1995; Gitlin 2003). The correlation between genotype and phenotype is complicated by the fact that many WD patients are compound heterozygotes meaning that they carry different ATP7B mutations on each allele. Patients with ATP7B homozygous severe mutations have an earlier onset of the disease (Thomas et al., 1995) wheras milder heterozygotes mutations may present with later-onset neurological disease (Forbes and Cox 2000). The most common mild form of Wilson disease is due to a mutated ATP7B which has lost its trafficking properties such has migration to the canalicular membrane to excrete Cu into the biliary duct but is still able to deliver Cu to ceruloplasmin at the Golgi apparatus. Other factors can influence the severity of the disease such as amount of Cu ingested with the diet or allelic variation of MT genes and these factors could explain why the common His1069Gln mutation is associated with a range of clinical presentations (Duc et al., 1998).

Crucial for the understanding of Cu genetic disorders in humans have been the animal models. For MD these include the mottled mice (Cecchi et al., 1997; Cecchi and Avner 1996) and the recently discovered zebafish *calamity* (Mendelsohn et al., 2006). For WD these are the toxic milk mouse (Theophilos et al., 1996) and Long Evans Cinnamon (LEC) rats (Terada et al., 1998; Terada et al., 1999).

1.5.5 Extracellular Copper transport

In the enterocytes, transport from the gut lumen to the blood stream involves Ctr1 (Nose et al., 2006a) and the Atox1-ATP7A axis (Yuan et al., 1995; Wernimont et al., 2000; Banci et al., 2007a; Larin et al., 1999), and potentially each one of these proteins could therefore regulate the absorption from the intestine to the blood stream. Indeed,

specific knock out of intestinal Ctr1 results in a phenotype very similar to the Menkes disease patients who have a mutated ATP7A protein (Nose et al., 2006a). Through the portal vein, Cu reaches the liver where it is delivered by Ctr1 (Puig and Thiele 2002) to the excretory pathway through the Atox1-ATP7B axis (Larin et al., 1999). At this level ATP7B regulates intracellular and consequently body status Cu concentration mediating Cu excretion into the bile (Linder et al., 1998; Guo et al., 2005).

Radiotracer studies have been used to follow dietary absorption, distribution and excretion of Cu in rats (Bissig et al., 2005; Linder et al., 1998). Most of the Cu absorbed by the intestine is delivered to the liver and less to kidney and other tissues whilst biliary excretion of excess Cu occurs after 15 min. Moreover Cu is excreted in the bile in a less re-absorbable form (Linder et al., 1998). The dose of radioactive Cu in the plasma over time appears in two waves of distribution. In this first wave Cu exiting the intestine is mainly bound to albumin and transcuprein (also known as macroglobulin α1-inhibitor III) (Linder et al., 1998; Lui et al., 2007) and plasma levels drop within 30 min due to absorption by the liver and kidney. Whilst not mechanistically proven, the main candidate for delivering Cu to the liver, considering that the bulk of Cu derived from the intestine is mainly bound to the transcuprein, a 270 kDa plasma protein which bind Cu^{2+} with high efficiency and to albumin, with which it readily exchanges Cu^{2+}, is transcuprein. Moreover, transcuprein gene expression has been shown to be influenced by nutritional Cu and iron status (Lui et al., 2007) although conclusive evidence for a central role in Cu transport is still lacking. The liver is the site of synthesis of the major plasma cuproprotein, ceruloplasmin (CP). In fish the liver is also the major organ involved in Cu homeostasis (Grosell et al., 1997; Grosell et al., 2000; Kamunde et al., 2001; Kamunde et al., 2002a) as it accumulates a large proportion of the Cu absorbed from diet or water and it is the site for the synthesis of the most abundant Cu-containing plasma protein;

ceruloplasmin. After 6 h Cu re-emerges in the plasma from the liver bound to ceruloplasmin (Linder et al., 1998). Ceruloplasmin is synthesized as a holo-protein with six atoms of Cu (possibly Cu^{+}) transferred by ATP7B incorporated during its biosynthesis (Hellman et al., 2002). It is then secreted into the blood (Hellman and Gitlin 2002). 95% of plasma Cu is present as CP and the primary physiologic function of CP is to oxidize Fe^{2+} to Fe^{3+}, enabling it to be bound to the plasma iron binding protein, transferrin which can then be taken up by cells, thus providing them with Fe (Frieden 1980). Lack of ceruloplasmin in the blood results in hepatic iron accumulation and this is one of the symptoms of Wilson's Disease (Gitlin 2003). CP has also free radical scavenging properties (Gutteridge 1985). For many years ceruloplasmin was thought to have a role in delivering Cu to tissues, however, CP knock out mice do not show abnormalities in tissue Cu absorption or CuZn-SOD activity suggesting that ceruloplasmin does not have an essential role in Cu transport and metabolism (Meyer et al., 2001).

A mutation on the gene coding for the protein ceruloplasmin, results in a medical condition known as aceruloplasminemia, which is one of the rarest Cu-related disorders (Miyajima et al., 1987). Aceruloplasminemic individuals have no oxidase-detectable or immunoreactive ceruloplasmin in their serum (Miyajima et al., 1987). The absence of ceruloplasmin does not produce marked changes in Cu metabolism, it does, however, produce a gradual accumulation of iron in liver and other tissues (Yoshida et al., 1995; Gitlin 1998) and Cu in liver (Meyer et al., 2001). However, Cu bound to ceruloplasmin was shown to be absorbed more efficiently than Cu bound to albumin and transcuprein by the rat fetus (Lee et al., 1993), therefore its role in Cu delivery to tissues can not be excluded. Previous studies have identified ceruloplasmin as a ferroxidase and are remarkably consistent with studies on the essential role of a homologous Cu oxidase (FET3) in iron metabolism in yeast (Harris et al., 1995). Consistently, ceruloplasmin

knockout mouse shows progressive accumulation of iron predominantly in the organs of the reticuloendothelial system, and an increase in serum ferritin, a marker of increased iron storage (Harris et al., 1999; Meyer et al., 2001). The predominant clinical syntoms in patients with aceruloplasminemia are neurological and suggest that ceruloplasmin plays an essential role in normal brain iron metabolism (Waggoner et al., 1999).

A diagram for human Cu absorption and distribution at the organ and tissue level is represented in Figure 1-5.

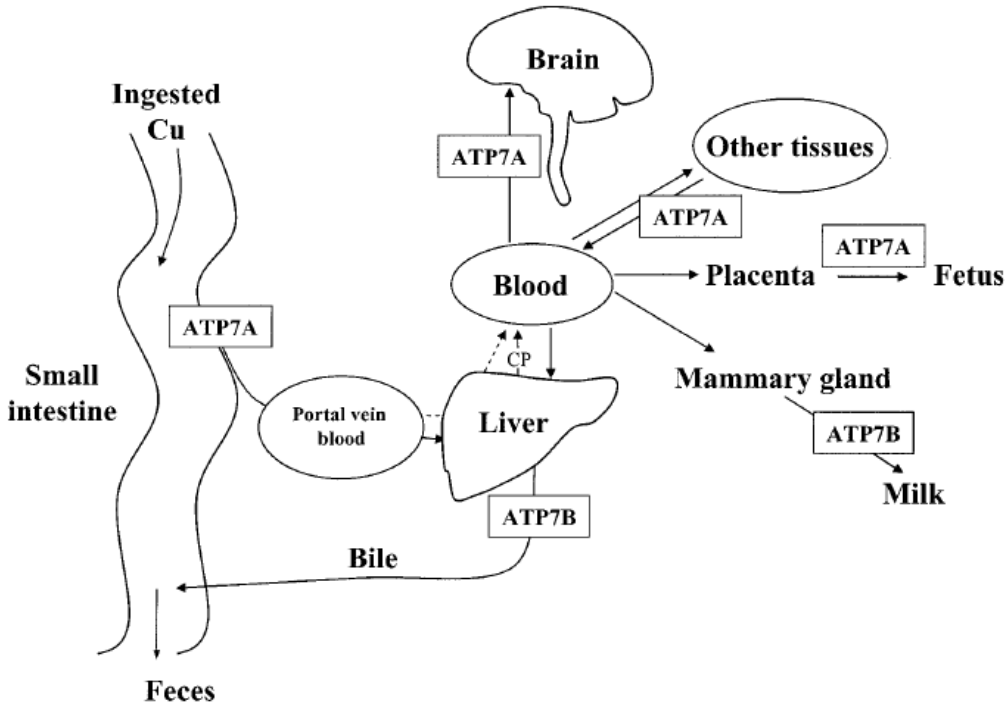

Figure 1-5 Model for human copper absorption and distribution at the organ and tissue level. (from Mercer and Llanos, 2003).

1.5.6 Other diseases related to copper metabolism

Indian childhood cirrhosis (ICC) presents a similar phenotype to the CT in Bedlington terriers and is commonly a fatal disease characterized by massive Cu

accumulation in the liver lysosomes (George, unpublished). It is primarily due to ingestion of high quantities of Cu from old cooking pots during infancy, however, once Cu intake is reduced, the liver condition returns to normal (O'Neill and Tanner 1989). Whilst it is not attributable to a genetic defect in ATP7B, nevertheless, like the CT in Bedlington terriers, it shows an autosomal recessive pattern of inheritance. Thus the non-Wilsonian hepatic Cu toxicoses are ecogenetic disorders requiring the involvement of both genetic and environmental factors for the disease to become manifest (Muller et al., 1999; Muller et al., 1996).

Copper has also been strongly implicated in neurodegenerative diseases such as Alzheimer`s Disease, Parkinson's Disease, spongiform encephalopathies (prion diseases) and familial amyotropic lateral sclerosis (FALS) (Waggoner et al., 1999; Strausak et al., 2001). A common theme that emerges is the possibility of Cu-induced free radical production leading to neurological damage. Copper disease highlights the equally devastating result of deficiency and excess and the consequences of breakdown of homeostatic mechanisms.

1.5.7 Copper homeostasis in fish

Fish represent a unique model among vertebrates because they can absorb Cu through two routes; waterborne Cu can enter through the gills while dietary Cu is absorbed through the intestine (Kamunde et al., 2002b; Grosell and Wood 2002). In fish, diet is the main source of Cu under optimal growth conditions (Handy 1996; Kamunde et al., 2002b; Kamunde et al., 2002a) but in conditions of low Cu in the diet, fish are able to absorb Cu from the water through the gill (Miller et al., 1993; Kamunde et al., 2002b). These observations might therefore suggest the presence of Cu transporters in fish that respond to body Cu status. In mammals, regulation of total body Cu is mediated by

regulating the absorption of Cu in the intestine and the rate of hepatic excretion of Cu into the bile (Turnlund 1998). There is evidence that the intestinal regulation of Cu absorption in fish is, similarly to mammals, dependent on the Cu concentration in the diet and the rate limiting step is extrusion through the basolateral membrane of intestinal epithelium cells (Clearwater et al., 2000; Handy et al., 2000). It has been proposed that the intestine regulates Cu absorption, at the basolateral membrane, by a Cu P-type ATPase and by a Cu/anion symporter (Handy et al., 2000). Even though ATP7B, responsible for excess Cu excretion in mammals, is highly expressed in both liver and kidney (Yamaguchi et al., 1993) the main site for excretion of excess Cu is *via* bile and very little Cu is excreted in the urine (Linder et al., 1998). In fish, a similar situation has been reported (Grosell et al., 1998; Grosell et al., 2000). Therefore, intestinal basolateral membrane extrusion and biliary excretion in fish, like in mammals, are the main homeostatic mechanisms for controlling and regulating body Cu levels. In contrast to freshwater fish species, following branchial exposure, marine fish species can accumulate Cu in the kidney, although at considerably lower levels than liver (Grosell et al., 2003; Grosell et al., 1997). Significant amounts of Cu accumulate in the gills of FW fish fed elevated dietary concentrations even when water concentrations are normal inferring the presence of active Cu transport proteins and a role for the gill in the homeostatic response to excess dietary Cu (Handy 1996; Kamunde et al., 2001). Indeed this hypothesis has been supported by the identification of a vanadate-sensitive Cu transporting ATPase in fish gills (Campbell et al., 1999) which may be effective in basolateral Cu transport. Branchial uptake contributes approximately 60% of the body Cu load during deficiency, but diet is the preferred source of Cu under normal dietary and waterborne conditions, contributing more than 90% of the body intake (Kamunde et al., 2002a). These findings suggest a key role for the gills in Cu

homeostasis in fish and provide evidence of the gill as an organ of nutritional regulation under certain conditions (Kamunde et al., 2002b; Kamunde et al., 2002a).

Bunton et al., (1987) have shown that in a fish species white perch (*Morone americana*) there is an abnormal, age related, accumulation of Cu in hepatic lysosomes (up to 1000 μg Cu g^{-1} wet weight) which is pathologically similar to other Cu toxicosis. Since this pathology is not present in a closely related species (*Morone saxatilis*) obtained from the same site it is possible that the white perch may have a genetic or ecogenetic defect in one of the molecular components of the Cu metabolizing and trafficking machinery. The availability of this model may prove useful for fundamental studies on Cu homeostasis and regulation in fish.

1.6 Thesis aim and objectives

The physiologic evidence discussed in 1.5.7 suggests that fish, similarly to mammals, possesses dedicated molecular mechanisms to regulate Cu homeostasis in different environmental conditions. The overall aim of these studies is to understand the molecular basis of the observed physiological uptake, transport and excretion of Cu in fish. In addressing this aim the following specific objectives are defined:

1. **Identify clones homologues for Cu transporters in sea bream**: the high level of conservation in Cu transporters genes between yeast and mammals indicates that specific Cu transporters must also be present in fish. In order to understand the molecular basis of Cu homeostasis in fish it is essential to study these Cu transporter genes and therefore suitable probes are required.

2. **Measure the tissue expression profiles of Cu transporters in sea bream**: the measurement of Cu transporters mRNA expression in a wide range of tissues may be relevant to understand their function and their role in Cu homeostasis.
3. **Evaluate the expression of Cu transporters following dietary or waterborne Cu exposure**: previous studies (see 1.5.7) have shown that fish regulate body Cu homeostasis in response to dietary and waterborne Cu concentration however the molecular basis of these homeostatic processes are currently unknown. Therfore evaluation of their transcriptional regulation is important**.**
4. **Identify a suitable *in vitro* system for Cu homeostasis studies**: in sea bream few cell lines are available, however an established cell line, the *Sparus aurata* fibroblast (SAF1), is known. The use of a cellular model enables more rigorous control of experimental conditions, enabling the application of transcriptomic (microarray) tools to enable a broader and more comprehensive investigation of the response to excess Cu and potentially can lead to the discovery of new candidate genes and pathways involved in Cu homeostasis.

Chapter 2. Synthesis of sea bream cDNAs for copper homeostasis genes

2.1 Introduction

In attempting to understand the mechanisms of Cu homeostasis in fish, whether under conditions of deficiency, sufficiency or excess, it is essential to consider known Cu-homeostasis specific proteins. The first step toward this goal is to synthesise the cDNAs for the fish homologues of the characterized mammalian Cu homeostasis genes. This enables prediction of the protein sequences and the generation of molecular probes to measure the expression of these genes in different tissues and under different Cu environmental conditions.

In mammals, Cu body status is maintained mainly through the regulation of intestinal absorption and hepatic biliary excretion (1.5.5). Therefore, the following candidates for the regulation of these homeostatic functions were targeted in sea bream for further studies:

- the high affinity Cu transporter 1 (Ctr1) responsible for cellular Cu uptake (Petris 2004);
- the Cu chaperone Atox1 responsible for delivering Cu to the ATPases ATP7A and ATP7B (Field et al., 2002);

- the ATPase ATP7A responsible for the regulation of Cu absorption through the intestine and other tissues (Lutsenko et al., 2007a);
- the ATPase ATP7B responsible for the regulation of excretion of excess Cu into the bile (Lutsenko et al., 2007a).

Furthermore, to better understand the intracellular molecular response to excess Cu, the sea bream cDNAs of genes involved in Cu sequestration and storage such as MT (Coyle et al., 2002) and antioxidant defence such as CuZn-SOD (Fridovich 1995) and glutathione reductase (Winston and Di Giulio 1991) were targeted. In addition three well-known reference genes (Olsvik et al., 2005) such as: β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 1α (EF1 α) were targeted to enable normalization of quantitative measures of gene expression (Vandesompele et al., 2002).

2.1.1 Gene isolation strategy

In the last twenty years a considerable effort has been made to sequence the complete genome of a variety of organisms including human, and other mammals but also for model fish species such as fugu (*Fugu rubripes*) and the zebrafish (*Danio rerio*) (Roberts et al., 2001). The isolation of genes of interest in fish species where complete cDNA and EST's (Expressed Sequence Tags) libraries are not available rely on other fish or other vertebrate gene data bases. The most complete and accurate gene databases available online are the NCBI (National Centre for Biotechnology Information) (www.ncbi.nlm.nih.gov/Genbank/) and the EMBL (European Molecular Biology Labs) (www.ebi.ac.uk/Databases/). These websites contain bioinformatic tools such as BLAST which enable the search of the database against a homologous known gene sequence from another species allowing the identification of orthologous genes.

The level of conservation of the genes investigated, the knowledge of mammalian and yeast functionally characterised Cu homeostasis genes (Puig et al., 2002; Hamza et al., 2003; Voskoboinik and Camakaris 2002), and the availability of fish gene databases have enabled the isolation of sea bream Cu homeostatic genes and other genes of interest. The technique used for the isolation of the cDNA of interest in this study was based on PCR (Polymerase Chain Reaction). The information, along with the sequences of Cu homeostasis genes from mammals and other organisms that have periodically appeared in the Genbank database, enabled alignments to be made between deduced protein sequences. These alignments enable the structurally and functionally important regions to be identified, based on highest conservation of sequence across species. Such alignments also enable the design of oligonuclotide primers which can be used to isolate the homologous genes from uncharacterised species, such as sea bream.

2.1.2 Primer design

Even though the efficiency of a PCR reaction is dependent on many variables, the key point to isolate the cDNA corresponding to the gene of interest is the accuracy with which the PCR primers are designed. In this study the software PrimerSelect 6.1, DNASTAR, USA was used. This software was particularly useful when designing primers on known sequence templates and takes into consideration several variables which influence priming efficiency, such as: GC content, primer length, formation of primer secondary structures (duplex structure with each other or hairpin loops within themselves) and annealing temperature (Tm).

The strategy used to clone the sea bream sequences, coding for the genes of interest, was based on designing degenerate primers predicted from multiple sequence alignments of characterised genes in mammals, reptile, amphibian and fish if available.

These primers have then been used to amplify Cu homeostasis genes by PCR. Degenerate primers are mixtures of oligonucleotide sequences which only differ in having alternative nucleotides at key positions predicted to vary based on known of potential differences in cross-species target alignments (Kwok et al., 1994) (Appendix 1). Even though software exists and it has been used in this study to design degenerate primers such as CODEHOP (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) the best results were achieved designing primers manually and using a few simple rules. The first step was to identify functionally conserved regions of the peptide sequence alignment. Then by analysing the corresponding nucleotide sequence, identify part of the consensus sequence that is the result of the least redundant overlap of all the aligned sequences. A maximum level of degeneracy was set such that only up to 16 possible oligonucleotides were possible. The general rules used to design primers were (Chen et al., 2002):

- Sequence length 20-28 with CG content of (45-60%).
- Select primers with Tm between 55 and 70˚C.
- The 3' end of the primer should be less redundant as possible and containing not more that 3 G or C in the 3'-most 5 bases.
- Avoid sequences with 3' ends that can self-hybridize or hybridize to the 3' ends of other primer in the PCR (forming primer dimers).

2.1.3 PCR strategies

The combination of degenerate primers with touch down PCR and nested PCR is a very powerful strategy to isolate cDNAs from species where sequence information is limited or not available (see 2.1.1).

2.1.3.1 Touch down PCR

Touchdown PCR is a modification of conventional PCR that may result in a reduction of nonspecific amplification. It involves the use of primers with differing optimum annealing temperatures and using a high temperature in early PCR cycles. The annealing temperature is decreased by 1 or 2°C every cycle or every five cycles until the optimum annealing temperature for the second primer or 'touchdown' temperature is reached. The touchdown temperature is then used for the remaining number of cycles. This allows for the enrichment of the desired product primed by a single primer early in the reaction, thus increasing the likelihood of synthesizing target after completion of the PCR reaction (Don et al., 1991).

2.1.3.2 Nested PCR

Nested PCR is another PCR strategy which is particularly useful when, after first round PCR, low or undetectable target PCR products is obtained. A second round PCR is performed on the product of the first PCR reaction using primers which are internal to the original primers. This improves sensitivity without impairing specificity (McPherson et al., 1991).

2.1.3.3 RACE PCR

Rapid Amplification of cDNA Ends (RACE) is a PCR-based technique which facilitates the cloning of full-length cDNA sequences when only a partial cDNA sequence is available (Frohman 1994). In this study two RACE kits have been used; the FirstChoice® RLM-RACE (Ambion, UK) and the SMART™ RACE (Clontech Laboratories, USA). Briefly, the two kits use two very different approaches to extend the 5'end of the cDNA, whilst at the 3' both kits use the same strategy based on oligo dT

reverse primers equipped with an adaptor sequence. The cDNA produced is then subjected to one or two (nested) rounds of PCR using the gene specific forward primer and the reverse kit primer complementary to the 3'adaptor. The Ambion strategy to extend the 5' end of the cDNA relies on an enzymatic treatment which selectively ligates a 5`adaptor sequence to the full-length (capped) mRNA. A primer complementary to the adaptor sequence and a target-specific primer is then used to amplify the 5' end, either directly or after nested PCR using a second target-specific primer. The Clontech kit relies on the fact that Murine Leukemia Virus Reverse Transcriptase (MMLV RT) exhibits terminal 5' transferase activity, adding 3-5 deoxycytidine (dC) residues to combine with a SMART (Switching Mechanism At 5' end of RNA Transcript) oligonucleotide primer (ribo-guanosine template-switch oligo) which priming on the dC stretch generate a modified cDNA. The cDNA produced can then be subjected directly to 5' RACE PCR using gene specific reverse primers and kit forward primers directed against the SMART sequence (Matz et al., 1999).

2.1.4 Structural characteristics of copper homeostasis genes

Copper homeostasis proteins in species as different as bacteria and humans are characterized by common structural motifs which enable a specific and tight binding to prevent Cu from engaging unwanted interactions but at the same time flexible to allow ease and efficient transfer to target proteins that require Cu to carry out vital biological processes (Finney and O'Halloran 2003). These structural motifs are the signature of this family of proteins (Huffman and O'Halloran 2001) (1.5) and can be utilized to putatively identify candidate Cu transporter proteins.

2.1.4.1 Copper transporter 1 - Ctr1

All Ctr family members contain three predicted transmembrane regions, characterized by stretches of hydrophobic amino acids. The amino terminal (Nt) region is rich in methionines that are arranged as MxxM and MxM motif (Mets motif shown in blue in Figure 1-3b). This domain is highly conserved and appears in other proteins involved in Cu trafficking and homeostasis (Jiang et al., 2005). In addition, the Met motifs seem to be involved in Cu sensing rather than transport, a role that is delegated to the second transmembrane domain MxxxM (Guo et al., 2004). Finally, the carboxyl-terminal (Ct) portion of Ctr1 is rich in charged amino acids and contains highly phylogenetically conserved cysteines and histidines (Cys/His motifs shown in green in Figure 1-3b). The function of this domain is not clear, however there is some evidence of protein-protein interaction with the chaperone Atx1 in yeast (Xiao and Wedd 2002; Xiao et al., 2004). On the basis of computer algorithm analyses the proposed topological structure of Ctr1 family members, would locate the Met motifs outside the cell and the Cys/His motif in the cytosol (Figure 1-3b). Crystallography and electron microscopy demonstrate that human Ctr1 forms a symmetrical homotrimer with a putative pore between the subunit interfaces (Aller and Unger 2006). Moreover the GG4 motif (Gly-X-X-X-Gly) present on the third transmembrane domain has been shown to be important for multimerization and for proper localization in the yeast plasma membrane (Aller et al., 2004). This architecture reveals that Ctr1 proteins have a structure closely related to ion channel proteins, where the metal binding domains coordinate and probably drive Cu atoms to the pore (Nose et al., 2006b) (Figure 2-1).

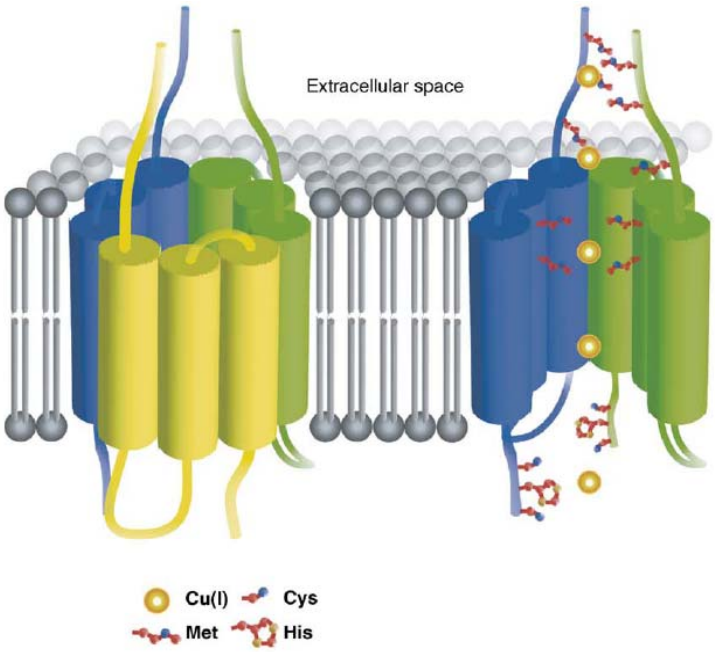

Figure **2-1** Interpretation of the structure and functional model of Ctr1. Each monomer is represented in a different colour. Copper atoms (orange spheres) and the side chains of methionine, histidine and cysteine are indicated (from Nose et al., 2006).

2.1.4.2 Copper chaperone Atox1

Atox1 is a protein of 68 amino acids characterised by a highly phylogenetically conserved Cu binding motif MxCxxC. Structurally Atox1 adopts a βαββαβ folding with the Cu binding motif located in the connecting loop between the first β-sheet and the first α-helix (Ralle et al., 2003). Crystallographic studies show Atox1 as a homo-dimer situated around a single atom of Cu coordinated by four cysteines (Figure 2-2 A). Moreover, the transfer process between the Atox1 Cu binding motif and the similar Cu binding domain (CxxC) located at the N terminus of Cu-ATPases occurs through a three coordinate intermediate (Wernimont et al., 2000) (Figure 2-2). *In vivo*, Cu-Atox1 has been demonstrated to transfer Cu to the N-terminus of ATP7B in a direct and reversible manner (Achila et al., 2006; Banci et al., 2007a).

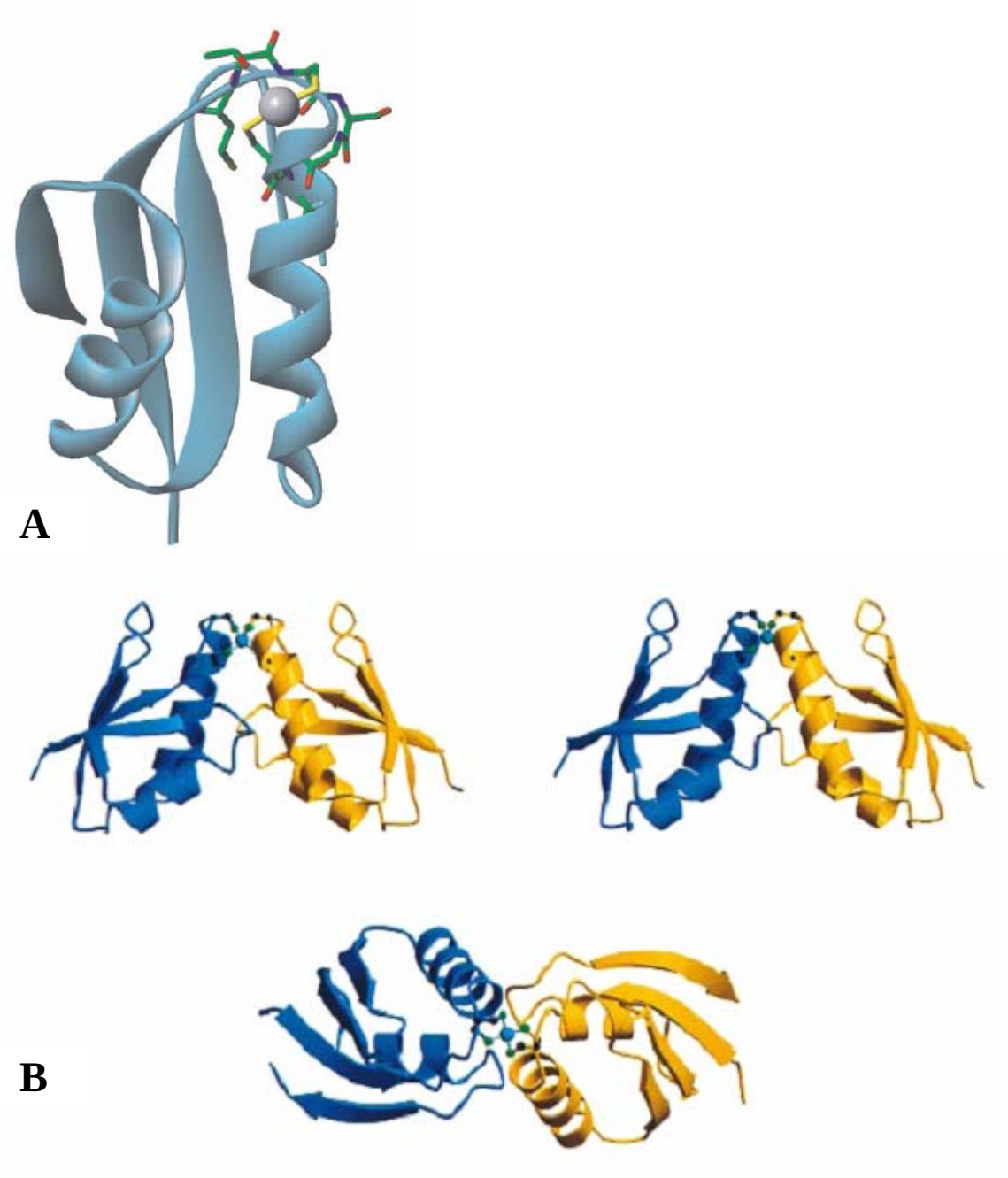

Figure 2-2 Protein crystal structure of Atox1. A Atx1 monomer showing the mercury atom linearly coordinated to two cysteine residues (Hg^{2+}-Atx1 is known to mimic Cu^{1+}-Atx1 coordination state), Cys15 (exposed to solvent) and Cys18 (part of helix 1) (from Ralle et al., 2003) B Atox1 dimer, the Cu ion is shown as cyan sphere and the four Cys residues in the two MxCxxC motif are shown as a ball and stick representation (from Wernimont et al., 2000).

2.1.4.3 Cu-ATPase ATP7A and ATP7B

The human proteins ATP7A and ATP7B have 67 % common amino acid identity. Both ATP7A and ATP7B have six metal binding motifs (GMxCxxC) in the N-terminal region; these are presumed to accept metals from cytoplasmic carriers and/or assist in delivering Cu to the channel (Mercer 2001; Achila et al., 2006). Each of these sequences folds into a stable βαββαβ (like Atox1) and binds a single Cu ion in the reduced Cu^{+} form via two cysteine residues.

The transmembrane portion has eight trans-membrane domains (TMD) which forms the ion channel. The ATP-binding domain located between TMD 6 and TMD 7 is composed of two independent parts: the nucleotide-binding domain (N-domain), which is involved in coordination of the adenine moiety, and the phosphorylation domain (P-domain), which contains residues directly involved in catalytic reaction, including an invariant aspartic acid (D) residue within a conserved DKTG motif (Lutsenko et al., 2007b). Another important motif in Cu-ATPases structure is the DxxK motif located between TMD6 and TMD7 at the end of the flexible linker connecting the N- and P-domains. Mutation of these residues results in a significant decrease of ATP7A Cu transport activity (Voskoboinik et al., 2003).

The amount of data available on the structural importance of the C-terminus of Cu ATPases is less compared to other domains of these proteins, however it has been demonstrated that it has a role in stability and the regulation of Cu pumping. Deletion of the entire C-terminal tail in ATP7A and ATP7B has a detrimental effect on protein stability (Dagenais et al., 2001; Hsi et al., 2004). Moreover Hsi et al., (2004) suggest that the C-terminus may interact with other protein regions promoting its correct folding and/or protecting this region from intracellular proteases. In addition the leucine - leucine

$L^{1487}L^{1488}$ motif located at the C-terminus of ATP7A contributes to the trafficking control of the transporter, maintaining the steady-state localization of the protein within the TNG. Deletion of this LL motif results in migration of the protein from the perinuclear region towards the vesicles and the plasma membrane (Petris et al., 1998). Similarly mutation of the tri-leucine $LLL^{1454-1456}$ at the C-terminus of ATP7B causes its constitutive localization to vesicles and not to the plasma membrane, suggesting that a vesicular compartment(s) is the final trafficking destination for ATP7B, however the significance of this result is not completely clear (Cater et al., 2006). The Cu-ATPase protein structural features are represented in Figure 2-3.

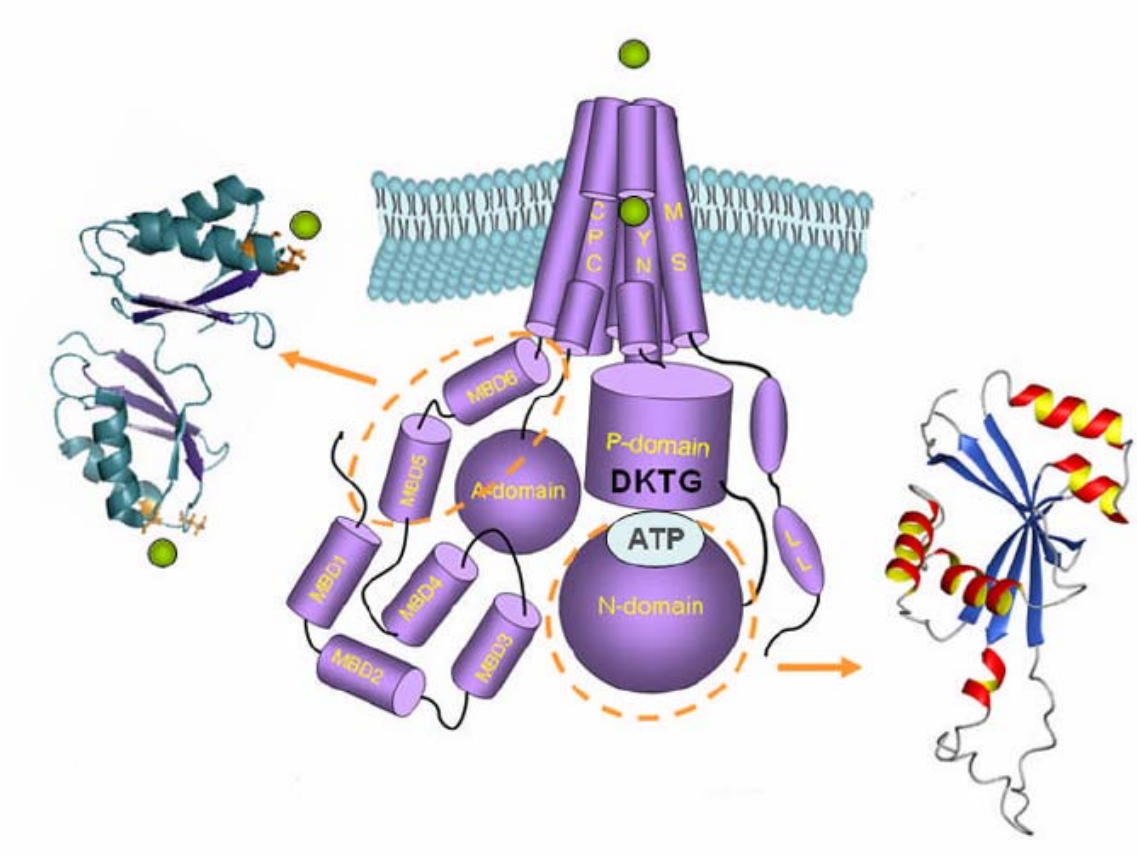

Figure 2-3 Protein structure of human Cu-ATPases. Interaction with Atox1 shown on the left of the picture, Cu represented as a green sphere. The MBD 1 to 6, the A domain which is involved with the N domain in conformational changes are indicated as well as the trans-membrane residues predicted to be involved in Cu coordination (CPC, YN, MxxS). The ATP binding domain consists

of the P and N domain. The two leucines at the C-terminus tails required for endocytosis and/or return to TGN, are indicated. The parts of the protein which structure has been experimentally determined (by NMR) are indicated by dashed circles and corresponding folding structure are shown (form Lutsenko et al., 2007).

2.1.4.4 Metallothionein

Metallothioneins constitute a protein superfamily structurally characterised by a high content in cysteines (up to one third of the total) the positions of which are highly conserved (Figure 2-4). 2D Nuclear magnetic resonance (NMR) spectroscopy shows that despite different amino acid sequences MTs have similar spatial structures with two metal-thiolate clusters containing three and four bivalent metal ions, respectively (Braun et al., 1992). These clusters bind different metal ions depending on the stability of the clusters (A>B) and on the affinity of the metal for cysteines. The relative levels of affinity for various metals is: Zn(II)< Pb(II)< Cd(II)< Cu(I), Ag(I), Hg(II) (Kagi and Kojima 1987).

The MT gene family in mammals consists of four subfamilies (MT-1 – MT-4). MT-1 and MT-2 are widely expressed in all the body tissues and seem to be involved in Zn metabolism, MT-3 is expressed mainly in the brain and MT-4 is mostly present in certain squamous epithelia (Coyle et al., 2002). In fish, one or two MT genes, coding for different MT isoforms, have been reported depending on the fish species (Knapen et al., 2005; Kille et al., 1991). The translated part of the two MT gene isoforms, within the same species, is nearly identical (one or two amino acids different), the main differences are in the 5' and 3' untranslated regions (UTR). Moreover, in Antarctic fish (*Chionodraco hamatus*), the two MT isoforms are differently regulated, one is constitutively expressed and the other is more metal inducible, suggesting that they have differing physiological roles (Carginale et al., 1998; Olson et al., 1995; Bargelloni et al., 1999). In addition, the

differences in expression of the two MT isoforms could be due to different numbers of MREs in the promoter region of the genes (Scudiero et al., 2001; Mayer et al., 2003).

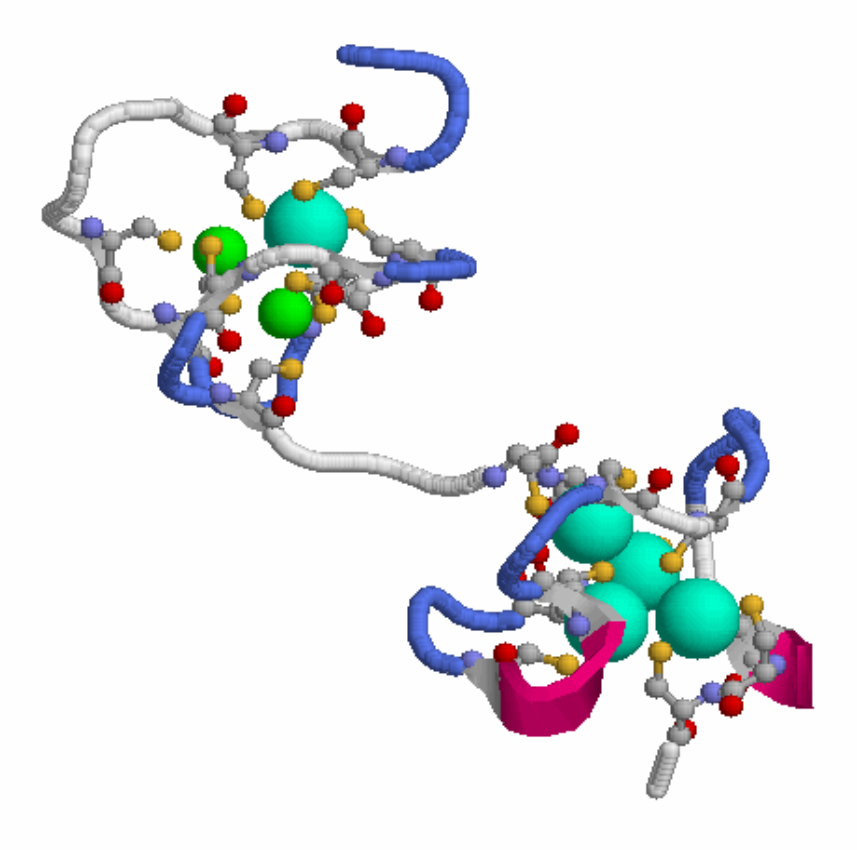

Figure 2-4 MT structure. Rat MT-II binding 4 atoms of Cd and 2 of Zn represented in cyan and in green spheres respectively. The cysteines' sulphur atoms are represented in orange spheres. (PDB from Braun et al., 1992; elaborated using Rasmol software).

2.1.4.5 CuZn-SOD

As briefly described in 1.4, CuZn-SOD is a metalloenzyme which protects the cell from oxygen toxicity by catalyzing the dismutation of superoxide ($O_2^{\cdot-}$) into molecular oxygen and hydrogen peroxide (Fridovich 1995).

Human CuZn-SOD is a protein of 154 amino acids. The functional protein is a homodimer; each monomer binds one Cu and one Zn ion and displays the Greek key β-barrel folding topology (Figure 2-5). Crystallographic studies show that Cu and Zn ions

are bridged by an imidazole ring of histidine[61]. Copper is coordinated by four histidines residues and a water molecule and Zn is coordinated by three histidines residues and an aspartate (Hough and Hasnain 1999) (Figure 2-21).

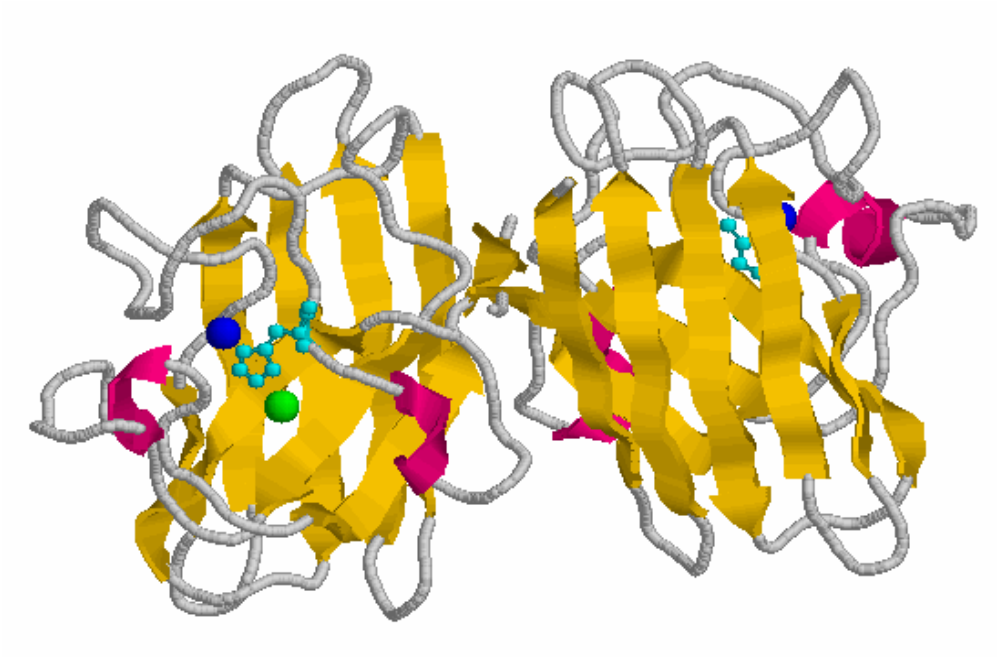

Figure 2-5 CuZn-SOD structure. Human CuZn-SOD. Cu and Zn atoms are represented in green and blue respectively the His 63 in displayed in blue ball and stick. (PDB from Hart et al., 1998 elaborated using Rasmol software).

2.1.4.6 Glutathione reductase

Glutathione reductase (GR) has a role in the cellular oxidative defence maintaining the optimum level of oxidized and reduced glutathione (GSH: GSSG) under oxidative stress conditions (Winston and Di Giulio 1991).

Human GR is a protein of 479 amino acids. GR is one of most studied FAD-containing proteins. All GRs family members adopt the Rossmann fold ($\beta_1\alpha_1\beta_2\alpha_2\beta_3$) (Dym and Eisenberg 2001) (Figure 2-6). FAD-containing proteins share some motifs. The most conserved sequence motif is part of the Rossmann fold and is found at the protein's N-

terminus xhxhGxGxxGxxxhxxn(x)$_8$hxhE where x is any residue and h is an hydrophobic residue. This consensus is known as the dinucleotide-binding motif (DBM) and is a common motif among FAD and NAD(P)H-dependent oxidoreductases. A variation of the glycine-rich sequence motif is hhhxGxGxxGxE and it is part of the NAD(P)h- binding domain. Another highly conserved FAD-binding sequence motif is T(S)xxxxxF(Y)hhGD(E) the hydrophobic residues belong to the seventh strand of the FAD-binding domain near the C-terminus of the protein. In addition there are some other partially conserved sequences D(x)$_6$GxxP located at the interface between the NAD(P)H and FAD-binding domains and one of the x residues, usually an Arg, between Gly and Pro makes a polar contact with the isoalloxazine ring of FAD (Dym and Eisenberg 2001) (Figure 2-23).

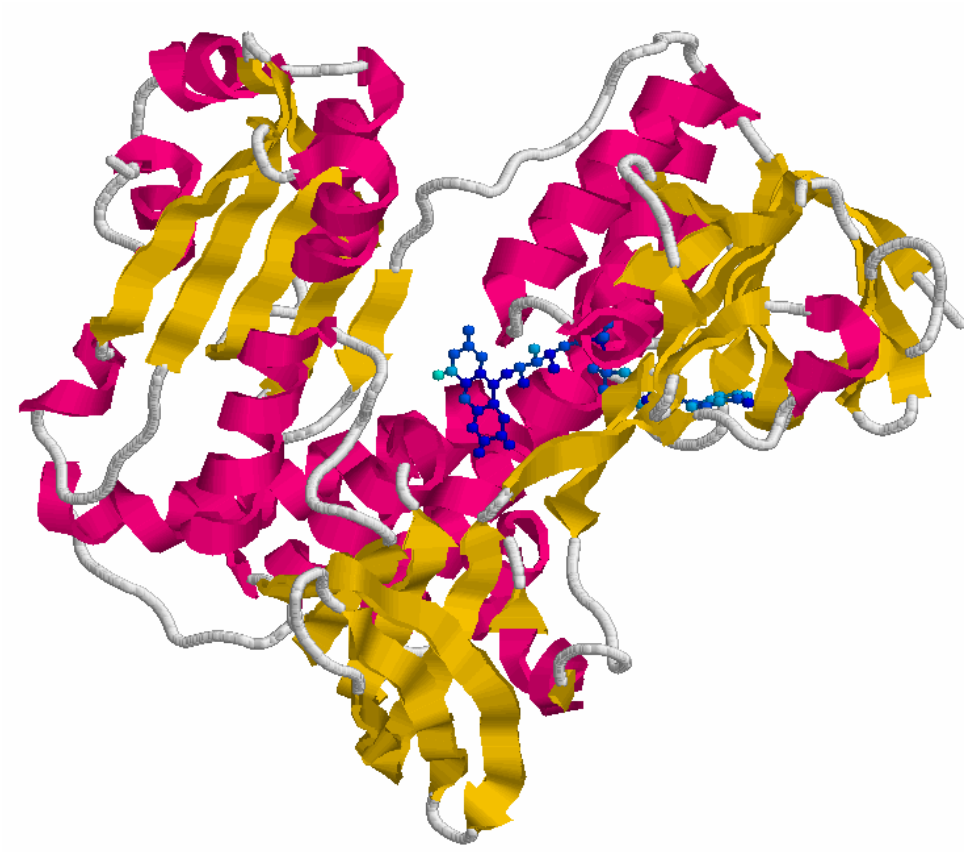

Figure 2-6 Glutathione reductase structure. Human GR, FAD is displayed in blue ball and stick (PDB from Stoll et al., (1997) elaborated using Rasmol software).

2.2 Materials and Methods

2.2.1 Total RNA extraction

Total RNA was extracted from all tissues with the same method. Following fish sacrifice, about 100-200 mg of tissue was dissected and immediately homogenised on ice for 10 sec in 2 ml of TRI Reagent® RNA extraction buffer (Sigma, UK) using a rotating probe homogeniser (Ultra-Turrax®). In addition, to accelerate the sampling procedure and avoid cross contamination two rotating probes were used, one for the head (brain, gill and heart) and one other for the rest of the body (liver, intestine, kidney and muscle) and the rotating probe and sampling scalpels and tweezers were washed between samples. The homogenate samples were then stored at -80 °C until extraction. To remove the insoluble material (extracellular membranes, polysaccharides and high molecular weight DNA) the 12 ml tubes containing the homogenate samples were thawed and centrifuged at 5,000 x *g* for 35 min at 4 °C (Sigma 4K 15, SciQuip, UK). To ensure complete dissociation of nucleoprotein complexes, the homogenate was subsequently incubated for 5 min (min) at room temperature and then 1 ml of the supernatant transferred into a clean (DNA and RNAase free) 1.5 ml tube containing 0.2 ml of chloroform (Sigma, UK). The tubes were then mixed with a vortex mixer for 10 seconds (sec), incubated for 10 min at room temperature before centrifugation at 12,000 x *g* for 15 min at 4 °C. The aqueous phase was transferred to a clean 1.5 ml tube containing 50 µl of isopropanol (Sigma, UK), mixed, incubated at room temperature for 5 min and centrifuged at 12,000 x *g* for 10 min 4 °C. This extra step minimizes the possibility of DNA contamination. The supernatant was then transferred in a new clean tube containing 450 µl of isopropanol. The mixture was then incubated for 10 min at room temperature after which it was centrifuged at 12,000 x *g* for 10 min at 4 °C. The supernatant was discarded, the pellet washed with 1 ml of 75 %

ethanol and then centrifuged at 7,500 x *g* for 5 min at 4 °C. This washing procedure was performed twice. The samples were then stored at -80 °C until quality check and cDNA synthesis was performed.

2.2.1.1 RNA quality check

RNA pellets were reconstituted in 50 µl or more of MilliQ water depending on the size of the pellet. Samples which were difficult to dissolve were incubated at 60 °C for 10-15 min. RNA measurements and quality checks were performed with a ND-1000 Nanodrop spectrophotometer (Labtech Int., UK) to evaluate the absorbance ratio at 260/280. A ratio of absorbance at 260/280 > 1.8 indicates a high level of purity and that the sample is not contaminated by protein (McKenna et al., 2000).

Furthermore, RNA degradation was checked by running 2 µg of total RNA on an agarose denaturing RNA gel electrophoresis. The quality of the total RNA can be inferred from the integrity of the rRNA (18s and 28s) which is detectable by ethidium bromide after separation by gel electrophoresis. The sample denaturing mix consisted of 400 µl of dimethyl sulfoxide (DMSO) (AnalaR®, BDH, UK), 250 µl of glyoxal (Fluka, Biochemika, Germany) 25 µl of MOPS buffer 25X (Sigma, UK) and 20 µl of blue loading dye 6X (Fermentas, UK). 2 µl (of a 1 µg/µl solution of RNA) were mixed with 4 µl of the denaturizing mix and incubated for 1 hour at 55 °C. The agarose gel was prepared by mixing 0.8 g of agarose (Invitrogen, UK) with 80 ml of distilled water, the solution was then microwaved for 50 sec and when cooled to about 50-60 °C 1 ml of MOPS 25X and 1.5 µl of ethidium bromide (Sigma, UK) was added. The gel was then poured into a gel casting tray and left to set for 30 min. 30 µl of ethidium bromide was added to the running buffer MOPS 1X (500 ml). Finally the samples were run for 45 min at 100 V and then the

gel visualized on a UV transilluminator (In Genius bio imaging, SYNGENE, UK), photographed and the picture saved electronically.

2.2.2 Synthesis of sea bream cDNA

Complementary DNA (cDNA) is typically generated from mRNA by action of a retroviral reverse transcriptase which reverse transcribes a single strand molecule of RNA into single stand cDNA. cDNA synthesis could be initiated by different primers: oligo dT which primes on the poly A tail of mature mRNA, random hexamers which prime randomly to any RNA (rRNA, tRNA and mRNA), or gene specific reverse primers which will reverse copy only the gene of interest. cDNA was prepared from 3 µg of total RNA as follows: RNA was first incubated with 25 µM of anchored oligo dT_{20} (Invitrogen, UK) at 70 °C for 5 min in a volume of 11 µl to denature RNA secondary structure and then quickly chilled on ice to let the primer anneal to the RNA. Then the other components of the reaction were added including 500 µM dNTPs (Invitrogen, UK), 200 units of SuperScript™ II RT reverse transcriptase with provided buffers 0.1 M Dithiothreitol (DTT) and 5X First-Strand Buffer (Invitrogen, UK) in a final volume of 20 µl. Reactions were then incubated for 60 min at 42 °C followed by 70 °C for 15 min to inactivate the enzyme and stored at -20 °C.

2.2.3 Cloning method

PCR cloning is a technique convenient for many reasons including:

- A PCR product cloned into a cloning vector can be conserved and when necessary re-amplified.

- Cloning vectors contain primers before and after the cloning site which are particularly useful for sequencing purposes.
- Enable the selection of one single molecule.
- PCR product cloned into a plasmid vector can be used as stable qPCR standards.

The cloning vector used in this study was pCR 2.1-TOPO® (Invitrogen, UK) (Figure 2-8).

Prior to cloning, the PCR product was purified by cutting a DNA fragment of the predicted molecular weight from a gel and processing it with a SV mini column PCR purification kit (Promega, UK) according to the manufacturers instructions. When the PCR produced a single fragment the PCR product was purified directly through the mini column to exclude short PCR products, dNTPs and buffer. The mini column was eluted with 20-50 µl of milliQ water, depending on the size of the band.

The purified PCR product concentration was then measured using a Nanodrop spectrophotometer. The amount of PCR product to include in the cloning reaction was calculated using the following formula:

$$\text{X ng of PCR product} = \frac{(\text{Y size in bp of PCR product})\ (\text{pCR 2.1 weight -25ng})}{\text{Size in bp of 2.1 pCR vector} - 3.9\text{Kb}}$$

The cloning reaction was executed as described in the Invitrogen cloning kit manual. The ligation of the PCR product was performed by the intrinsic topoisomerase activity associated with the commercial cloning vector preparation. The cloning reaction was performed at room temperature gently mixing the PCR product with the cloning vector in

a proportion of 3:1 then the reaction was incubated at room temperature for 20 min. The plasmid containing the ligated PCR product was then transformed into a One Shot® chemically competent *Escherichia coli* cell (TOP 10 Invitrogen, UK) by heat shock for 30 sec. 250 µl of SOC medium (provided with the kit) was then added and the mixture was incubated at 37 ˚C for 1 hour on a shaker mixer. The mixture containing the transformed cell were then spread on a preheated selective LB agar plate and incubated overnight at 37 ˚C. The selective LB agar plates were prepared following the kit manual instructions. The selective components contained in the LB agar plate were ampicillin (100 µg/ml), kanamycin (50 µg/ml) and X-gal (40 mg/ml in dimethylformamide). As a result, only the cells which contained the plasmid which contained ampicillin and kanamicin resistance genes would grow. Moreover the selection of the cells containing a ligated PCR fragment was based on the colorimetric metabolic reaction of X-gal by LacZα gene as the ligation of the PCR product would result in disruption of the LacZα reading frame leading to the production of white colonies. The white colonies were picked and grown over night in 2 ml of LB containing ampicillin (100 µg/ml). The plasmid-vector was purified using the GenElute™ Plasmid Miniprep Kit (Sigma, UK) as described in the manual. The column was eluted with 100 µl of milliQ water and samples were stored at -20°C. Before sequencing to check that the plasmid vector contained the right size PCR product, the plasmid was digested with EcoRI (Invitrogen, UK) restriction enzyme, which cut before and after the cloning site enabling an evaluation of the size of the insert by gel electrophoresis (Figure 2.8).

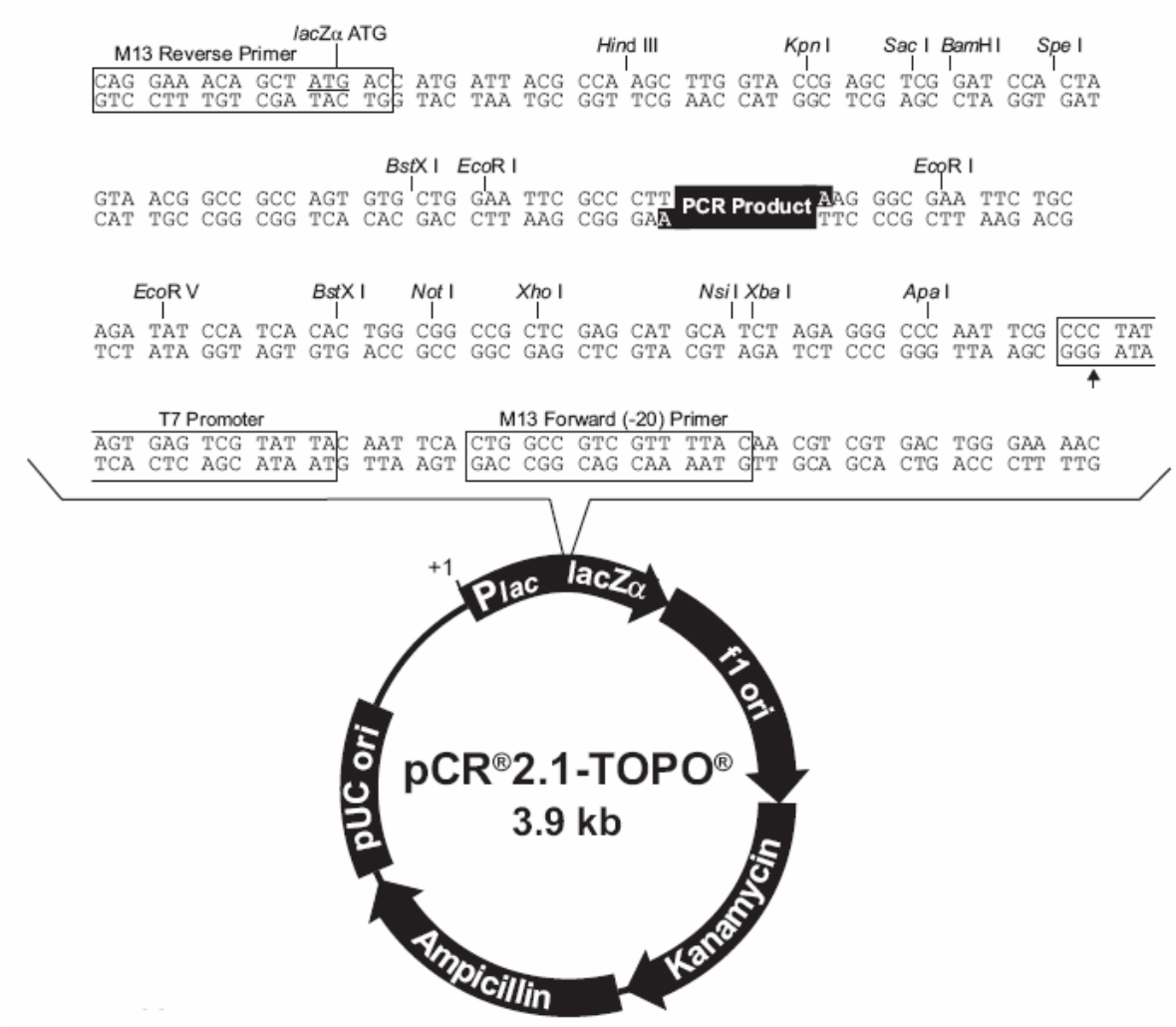

Figure 2-7 Map of pCR 2.1-TOPO cloning vector.

2.2.4 Sequencing analysis

Sequencing was performed using a Beckman 8800 autosequencer **(**Beckman Coulter, UK). The Beckman sequencing system included a sequencing reaction kit GenomeLab DTCS Quick Start Kit. The recommended amount of purified plasmid DNA was added to 2 µl (one fourth of the full amount used in the original protocol) of the sequencing mastermix including 1 µl of M13 F or M13R (4 µM) primer (Figure 2-7) in a final volume of 5 µl. After the sequencing reaction (30 cycles, 96 ˚C 20 sec, 50 ˚C 20 sec, 60 ˚C 4 min) 15 µl of milliQ water was added. Then the reaction was stopped by adding 5

µl of stop/glycogen solution which consisted of 2µl of EDTA (pH 8) (Sigma, UK), 2µl of 3M sodium acetate (pH 5.2) and 1 µl of glycogen (supplied with the Beckman sequencing kit). DNA precipitation was achieved by adding 60 µl of cold (from -20°C) 95% ethanol, mixing the solution thoroughly and then centrifugation at 14000 *g* for 15 min. The pellet was rinsed two times with 150 µl of 70 % ethanol and then let to air dry for 15 min. After that each sample was resuspended in 30 µl of samples loading solution (supplied with the Beckman sequencing kit) and loaded in the sequencing plate. Lasergene SEQman software (DNASTAR, USA) was used to edit and assemble DNA sequences.

In order to predict full length polypeptide sequences derived from Cu-homeostasis genes in other fish species with sequenced genomes, the sea bream cDNA sequences coding for Cu-homeostasis 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 as a key to generate predicted polypeptide sequences. ClustalW (Thompson et al., 2000) was used to generate multiple alignments of deduced protein sequences. MEGA version 4 was used (Tamura et al., 2007) to deduce and bootstrap phylogenetic trees using the neighbor-joining method (Saitou and Nei 1987). To verify if the neighbor-joining method is suitable to undertake phylogenetic inference and bootstrapping the average pairwise distance (Jukes Canton method) should be < 1 (Hall 2007). In all gene sequences analysed, the average pairwise distance (JC) was < 1.

Rasmol software (Sayle and Milner-White 1995) was used to elaborate the protein's 3D structure contained in PDB files (Protein Data Bank) www.rcsb.org/pdb/home .

2.2.5 Sea bream Ctr1 cDNA synthesis

A partial Ctr1 cDNA was generated using PCR primers, Ctr1F1 and Ctr1R2 (Table 2-1), which were designed by selecting conserved areas from alignments of protein sequences derived from previously described mRNA sequences of human (*Homo sapiens*) (BC013611), rat (*Rattus norvegicus*) (BC078745) and zebrafish (NM 205717) Ctr1 (see 2.1.2).

To optimize PCR, an annealing temperature gradient of 40-60 ˚C and a magnesium (Mg) concentration gradient (1-5 mM $MgCl_2$) were applied. The annealing temperature and Mg concentration that gave the most homogenous and largest amount of product was 52˚C and 2 mM $MgCl_2$ respectively. The PCR was performed in the following way: primers Ctr1F1 and Ctr1R2 were included, to a concentration of 0.5 µM, in a reaction containing 0.2 µM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 2mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 µl) of the cDNA (synthesised from intestinal total RNA) reaction in a final volume of 25 µl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 52 ˚C 30 sec – 72 ˚C 30 sec followed by 72 ˚C for 7 min.

RACE-PCR (Rapid Amplification of cDNA Ends) was performed using the FirstChoice RLM-RACE kit (Ambion,UK). RACE cDNA was generated as described in the Ambion manual from 10 µg of intestinal sea bream RNA. The PCR components used

to generate RACE amplicons included 0.5 μM primers, designed from the sequence of the initial partial cDNA (for 5'RACE, 5'Ctr1-R1, 5'Ctr1-R2 and for 3'RACE, 3'Ctr1F1; Table 2-1) and all the other Ambion components as described in the manual including one unit of SuperTaq-Plus (Ambion, UK). The PCR strategy for 5'RACE consisted of a first round of touch down PCR using the outer RACE primer (Ambion, UK) with the gene specific primer 5'Ctr1-R1. The PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 2 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 2 min, 25 cycles 95 ˚C 20 sec, 60 ˚C 20 sec, 72 ˚C 2 min and 1cycle at 72 ˚C for 7 min. Then, using 1 μl of the first round PCR and the nested primers (inner RACE Ambion and 5'Ctr1-R2) a second round of nested PCR was performed and the thermocycling conditions were: 94 ˚C for 2 min followed by 30 cycles 94 ˚C 20 sec – 60 ˚C 20 sec – 72 ˚C 2 min followed by 72 ˚C for 7 min. For 3'RACE the first round of touch down PCR using the same condition of 5'RACE but with 3'outer RACE primer (Ambion, UK) and 5'Ctr1-F1 was sufficient to generate a homogenous product.

The final full length PCR product was obtained using 0.5 μM primers (Ctr1Full-F and Ctr1Full-R Table 2-1, designed from the 5' and 3' RACE products), 0.2 μM dNTPs (Invitrogen, UK), one unit of SuperTaq-Plus with supplied buffer (Ambion, UK) and one eighth (2.5 μl) of the cDNA (synthesised from intestinal total RNA) reaction in a final volume of 25 μl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 60 ˚C 30 sec – 72 ˚C 60 sec followed by 72 ˚C for 7 min.

The cDNA cloning strategy is shown in Figure 2-8.

Every PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

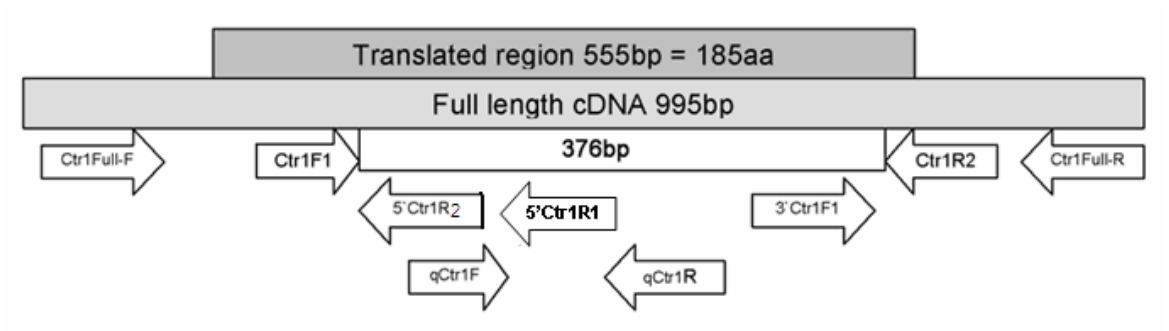

Figure 2-8 Graphic representation of the cloning strategy for Ctr1 cDNA isolation**.** Open box arrows represent primers and direction of DNA synthesis. Primer sequences are given in Table 2-1.

2.2.6 Sea bream Atox1 cDNA synthesis

A partial Atox1 cDNA was generated using PCR primers, Atox1-F2 and Atox1-R3 (Table 2-1), which were designed by selecting conserved areas from alignments of protein sequences derived from mRNA or predicted gene sequences of human (NM_004045), rat (AF177671), chick (XM_001233562) and puffer fish (*Tetraodon negroviridis*) (Ensemble transcript ID: GSTENT00024266001) Atox1 (see 2.1.2).

PCR optimization conditions consisted of a gradient 50-60˚C of the annealing temperature. The annealing temperature that gave the most homogenous product was 56˚C. The PCR was performed in the following way: primers Atox1F2 and Atox1R3 were included, at a concentration of 0.5 µM, in a reaction containing 0.2µM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 1mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 µl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25 µl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were 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.

RACE-PCR (Rapid Amplification of cDNA Ends) was performed using the FirstChoice RLM-RACE kit (Ambion, UK). RACE cDNA was generated as described in the manual from 10 μg of liver sea bream RNA. PCR components used to generate RACE amplicons included 0.5 μM primers, designed from the sequence of the initial partial cDNA (for 5'RACE, 5'Atox1-R1, 5'Atox1-R2 and for 3'RACE, 3'Atox1-F1, 3'Atox1-F2;Table 2-1) and all the other Ambion components as described in the manual including one unit of SuperTaq-Plus (Ambion, UK). The PCR strategy for both 5'RACE and 3' RACE consisted of a first round of touch down PCR using the outer RACE primer 5' or 3' (Ambion, UK) with the gene specific primer 5'Atox1-R1 or 3'Atox1-F1 respectively. The PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 2 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 2 min, 25 cycles 95 ˚C 20 sec, 62 ˚C 20 sec, 72 ˚C 2 min and 1 cycle at 72 ˚C for 7 min. Then, using 1 μl of the first round PCR and the nested primers, inner RACE 5' or 3' (Ambion) with 5'Atox1-R2 or 3'Atox1-F2 respectively, were subjected to a second round of PCR and the thermocycling conditions were: 94 ˚C for 2 min followed by 30 cycles 94 ˚C 20sec – 60 ˚C 20 sec – 72 ˚C 2 min followed by 72 ˚C for 7 min.

The final full length PCR product was obtained using 0.5 μM primers (Atox1Full-F and Atox1Full-R Table 2-1, designed from the 5' and 3' RACE products), 0.2 μM dNTPs (Invitrogen, UK), one unit of SuperTaq-Plus with supplied buffer (Ambion, UK) and one eighth (2.5 μl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25 μl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were 94 ˚C for 3 min followed by 30 cycles 9 4˚C 30sec – 60 ˚C 30 sec – 72 ˚C 60 sec followed by 72 ˚C for 7 min.

The cDNA cloning strategy is similar to that shown in Figure 2-8.

Every PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

2.2.7 Sea bream ATP7A cDNA synthesis

Compared to Ctr1 and Atox1, ATP7A is a much larger protein, therefore different sets of primers were designed and three partial cDNAs were generated using the following primer combinations (Table 2-1):

1. ATP7AF2 with ATP7AR5
2. ATP7AF5 with ATP7AR6
3. ATP7AF7 with ATP7AR9

As described in (2.1.2) degenerate primers were designed by selecting conserved areas from alignments of protein sequences derived from mRNA or partial gene sequences of human (NM_000052), chick (XM_420307), zebrafish (Ensemble transcript ID: ENSDART00000054977) and puffer fish (Ensemble transcript ID: GSTENT00017010001) ATP7A.

PCR optimization conditions consisted of a gradient 50-60 ˚C of the annealing temperature. The annealing temperature that gave the cleanest and strongest band was 52, 50 and 56 ˚C for reactions 1, 2 and 3 respectively. PCRs were performed in the following way: primers combination (1-3) were included, at a concentration of 0.5 μM, in a reaction containing 0.2 μM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 1 mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 μl) of the cDNA reaction (synthesised from intestinal total RNA) in a final volume of 25 μl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were:

1. 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30sec – 52 ˚C 30 sec – 72 ˚C 2 min followed by 72 ˚C for 7 min.

2. 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30sec – 50 ˚C 30 sec – 72 ˚C 1 min followed by 72 ˚C for 7 min.

3. 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30sec – 56 ˚C 30 sec – 72 ˚C 1.5 min followed by 72 ˚C for 7 min.

RACE-PCR (Rapid Amplification of cDNA Ends) was performed using the SMART™ RACE (Clontech, USA). RACE cDNA was generated as described in the Clontech manual from 1 μg of intestinal sea bream total RNA. The PCR component used to generate RACE amplicons included 0.5 μM primers, designed from the sequence of the initial partial cDNAs (for 5'RACE, 5'ATP7A-R1, 5'ATP7A-R2 and for 3'RACE, 3'ATP7A-F1;Table 2-1) and all the other kit components as described in the manual including Advantage® Taq (Clontech, USA). The PCR strategy for 5'RACE consisted of a first round of touch down PCR using the universal RACE primer (Clontech) with the gene specific primer 5'ATP7A-R1 and the PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 3 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 3 min, 25 cycles 95 ˚C 20 sec, 60 ˚C 20 sec, 72 ˚C 3 min followed by 72 ˚C for 7 min. Then, using 1 μl of the first round PCR and the nested primers (Universal primer and 5'ATP7A-R2) a second round of nested PCR was performed and the thermocycling conditions were: 94 ˚C for 2 min followed by 30 cycles 94 ˚C 20 sec – 60 ˚C 20 sec – 72 ˚C 3 min followed by 72 ˚C for 7 min. For 3'RACE the first round of touch down PCR using the same conditions of 5'RACE but with the universal primer (Clontech, USA) and the gene specific 3'ATP7A-F1 was sufficient to generate a homogenous product.

The final full length PCR product was obtained using 0.5 µM primers (ATP7AFull-F1, ATP7AFull-F2 and ATP7A-R1, ATP7A-R2 Table 2-1, designed from the 5' and 3' RACE products), 0.2 µM dNTPs (Invitrogen, UK), one unit of SuperTaq-Plus with supplied buffer (Ambion, UK) and one eighth (2.5 µl) of the cDNA (synthesised from intestinal total RNA) reaction in a final volume of 25 µl. The thermocycling conditions (Tgradient, Whatman Biometra, Germany) were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30sec – 60 ˚C 30 sec – 72 ˚C 60 sec followed by 72 ˚C for 7 min. Following the first round of PCR, 0.5 µl of PCR product was subjected to a second nested PCR using ATP7AFull-F2 and ATP7AFull-R2 with the same PCR component and PCR conditions of the first round.

The cDNA cloning strategy is shown in Figure 2.10.

Every PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

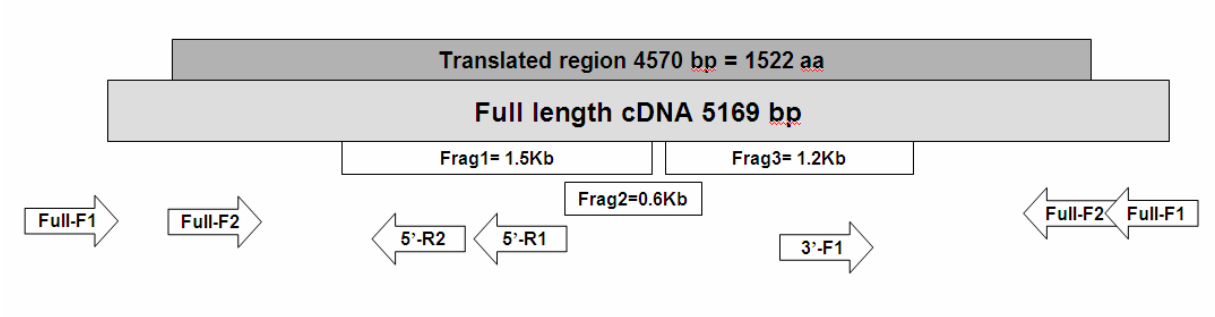

Figure 2-9 Graphic representation of the cloning strategy for ATP7A cDNA isolation**.** Open box arrows represent primers and direction of DNA synthesis. Primers ID used for initial fragment 1, 2 and 3 are reported in the text above. Primer sequences are reported in Table 2-1.

2.2.8 Sea bream ATP7B cDNA synthesis

ATP7B cDNA was synthesised in three steps:

The first fragment of ATP7B synthesised was the 3`end, generated using the FirstChoice RLM-RACE kit (Ambion, UK). The degenerate forward primers were designed by selecting conserved areas from alignments of protein sequences derived from mRNA or partial gene sequences of human (NM_000053), zebrafish (Ensemble transcript ID: ENSDART00000030246), puffer fish (Ensemble transcript ID: GSTENG00020077001) and stickleback (Ensemble transcript ID: ENSGACG00000014945), ATP7B. RACE cDNA was generated as described in the Ambion manual from 1 μg of inestinal sea bream total RNA. The PCR component used to generate RACE amplicons included 0.5 μM primers (3'ATP7B-F2 and 3'ATP7B-F1;Table 2-1) and all the other kit components as described in the manual including one unit of SuperTaq-Plus (Ambion, UK). The PCR strategy for 3'RACE consisted of a first round of touch down PCR using the RACE reverse outer primer (Ambion) with the gene specific forward primer 3'ATP7B-F2 and the PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 2 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 2 min, 25 cycles 95 ˚C 20 sec, 60 ˚C 20 sec, 72 ˚C 2 min followed by 72 ˚C for 7 min. Then, using 1 μl of the first round PCR and the nested primers inner reverse RACE (Ambion) and 3'ATP7B-F1 a second round of nested PCR was performed and the thermocycling conditions were: 95 ˚C for 2 min followed by 30 cycles 95 ˚C 20sec – 58 ˚C 20 sec – 72 ˚C 2 min followed by 72 ˚C for 7 min.

The second fragment of ATP7B synthesised was obtained designing reverse primers on the ATP7B 3` end fragment and forward degenerate primers on the same ATP7B sequences described above. The PCR included 0.5 μM of the primers (ATP7B-F1

and ATP7B-R5; Table 2-1), 0.2 µM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 1mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 µl) of the cDNA reaction (synthesised from intestinal total RNA) in a final volume of 25 µl. The PCR strategy consisted of a first round of touch down PCR and the PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 5 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 5 min, 25 cycles 95 ˚C 20 sec, 58 ˚C 20 sec, 72 ˚C 5 min followed by 72 ˚C for 7 min. Then, using 1 µl of the touch down PCR and the nested primers ATP7B-F3 and ATP7B-R4 a second round of nested PCR was performed using the same PCR component mention above and the same primers concentrations. The thermocycling conditions were: 95 ˚C for 2 min followed by 30 cycles 95 ˚C 20 sec – 53-63 ˚C 20 sec – 72 ˚C 3 min followed by 72 ˚C for 7 min. The optimum annealing temperature was 59 ˚C.

The third fragment of ATP7B synthesised was the 5'end generated using the SMART™ RACE (Clontech, USA). RACE cDNA was generated as described in the Clontech manual from 1µg of intestinal sea bream total RNA. The PCR components used to generate the 5' RACE amplicon included 0.5 µM reverse primers, designed from the sequence of the second partial cDNAs (5'ATP7B-R1, 5'ATP7B-R2; Table 2-1) and all the other kit components as described in the manual including Advantage® Taq (Clontech, USA). The PCR strategy for 5'RACE consisted of a first round of touch down PCR using the universal RACE primer (Clontech) with the gene specific primer 5'ATP7B-R1 and the PCR thermocycling conditions were: 1 cycle at 95 ˚C for 2 min, 5 cycles 95 ˚C 20 sec, 72 ˚C 3 min, 5 cycles 95 ˚C 20 sec, 70 ˚C 20 sec, 72 ˚C 3 min, 25 cycles 95 ˚C 20 sec, 62 ˚C 20 sec, 72 ˚C 3 min followed by 72 ˚C for 7 min. Then, using 1 µl of the first round PCR and the nested primers (Universal primer and 5'ATP7B-R2) a second round of PCR was

performed and the thermocycling conditions were: 95 ˚C for 2 min followed by 30 cycles 95 ˚C 20sec – 60 ˚C 20 sec – 72 ˚C 3 min followed by 72 ˚C for 7 min.

The final full length PCR product was obtained using 0.5 µM primers (ATP7BFull-F1, ATP7BFull-F2 and ATP7B-R1, ATP7B-R2; Table 2-1, designed from the 5' and 3' RACE products), 0.2 µM dNTPs (Invitrogen, UK), one unit of SuperTaq-Plus with supplied buffer (Ambion, UK) and one eighth (2.5 µl) of the cDNA (synthesised from intestinal total RNA) reaction in a final volume of 25 µl. The thermocycling conditions in the first round PCR, using ATP7BFull-F1 and ATP7BFull-R1, were 94˚C for 3 min followed by 30 cycles 94 ˚C 30sec – 55 ˚C 30 sec – 72 ˚C 4.5 min followed by 72 ˚C for 7 min. Following the first round of PCR, 0.5 µl of PCR product was subjected to a second nested PCR using ATP7BFull-F2 and ATP7BFull-R2 with the same PCR component and PCR conditions of the first round.

The cDNA cloning strategy is shown in Figure 2-10.

Every PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

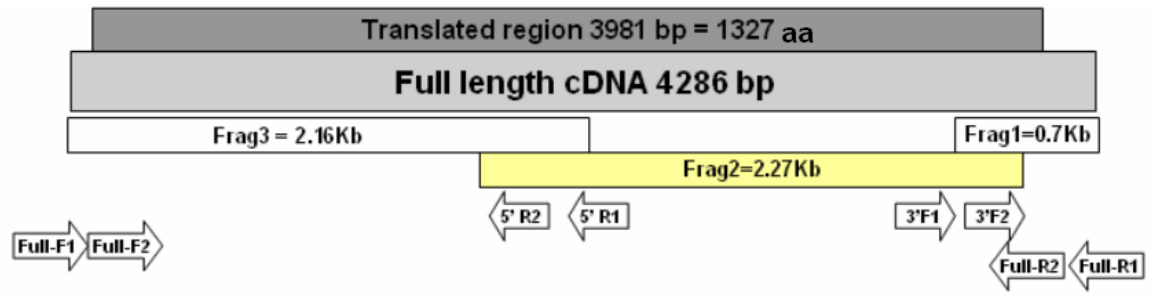

Figure 2-10 Graphic representation of the cloning strategy for ATP7B cDNA isolation**.** Open box arrows represent primers and direction of DNA synthesis. Primers ID used for initial fragment 1, 2 and 3 are reported in the text above. Primer sequences are reported in Table 2-1.

2.2.9 Sea bream MT, oxidative stress related genes and reference genes

2.2.9.1 Cloning of partial MT cDNA

Based on the existing complete cDNA sequence of sea bream MT (U58774) one set of primers was designed (Table 2-1). The PCR product was obtained using 0.5 μM primers (MT-F1, MT-R1 Table 2-1), 0.2 μM dNTPs (Invitrogen, UK), Taq polymerase with supplied buffer IV, 2mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 μl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25μl. The thermocycling conditions were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 51 ˚C 30 sec – 72 ˚C 40 sec followed by 72 ˚C for 7 min. Then the PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

2.2.9.2 Cloning of partial CuZn-SOD cDNA

Based on very highly conserved CuZn-SOD fish cDNA sequences such as: Red seabream (*Pagrus major*) (AF329278), Chinese perch (*Siniperca chuatsi*) (AY909486) and European flounder (*Platichthys flesus*) (AJ291980) one set of primers was designed (Table 2-1). The PCR product was obtained using 0.5 μM primers (CuZnSOD-F1, CuZnSOD-R1 Table 2-1), 0.2 μM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 2 mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5μl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25 μl. The thermocycling conditions were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 55 ˚C 30 sec – 72 ˚C 40 sec followed by 72 ˚C for 7 min. Then the PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

2.2.9.3 Cloning of partial GR cDNA

Based on highly conserved GR cDNA sequences such as: European flounder (AJ578037), mouse (*Mus musculus*) (NM_010344) and human (NM_000637) one set of primers was designed (Table 2-1). The PCR product was obtained using 0.5 μM primers (GR-F1, GR-R1 Table 2-1), 0.2 μM dNTPs (Invitrogen, UK), Taq polymerase with supplied buffer IV, 2mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 μl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25 μl. The thermocycling conditions were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 55 ˚C 30 sec – 72 ˚C 1.5 min followed by 72 ˚C for 7 min. Then the PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

2.2.9.4 Reference gene cloning

Partial cDNAs of the reference genes (ref. genes) were amplified by PCR using primers designed on the sea bream sequences: β-actin (X89920), GAPDH (DQ641630), EF1-α (AF184170) (Table 2-1). The PCR product was obtained using 0.5 μM primers (Table 2-1), 0.2 μM dNTPs (Invitrogen, UK), one unit of Taq polymerase with supplied buffer IV, 2 mM $MgCl_2$ (Taq DNA Polymerase, ABgene, UK) and one eighth (2.5 μl) of the cDNA (synthesised from liver total RNA) reaction in a final volume of 25 μl. The thermocycling conditions for all reference genes were 94 ˚C for 3 min followed by 30 cycles 94 ˚C 30 sec – 60 ˚C 30 sec – 72 ˚C 60 sec followed by 72 ˚C for 7 min. Then the PCR product was purified, cloned and sequenced as described in (2.2.3; 2.2.4).

Table 2-1 Primers used for cDNA isolation.

ID	*Sequence 5`→3`*	*length*	*Tm*	*GC%*
Ctr1F1	gccgcaaatgaccttctactt	21	57.9	47.6
Ctr1R2	actgccttcttccagctgaa	20	57.3	50
5'Ctr1-R1	agcgatgcagaggtaagcgttgta	24	62.7	50

5'Ctr1-R2	tggagttgtagcggacgttgacctgactgc	30	70.9	56.7
3'Ctr1-F1	gctgatggagacgcacaagact	22	62.1	54.5
Ctr1Full-F	ctcgcgactttgtgagtttcgtgt	24	62.7	50
Ctr1Full-R	caaggtgttggttaccacggttac	24	62.7	50
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		
ATP7AF5	cgmtggctggarcaratagc	20		
ATP7AF7	cgmttygccttccargcctc	20		
ATP7AR5	gctggatrggmgcctttga	19		
ATP7AR6	gaggcytggaaggcraakcg	20		
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-F3	grtytacatgatggtgatggaca	23		
ATP7B-R4	cctgcagcgacgggtattcc	20	63.4	65
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
MT-F1	acgcaatcacatctcacgaa	20	55.2	45
MT-R1	ctggacatttgctactgcaat	21	55.9	42.9
CuZnSOD-F1	gagtgcaggacctcacttcaatcc	24	64.4	54.2
CuZnSOD-R1	ccagcattgcccgtctttagact	23	62.4	52.2
GR-F1	gcgtgaatgttggatgtgtccc	22	62.1	54.5
GR-R1	cctgaagcatctcatcacag	20	57.3	50
β-actin-F1	atgaaatcgccgcactggttg	21	59.8	52.4
β-actin-R1	gatgtcacgcacgatttccctctc	24	64.4	54.2
GAPDH-F1	gacccctcatcgacctggagta	23	64.2	56.5
GAPDH-R1	tgcagccttgacgaccttcttgat	24	62.7	50
EF1α-F1	cgtcgtggatacgtcgctggtgacag	26	69.5	61.5
EF1α-R1	gagaagatgatccaggatgggggtaagg	28	68	53.6

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

2.3 Results

Cu homeostatic genes Ctr1, Atox1, ATP7A and ATP7B have not previously been reported from sea bream and in this study it was important to isolate full length cDNAs to confirm their identities. Moreover the oxidative stress response genes CuZn-SOD and GR have not been previously reported in sea bream and therefore fragments of the cDNA sequences were isolated. In addition cDNA fragments from the known sequences MT, β-actin, GAPDH and EF1-α were also isolated for subsequent QPCR analysis.

2.3.1 Sea bream Ctr1 cDNA

Following the first round of RT-PCR a DNA fragment of 376 base pairs was generated which, on sequencing, showed 88 % identity to the zebrafish Ctr1 and 76 % identity to human Ctr1. To amplify the 5' end of the sea bream Ctr1 cDNA, two primers were synthesised based on the sequence of this fragment and used in a nested PCR procedure whereby a portion of the reaction mix after use of the 5'Ctr1-R1 was subjected to a further round of 5'-RACE using the 5'Ctr1-R2 primer. This yielded a fragment of 728 base pairs which overlapped with 100 % identity with the original sequence. 3'RACE using 3`Ctr1-F1 yielded a fragment of 398 bp which also showed 100 % identity with the original PCR product in the region of overlap. This allowed the inference of a full length cDNA for sea bream Ctr1 (saCtr1) which consisted of an open reading frame of 222 amino acids and 5' and 3' untranslated regions of 197 bp and 243 bp respectively. To confirm the saCtr1 sequence primers (Ctr1Full-F and Ctr1Full-R) were designed to amplify the entire sea bream Ctr1 ORF (Figure 2-8). The sequence obtained (saCtr1), submitted on gene bank with accession number AJ630205, was 100 % identical to the assembled sea bream sequence and showed 78 % identity with the human sequence and 89 % with the zebrafish sequence.

Alignment of the deduced amino acid sequence of saCtr1 with Ctr1 proteins from other fish species, frog, lizard, chick and human show a strong sequence conservation, notably the MXM Cu-binding motif, three trans-membrane domains (TMD) and the C-terminal HCH motif (Puig and Thiele 2002; Guo et al., 2004; Guo et al., 2006) (Fig. 1). Moreover, amino acids G34, N53, M69, K178 and D84 (of human Ctr1) which were shown by mutational analysis to affect Vmax and Km of Cu uptake (Eisses and Kaplan 2005) are completely conserved in sea bream and in all of the other vertebrate Ctr1 sequences (Figure 2-11). Furthermore the saCtr1 hydrophobicity plot, done by the online software (www.vivo.colostate.edu/molkit/hydropathy/index.html), confirmed the hydrophobicity of the 3 TMDs.

In view of the possibility that fish species may contain multiple genes for which only single mammalian examples exist, the sea bream cDNA sequence was used to search for similar sequences in the zebrafish, puffer fish, medaka and stickleback genomes. In each species a single Ctr1 gene was identified and the Ctr1 derived from the zebrafish genome was identical to that previously described (Mackenzie et al., 2004). Furthermore, phylogenetic comparison of all of the fish sequences with Ctr1 from other vertebrates indicated that the fish genes were monophyletic and that the sea bream Ctr1 was most closely related to the puffer fish proteins (Figure 2-12).

Figure 2-11 Alignment of vertebrate Ctr1 polypeptide sequences. Residues identical in all proteins are shaded. The positions of the methionine Cu binding motif (Met motif), transmembrane domains (TMD1-3) and the C-terminal HCH motif are indicated. The residues shown to be required for the activity of human Ctr1 are boxed.

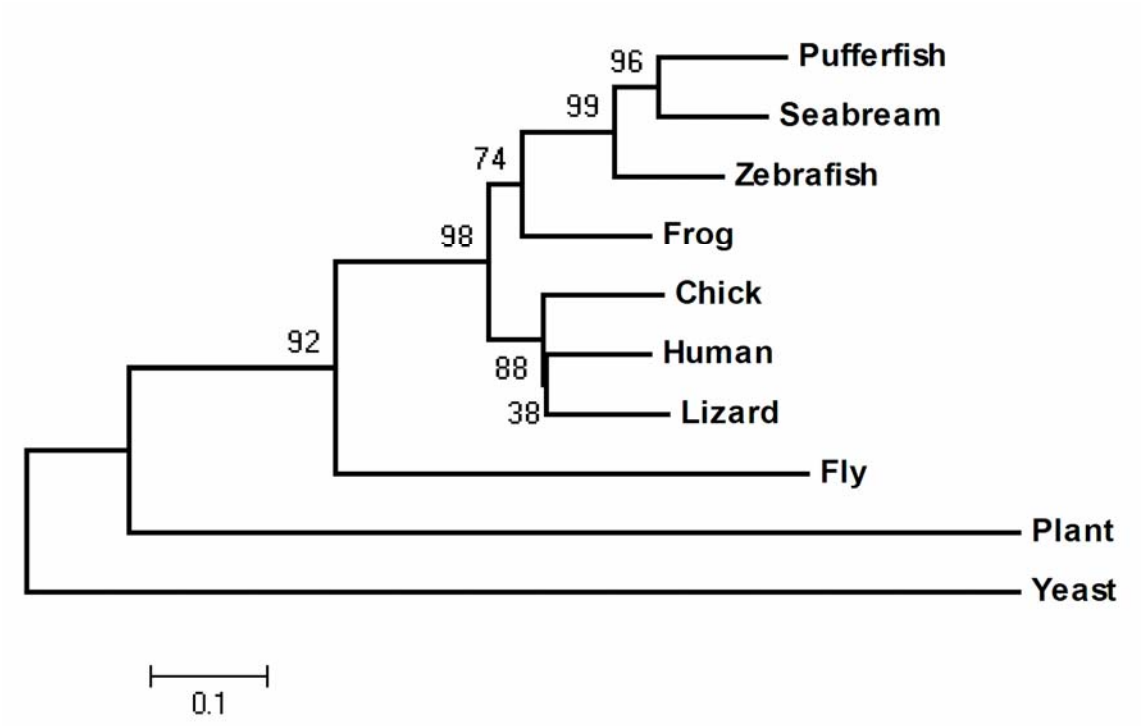

Figure 2-12 Ctr1 phylogenetic plot of vertebrate Ctr1 polypeptide sequences. Sea bream (*Sparus aurata*, AJ630205), Zebrafish, (*Danio rerio*, AY077715) and puffer fish, (*Tetraodon nigroviridis*, Ensembl genomic sequence scafold SCAF 14657) along with human (U83460), frog, (*Xenopus tropicalis,* NP001001238), lizard, (*Podarcis sicula,* AJ421475), chick (*Gallus gallus*, XM_415542), fly (*Drosophila melanogaster,* NP_572336.2), plant (*Arabidopsis thaliana,* AF466373) and yeast (*Saccharomyces cerevisiae,* AAA17369) Ctr1 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.

2.3.2 Sea bream Atox1 cDNA

The first round of PCR produced a fragment of 154 bp which after sequencing showed 61 % identity to zebrafish Atox1 and 56 % identity to human Atox1. The same strategy used to amplify the full length of Ctr1 was used for Atox1. Two rounds of nested PCR were applied to generate the 5'end, using primers 5'Atox1-R1 and 5'Atox1-R2 yielded a fragment of 150 bp which overlapped 100 % with the original sequence. The 3' end was obtained using 3`Atox1-F1 and 3`Atox1-F2 in two rounds of RACE nested PCR

and yielded a fragment of 1094 bp which also showed 100 % identity with the original PCR product in the region of overlap. This allowed the inference of a full length cDNA for sea bream Atox1 (saAtox1) which consisted of an open reading frame of 68 amino acids and 5' and 3' untranslated regions of 53 bp and 1084 bp respectively. To confirm the saAtox1 sequence, primers (Atox1Full-F and Atox1Full-R) were designed to amplify the entire sea bream Atox1 ORF. The sequence obtained (SaAtox1), submitted on gene bank with accession number AJ966735, was 100 % identical to the assembled sea bream sequence and showed 58 % identity with the human sequence and 61 % with the zebrafish sequence.

Alignment of the deduced amino acid sequence of saAtox1 with Atox1 proteins from other fish species, frog, chick, human, insects and yeast show strong sequence conservation. Remarkably the metal binding domain (MBD) (MxCxxC) is highly conserved from yeast to humans (Hung et al., 1998; Ralle et al., 2003; Wernimont et al., 2000). Moreover K65 which was demonstrated to be essential for Cu transport by mutational analysis is also conserved in all sequences (Portnoy et al., 2001) (Figure 2-13).

To verify if fish species contain multiple genes for which only single mammalian examples exist, the sea bream Atox1 cDNA sequence was used to search for similar sequences in the zebrafish, puffer fish, medaka and stickleback genomes. In each species a single Atox1 gene was identified. Furthermore phylogenetic comparison of all of the fish sequences with Atox1 from other vertebrates indicated that the fish genes were monophyletic and that the sea bream Atox1 was most closely related to the puffer fish proteins (Figure 2-14).

```
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
```

Figure 2-13 Atox1 sequence alignment. Residues identical in all proteins are shaded. The position of the metal binding domain (MBD) is indicated. The residues shown to be required for the activity of yeast Atx1 are boxed.

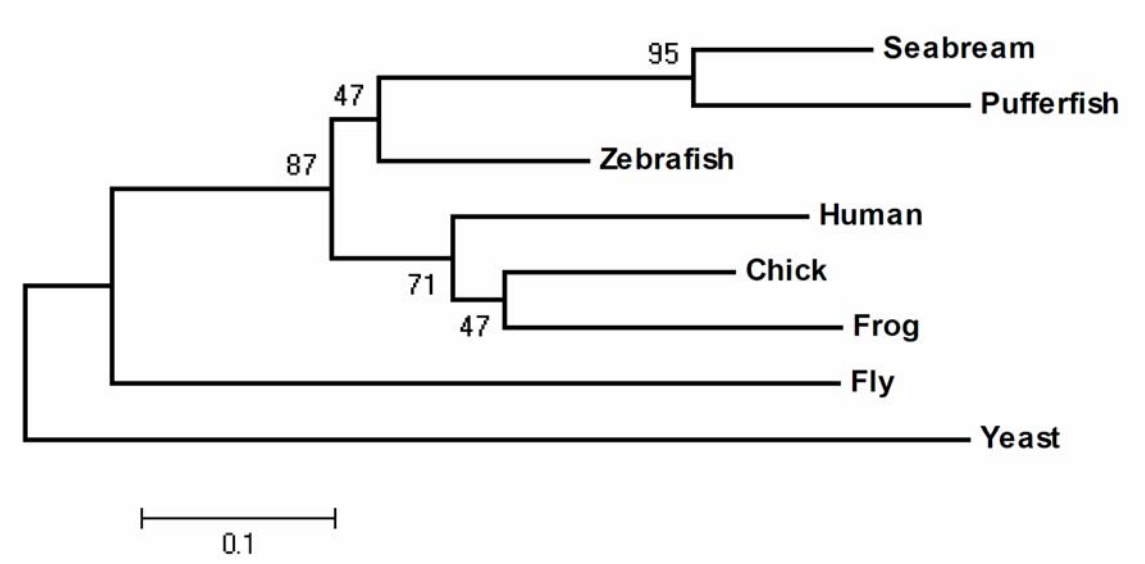

Figure 2-14 Atox1 phylogenetic tree. Human (NM 004045), chick (*Gallus galllus,* XM 001233562), frog (*Xenopus tropicalis,* NM 001045773), fly (*Drosophila melanogaster*, NM_168932), yeast (*Saccharomyces cerevisiae,* L35270), zebrafish (*Danio rerio*, XM 679218), puffer fish (Ensemble-Chr1 scaf14742) and the sea bream Atox1 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.

2.3.3 Sea bream ATP7A cDNA

As described in paragraph 2.2.7 and in the Figure 2-9, three PCR fragments of 1.5, 0.6 and 1.2 Kb, were generated following the first round PCRs. After sequencing and assembling they showed 88 and 77 % identity to zebrafish and human ATP7A respectively. The 5'end was obtained by two rounds of RACE nested PCR using primers 5'ATP7A-R1 and 5'ATP7A-R2 (designed on the sequence of previous fragments) which yielded a fragment of 1950 bp which overlapped with 100 % identity with the original sequence. 3' end was obtained using 3`ATP7A-F1 and 3`ATP7A-F2 in two rounds of RACE nested PCR and yielded a fragment of 924 bp which also showed 100 % identity with the original PCR product in the region of overlap. The final assembling of the cDNA fragments allowed the construction of the full length cDNA for sea bream ATP7A (saATP7A) which resulted in an open reading frame of 1526 amino acids and 5' and 3' untranslated regions of 294 bp and 305 bp respectively. To confirm the saATP7A sequence, primers (ATP7AFull-F1, ATP7AFull-F2 and ATP7AFull-R1, ATP7AFull-R2) were designed to amplify the entire sea bream ATP7A ORF. The sequence obtained (saATP7A), was 100 % identical to the assembled sea bream sequence and showed 63 % identity with the human sequence and 74 % with the zebrafish sequence.

Similarly to the other Cu homeostasis genes, alignments of the deduced amino acid sequence of saATP7A with ATP7A proteins from other fish species, bird and human but also from Cu-ATPases from insects and yeast show strong sequence conservation. The proteins' features which characterize Cu-ATPases such as the 6 MBDs, 8 TMDs and the C1000(P)C1002 were conserved (Solioz and Vulpe 1996b). Moreover with the exception of the D1497TAL1500 all the other functionally important motifs were conserved in saATP7A (Lutsenko et al., 2007a; Lutsenko et al., 2007b) (2.1.4.3) (Figure 2-15).

Furthermore the saATP7A hydrophobicity plot, done by the online software (www.vivo.colostate.edu/molkit/hydropathy/index.html), confirmed the hydrophobicity of the 8 TMD.

To verify if fish species contain multiple genes for which only single mammalian examples exist, the sea bream cDNA sequence was used to search for similar sequences in the zebrafish, puffer fish, medaka and stickleback genomes. In each species a single ATP7A gene was identified. Furthermore, similarly to Ctr1 and Atox1, phylogenetic comparison of all of the fish sequences with ATP7A from other vertebrates indicated that the fish genes were monophyletic and that the sea bream ATP7A was most closely related to the puffer fish protein.

```
Human          MDPSMGVNS----VTISVEGMTCNSCVWTIEQQIGKVNGVHHIKVSLEEKNATIIY 52
Chicken        MEARS--------IVIGVEGMTCHSCVQTIEQHVGKMNGIHNVKVSLEDKNAVIIY
Seabream       MTQKV----NLCTVSLGVEGMTCGSCVQSIEQRIGSLPGVIHIKVSLEQKIATIIF
Pufferfish     MTDKG----SLCLVSLGVEGMTCNSCVQTIQQRIGSFAGVIDVKVSLDQKNAAIIF
Zebrafish      MALSTNLCRNLCRVTLGVEGMTCGSCVQSIEGRIGGLPGVIHIQVSLEQNNATVTY
Seasquirt      MACDT------KEIMISITGMTCNSCVKTIESEVGKENGIHKIKVSLENMNAKVSF
FruitFly       MPSDERVEATMSTVRLPIVGMTCQSCVRNITEHIGQKSGILGVRVILEENAGYFDY
Yeast          ----------MREVILAVHGMTCSACTNTINTQLRALKGVTKCDISLVTNECQVTY
                                 *******
                                MBD 1
Human          DPKLQTPKTLQEAIDDMGFDAVIHN-PDPLPVLTDTLFLTVTASLTLPWDHIQSTLLKTK 111
Chicken        DSKLHTPATLQEAIYDMGFDATSAG-SNPQPVLPDTIFLTIPTQSALTSKELRSTLLKNK
Seabream       DHSQQSPESLSEAVEDMGFESSLSESSTATHVSTDTQLIPTSGLTPAAQQEALEKLSQIQ
Pufferfish     DSSRQSPESL--AIEDMGFESTLSETTPVAAVSVDTQVIPTPNLEAAAQQEALQKLAQIH
Zebrafish      DHTQHTPQSIADAIEDMGFESSLTN-ATSTPVQTETKVFSKAGCSADSVQQALSTLAQIK
Seasquirt      DPQVLTNKDVVELIEDLGFDAEVETGNTDTVVLN-----IDGMTCDACVNTIQSTLSGLK
FruitFly       DPRQTDPARIASDIDDMGFECSYPG---------------------------------
Yeast          DN-EVTADSIKEIIEDCGFDCEILR---------------------------------

Human          GVTDIKIYPQKRTVAVTIIPSIVNANQIKELVPELSL----DTGTLEKKSGACEDHSMAQ 167
Chicken        GILDVKVSSDQKSAVVTFLSSIINGKQIVQMVPGVDL----SISAPEVTPGTCEDPSWSQ
Seabream       GVLDVRESPAQMSLSVTFIPSLTSSQQLSEVMVSLMPLDIPTLSSPTYKGPSLSPSHTAG
Pufferfish     GVLDVREGPAGTGLSVTFVPSLTTSLQLNEAVASWIP-ESPAPGSPKHEAPGSSPSHTVV
Zebrafish      GVIETQESADNQGLAVTFVPSLVSEDQLGEVLKCLAP---DTACRPPLSPKEGSTSRSFS
Seasquirt      GIITTQISLQNKQGVVNYKPNILNPSLIVSTIEDMGF----EASVSEDGVRKRTKTD---
FruitFly       ----------------------------------------DAADPPETPASAWTN----
Yeast          -------------------------------------------DSEITAISTKEG----
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Human           AGEVVLKMKVEGMTCHSCTSTIEGKIGKLQGVQRIKVSLDNQEATIVYQPHLISVEEMKK 227
Chicken         TSSVLLRLKVEGMTCHSCTSTIEGRIGKLQGIQRIKVSLDNQEAAVVYQPHLITAEEIKC
Seabream        DGVSLLKLRIEGMTCHSCTTTIEGKIGKLKGIEKIKVVLETQEATIVYLPYLITVQTIID
Pufferfish      GGVSLLKLCIEGMTCHSCTTTIEGKIGKLKGIEKIKVVLETQEATLVYLPHLITVQTITD
Zebrafish       G-VEAVKMRIEGMVCLSCTTTIEGKIGKLKGVEKIKVSLESQEAAVVYLPYIITVDEIVK
Seasquirt       ------KISIEGMTCNSCVQTIQQQIGSYTGVESIKVSLENKEATLDYNPELIGLELVID
FruitFly        -------IRVVGMTCQSCVRNIEGNIGTKPGIHSIEVQLAAKNARVQYDPAQYDPAQIAE
Yeast           ------LLSVQGMTCGSCVSTVTKQVEGIEGVESVVVSLVTEECHVIYEPSKTTLETARE
                      *******
                      MBD 2
Human           QIEAMGFPAFVKKQPKYLKLGAIDVERLKNTPVKSSEGS---QQRSPSYTNDSTATFIID 284
Chicken         QIEAAGFTASFKKQPRPLKLNAVDLERLKNTQTKSSDTAP-LKENTRNVNDTKTVVFRID
Seabream        QIAVAGFKASVKSKPRPLQLSPSEIERFVDSQKATISSPSETSEESEIFIDTTLIMLRVK
Pufferfish      QIAVAGFKAFVKTKPRPLQLSSDEFQRFVDSEKQAISSPSDTSEETEIFIDTVPAMLRVK
Zebrafish       QIEVAGFKATVKSKPRQLKLSASEVERLLSAPKQTEEKLSEPPADS-----TVTTLFQVT
Seasquirt       AIEDMGFDAALKYGVVE-KPRNDDLITLSDNFDVKGQAMLGNRDSVNGDVEYTVVNIGVE
FruitFly        LIDDMGFEASVQ-------------------------------------------------
Yeast           MIEDCGFD-----------------------------------------------------

Human           GMHCKSCVSNIESTLSALQYVSSIVVSLENRSAIVKYNASSVTPESLRKAIEAVSPGLYR 344
Chicken         GMHCSSCVLNIQSTISTLPSVTSIVVSLENKSAIVKYNPNLITVDVLRSAIEAVSPQTFK
Seabream        GMHCRSCVVNIQDNISKLPGVSSVEVSLEEEKASICYDPLKVTVTQLQQAIEALPPGNFK
Pufferfish      GMHCRSCVVNIQDNISKLPGVSSVEVSLEEERASVCYDPLKVTLSQLQQAIEALPPGNFR
Zebrafish       GMHCNSCVVNIQDNISKLPAVTSVVVSLENRQASIQHNPKQVSAVELQKAIEALPPGNFK
Seasquirt       GMHCKSCVRKIEENMKTMKGVAKVKVSLDDKMATISYDSDKIKEEKLAEKIKDLSFKATL
FruitFly        ----------------------------EPRSPSQSPSPAPASSPKKRATPTPPPP----
Yeast           -------------------------------------------------------------
                *******
                 MBD 3
Human           VSITSEVESTSNSPSSSS--------------LQKIPLNVVSQPLTQETVINIDGMTCNS 390
Chicken         VSLLDKYENVALFPALAS--------------PLKS-VKDAGQPLTQVVVINIEGMTCSS
Seabream        TQSWDSSAPFSAVTPSATPALLSLRPAGASQTKPAASQPCFMQPLASEANIHIEGMTCNS
Pufferfish      AQPWESPDPVRSATTSPAPDFLLPQPAGSNKARPASSEPYFTQPLLSVITIHIEGMTCTS
Zebrafish       AI-----------------------------IPASPEPGFLQPLVSVAEIHIEGMTCGS
Seasquirt       PNGRS--------------------------FTPETATAVNGDALIKLPPPPNGSDNSS
FruitFly        -------------------------------------------------------------
Yeast           -------------------------------------------------------------
                                                                      ******
                                                                      MBD 4
Human           CVQSIEGVISKKPGVKSIRVSLANSNGTVEYDPLLTSPETLRGAIEDMGFDATLSDTNEP 450
Chicken         CVQSIEGVISQKAGVKSINVSLANHNGTIEYDPLQTCPEDLRSSIENMGFDASLPEKTEL
Seabream        CVESIEGMISQRKGVMSAHVSLADHKGMFEYDPLLTSPEELREAIEDMGFDAFLPGTNSL
Pufferfish      CVQSIEGMISQKKGVMSAQVSLTDHSGVFEYDPVLTTPAELREAIEDMGFDAFLPATNSL
Zebrafish       CVQSIEGTLSQKKGVRSAQVSLANHKGTFEYDPLLTSPEELRAAIEDMGFDAFLPAFNRE
Seasquirt       KISEAAAVLSVRSTNHSIGSTPSNKTRSSAGRGTVKKAVSKRKKVEKQNSRKIVESSKML
FruitFly        --------------------SYAQN----------------------GSAVAIPVEQEL
Yeast           -------------------------------------------------------------
                *
Human           LVVIAQP---SSEMPLLTSTNEFYTKGMTPVQDKEEGKNSSKCYIQVTGMTCASCVANIE 507
Chicken         PVGITQP---TSKEQLES--AEPTSKMLRSFVAEQESKSLSKCYIQVTGMTCASCVANIE
Seabream        LPEPDRSLSKSSSLAPVTKLKELDSELHRETPQGCNGEMHSKCYIQIGGMTCASCVANIE
Pufferfish      LPEPACKRSNSSSVALVR--NELNSSFRKEPPRDQDGASHSKCYIHIGGMTCASCVANIE
Zebrafish       VPSVVKSPSPSVRSSSLSPVRSAVKENEAESDAEPSTNTISKCFIQIGGMTCASCVANIE
Seasquirt       EMSVSMET----------------------------DVERCFINITGMTCASCVNNIE
FruitFly        LT-----------------------------------KCFLHIRGMTCASCVAAIE
Yeast           -------------------------------------------------------SNII
                                                              *******
                                                              MBD 5
```

```
Human          RNLRREEGIYSILVALMAGKAEVRYNPAVIQPPMIAEFIRELGFGATVIENADEGDGVLE 567
Chicken        RNLRREDGIHSVLVALMAGKAEVRYNPAVIHPSAIAELIRELGFGATVMENSGEGDGILD
Seabream       RNLKNETGIYSVLVALMASKAEVRYNPELIDPGKIAECVKELGFTASVMENYEGSDGNLE
Pufferfish     RNLKNETGIYSVLVALMASKAEVRYNPELIDPLKMAECVKELGFTASVMENYEGSDGNLE
Zebrafish      RNLKNEYGIHSVLVALMASKAEVRYSPSVIDPLRIAELIRELGFTATVMDNYDGSDGSLE
Seasquirt      RNIGREEGIVSILVGLMSGRAEVKYRPSLIEPDTIAQLIEDLGFGAAVLEGTGKG-GQVE
FruitFly       KHCKKIYGLDSILVALLAAKAEVKFNANVVTAENIAKSITELGFPTELIDEPDNGEAEVE
Yeast          MDGNGNADMTEKTVILKVTKAFEDESPLILSS----------------------------

Human          LVVRGMTCASCVHKIESSLTKHRGILYCSVALATNKAHIKYDPEIIGPRDIIHTIESLGF 627
Chicken        LVVRGMTSAACVHKIESTLMKTNGVLYCSVALATNKAHIKYDPEIIGPRDIIQVIKDLDF
Seabream       LVVRGMTCASCVHKIESSLMREKGIIYASVALATNKAHIKFDSEIIGPRDIIKLIENLGF
Pufferfish     LVVKGMTCASCVHKIESNLTKRKGIIYVSVALATNKAHVKYDVEIIGPRDIIKLIENLGF
Zebrafish      LVVRGMTCASCVHKIESNLMKQKGILYASVALSTNKAHIKYDPEVTGPRDIIRLIENMGF
Seasquirt      LNVTGMTCSSCVHAIESRLQEVAGVTYASVALATSSAVVKYDPEILGVRDIIASIESAGF
FruitFly       LEIMGMTCASCVNKIESHVLKIRGVTTASVTLLTKRGKFRYITEETGPRSICEAIEALGF
Yeast          ------------VSERFQFLLDLGVKSIEISDDMHTLTIKYCCNELGIRDLLRHLERTGY
                   *******
                    MBD 6
Human          EASLVK-KDRSAS--HLDHKREIRQWRRSFLVSLFFCIPVMGLMIYMMVMDHHFATLHHN 684
Chicken        TTALVK-KDRSAS--HLDHRQEIRQWRRSFFVSLVFCIPVMAMMIYMMVVDSQLSDAHQH
Seabream       EASLVK-RDRTAS--HLDHSKEIRQWRKSFLVSLIFCVPVMGMMTYMIIMDHQMSVSHQH
Pufferfish     EVTLVK-NDRTAN--HLDHSKEIQQWRWSFLVSLFFCVPVMGMMMYMIVMDYKMSVSHPH
Zebrafish      TASLVK-KDRPGS—-HLDHSREIRQWKRSFQISLFFCVPVMGMMIYMIVVDHMIDKYHQH
Seasquirt      GASPRSCDNRVGA---LDHRVAIQQWRRSFLTALIFGVPVMIIMIYYMASGAHNNP----
FruitFly       EAKLMTGRDKMAHN-YLEHKEEIRKWRNAFLVSLIFGGPCMVAMIYFMLEMSDKGHANMC
Yeast          KFTVFSNLDNTTQLRLLSKEDEIRFWKKNSIKSTLLAIICMLLYMIVPMMWPTIVQDRIF
                                                 TMD1
Human          QNMSKEEMINLHSSMFLERQILPGLSVMNLLSFLLCVPVQFFGGWYFYIQAYKALKHKTA 744
Chicken        LNMSNEEMEAIHSSMFLEHQLLPGLSVMNFLSFLLCVPVQIFGGWHFYIQAYKALKHRTA
Seabream       N-ATVEDRNQYHSTMFLERQLLPGLSIMNLLSFLFCVPVQFIGGRYFYIQAYKALKHRSA
Pufferfish     N-LTAEERNHYHSSMVLEWQVAPGLSIMNLLSFIFCIPVQFIGGRKFYIQAYKSVKHRSA
Zebrafish      HNATAEDRAKYHSTMFLEKQLLPGLSIMNLISFLFCVPVQFIGGRYFYCQAYKAVKHRTA
Seasquirt      ------------------YMIVPGLSLQNLLMFLLCTPVQVYGGRYFYIQAWAAVKHRMA
FruitFly       C-------------------LVPGLSMENLVMFLLSTPVQFFGGFHFYVQSYRAIKHGTT
Yeast          P--------------YKETSFVRGLFYRDILGVILASYIQFSVGFYFYKAAWASLKHGSG
                                  TMD2
Human          NMDVLIVLATTIAFAYSLIILLVAMYERAKV--NPITFFDTPPMLFVFIALGRWLEHIAK 802
Chicken        NMDVLIVLATSVAFVYSFVILLVAMAEKAKV--NPVTFFDTPPMLLAFISLGRWLEHVAK
Seabream       NMDVLIVLATSIAFTYSLIVLIVAMVEKAKV--NPITFFDTPPMLFVFISLGRWLEQIAK
Pufferfish     NMDVLIVLATSIAFTYSVVVLIVAMAEKAKV--NPITFFDTPPMLFVFISLGRWLEQIAK
Zebrafish      NMDVLIVLATTIAFTYSVVVLLVAMVERAKV--NPITFFDTPPMLFVFISLGRWLEQIAK
Seasquirt      NMDVLIVMTTVICYAYSVILLIISMIQQAKG--SPKTFFETPPMLFVFIALGRWLEHIAK
FruitFly       NMDVLISMVTTISYVYSVAVVIAAVLLEQNS--SPLTFFDTPPMLLIFISLGRWLEHIAK
Yeast          TMDTLVCVSTTCAYTFSVFSLVHNMFHPSSTGKLPRIVFDTSIMIISYISIGKYLETLAK
                      TMD3                              TMD4
Human          GKTSEALAKLISLQATEATIVTLDSDNILLSEEQVDVELVQRGDIIKVVPGGKFPVDGRV 862
Chicken        GKTSEALARLISLQATEATIVTLGPDNILLSEEQVDVELVQRGDIVKVVPGGKFPVDGRV
Seabream       SKTSEALSKLMSLQATEATVVTLGSDKSILSEEQVDVELVQRGDVVKVVPGGKFPVDGRV
Pufferfish     SKTSEALSKLMSLQATEATVVTLGSDNSILSEEQLDVDLVQRGDVVKVVPGGKFPVDGRV
Zebrafish      SKTSEALSKLMSLQATEATVVTLNEDMSVLSEEQVDVELVQRGDVVKVVPGGKFPVDGRV
Seasquirt      GKTSEALAKLMQLQATEAILVVFGDDKTTVSEESISVDLVQRGDYLRVPPGTKIPTDGKV
FruitFly       GKTSEALSKLLSLKAADALLVEISPDFDIISEKVISVDYVQRGDILKVIPGAKVPVDGKV
Yeast          SQTSTALSKLIQLTPSVCSIISDVERN---ETKEIPIELLQVNDIVEIKPGMKIPADGII
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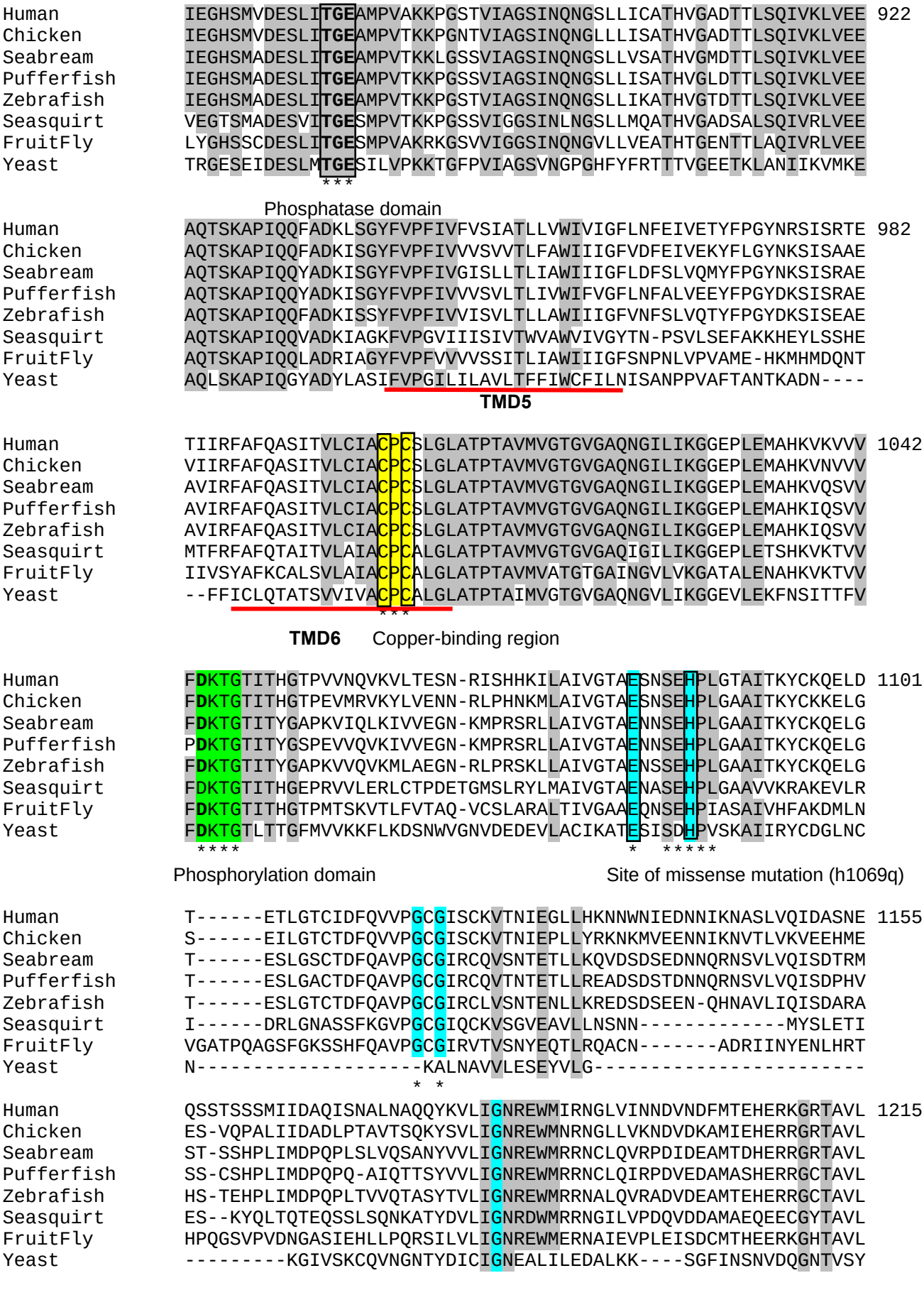
Human IEGHSMVDESLITGEAMPVAKKPGSTVIAGSINQNGSLLICATHVGADTTLSQIVKLVEE 922
Chicken IEGHSMVDESLITGEAMPVTKKPGNTVIAGSINQNGLLLISATHVGADTTLSQIVKLVEE
Seabream IEGHSMADESLITGEAMPVTKKLGSSVIAGSINQNGSLLVSATHVGMDTTLSQIVKLVEE
Pufferfish IEGHSMADESLITGEAMPVTKKPGSSVIAGSINQNGSLLISATHVGLDTTLSQIVKLVEE
Zebrafish IEGHSMADESLITGEAMPVTKKPGSTVIAGSINQNGSLLIKATHVGTDTTLSQIVKLVEE
Seasquirt VEGTSMADESVITGESMPVTKKPGSSVIGGSINLNGSLLMQATHVGADSALSQIVRLVEE
FruitFly LYGHSSCDESLITGESMPVAKRKGSVVIGGSINQNGVLLVEATHTGENTTLAQIVRLVEE
Yeast TRGESEIDESLMTGESILVPKKTGFPVIAGSVNGPGHFYFRTTTVGEETKLANIIKVMKE
Phosphatase domain
Human AQTSKAPIQQFADKLSGYFVPFIVFVSIATLLVWIVIGFLNFEIVETYFPGYNRSISRTE 982
Chicken AQTSKAPIQQFADKISGYFVPFIVVSVVTLFAWIIIGFVDFEIVEKYFLGYNKSISAAE
Seabream AQTSKAPIQQYADKISGYFVPFIVGISLLTLIAWIIIGFLDFSLVQMYFPGYNKSISRAE
Pufferfish AQTSKAPIQQYADKISGYFVPFIVVSVLTLIVWIFVGFLNFALVEEYFPGYDKSISRAE
Zebrafish AQTSKAPIQQFADKISSYFVPFIVVISVLTLLAWIIIGFVNFSLVQTYFPGYDKSISEAE
Seasquirt AQTSKAPIQQVADKIAGKFVPGVIIISIVTWVAWVIVGYTN-PSVLSEFAKKHEYLSSHE
FruitFly AQTSKAPIQQLADRIAGYFVPFVVVVSSITLIAWIIIGFSNPNLVPVAME-HKMHMDQNT
Yeast AQLSKAPIQGYADYLASIFVPGILILAVLTFFIWCFILNISANPPVAFTANTKADN----
TMD5
Human TIIRFAFQASITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVKVVV 1042
Chicken VIIRFAFQASITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVNVVV
Seabream AVIRFAFQASITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKVQSVV
Pufferfish AVIRFAFQASITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKIQSVV
Zebrafish AVIRFAFQASITVLCIACPCSLGLATPTAVMVGTGVGAQNGILIKGGEPLEMAHKIQSVV
Seasquirt MTFRFAFQTAITVLAIACPCALGLATPTAVMVGTGVGAQIGILIKGGEPLETSHKVKTVV
FruitFly IIVSYAFKCALSVLAIACPCALGLATPTAVMVATGTGAINGVLVKGATALENAHKVKTVV
Yeast --FFICLQTATSVVIVACPCALGLATPTAIMVGTGVGAQNGVLIKGGEVLEKFNSITTFV
TMD6
Copper-binding region
Human FDKTGTITHGTPVVNQVKVLTESN-RISHHKILAIVGTAESNSEHPLGTAITKYCKQELD 1101
Chicken FDKTGTITHGTPEVMRVKYLVENN-RLPHNKMLAIVGTAESNSEHPLGAAITKYCKKELG
Seabream FDKTGTITYGAPKVIQLKIVVEGN-KMPRSRLLAIVGTAENNSEHPLGAAITKYCKQELG
Pufferfish PDKTGTITYGSPEVVQVKIVVEGN-KMPRSRLLAIVGTAENNSEHPLGAAITKYCKQELG
Zebrafish FDKTGTITYGAPKVVQVKMLAEGN-RLPRSKLLAIVGTAENSSEHPLGAAITKYCKQELG
Seasquirt FDKTGTITHGEPRVVLERLCTPDETGMSLRYLMAIVGTAENASEHPLGAAVVKRAKEVLR
FruitFly FDKTGTITHGTPMTSKVTLFVTAQ-VCSLARALTIVGAAEQNSEHPIASAIVHFAKDMLN
Yeast FDKTGTLTTGFMVVKKFLKDSNWVGNVDEDEVLACIKATESISDHPVSKAIIRYCDGLNC
Phosphorylation domain
Site of missense mutation (h1069q)
Human T------ETLGTCIDFQVVPGCGISCKVTNIEGLLHKNNWNIEDNNIKNASLVQIDASNE 1155
Chicken S------EILGTCTDFQVVPGCGISCKVTNIEPLLYRKNKMVEENNIKNVTLVKVEEHME
Seabream T------ESLGSCTDFQAVPGCGIRCQVSNTETLLKQVDSDSEDNNQRNSVLVQISDTRM
Pufferfish T------ESLGACTDFQAVPGCGIRCQVTNTETLLREADSDSTDNNQRNSVLVQISDPHV
Zebrafish T------ESLGTCTDFQAVPGCGIRCLVSNTENLLKREDSDSEEN-QHNAVLIQISDARA
Seasquirt I------DRLGNASSFKGVPGCGIQCKVSGVEAVLLNSNN------------MYSLETI
FruitFly VGATPQAGSFGKSSHFQAVPGCGIRVTVSNYEQTLRQACN-------ADRIINYENLHRT
Yeast N--------------------KALNAVVLESEYVLG----------------------
Human QSSTSSSMIIDAQISNALNAQQYKVLIGNREWMIRNGLVINNDVNDFMTEHERKGRTAVL 1215
Chicken ES-VQPALIIDADLPTAVTSQKYSVLIGNREWMNRNGLLVKNDVDKAMIEHERRGRTAVL
Seabream ST-SSHPLIMDPQPLSLVQSANYVVLIGNREWMRRNCLQVRPDIDEAMTDHERRGRTAVL
Pufferfish SS-CSHPLIMDPQPQ-AIQTTSYVVLIGNREWMRRNCLQIRPDVEDAMASHERRGCTAVL
Zebrafish HS-TEHPLIMDPQPLTVVQTASYTVLIGNREWMRRNALQVRADVDEAMTEHERRGCTAVL
Seasquirt ES--KYQLTQTEQSSLSQNKATYDVLIGNRDWMRRNGILVPDQVDDAMAEQEECGYTAVL
FruitFly HPQGSVPVDNGASIEHLLPQRSILVLIGNREWMERNAIEVPLEISDCMTHEERKGHTAVL
Yeast ---------KGIVSKCQVNGNTYDICIGNEALILEDALKK----SGFINSNVDQGNTVSY

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Human          VAVDDELCGLIAIADTVKPEAELAIHILKSMGLEVVLMTGDNSKTARSIASQVGIT--KV 1273
Chicken        VAVDGVLCGLIAIADTVKPEAELAVYTLKNMGLEVVLMTGDNSKTARSIASQVGIS--KV
Seabream       VAVDDVLCAMIAIADTVKPEAELAVHTLTNMGLEVVLMTGDNNKTARAIAAQVGIR--KV
Pufferfish     VAIDNTLCAMIAISDKVKPEAELAVHTLTNMGLEVVLMTGDNSKTARAIAAQVGIR--TV
Zebrafish      VAVDNELCAMVAIADTVKPEAELAVHVLSAMGLEVVLMTGDNSKTARAIAAQVGIR--KV
Seasquirt      TAVNEKLEGMIAIADTVKSEAALAVYTLQHMGIDVILLTGDNKKTAKAIARQAGIK--NV
FruitFly       CALNGQLVCMFAVSDMVKPEAHLAVYTLKRMGIDVVLLTGDNKNTAASIAREVGIR--TV
Yeast          VSVNGHVFGLFEINDEVKHDSYATVQYLQRNGYETYMITGDNNSAAKRVAREVGISFENV
                                                      ****

Human          FAEVLPSHKVAKVKQLQEE--GKRVAMVGDGINDSPALAMANVGIAIGTGTDVAIEAADV 1331
Chicken        FAEVLPSHKVAKVKQLQDE--GKRVAMVGDGINDSPALAMANVGIAIGTGTDVAIEAADV
Seabream       FAEVLPSHKVAKVEQLQQA--GKRVAMVGDGVNDSPALAMADVGIAIGTGTDVAIEAADV
Pufferfish     FAEVLPSHKVAKVEQLQQA--GKRVAMVGDGVNDSPALAMADVGIAIGTGTDVAIEAADV
Zebrafish      FAEVLPSHKVAKVEQLQQE--GKRVAMVGDGVNDSPALAMADVGIAIGTGTDVAIEAADV
Seasquirt      YAEVLPSHKVDKVRQLQES--GHKVAMVGDGVNDSPALAQADVGVAIGTGTDVAVEAADV
FruitFly       YAEVLPSHKVAKIQRIQAN--GIRVAMVGDGVNDSPALAQADVGITIAAGTDVAAEASDI
Yeast          YSDVSPTGKCDLVKKIQDKEGNNKVAVVGDGINDAPALALSDLGIAISTGTEIAIEAADI
                                        ******
                                        ATP-binding site

Human          VLIR------NDLLDVVASIDLSRETVKRIRINFVFALIYNLVGIPIAAGVFMPIGLVLQ 1385
Chicken        VLIK------DDLMDVVASIDLSRKTVKRIRINFVFALIYNLVGVPIAAGVFLPIGLVLQ
Seabream       VLIR------NDLLDVVGSIDLSKKTVKRIRINFVFALIYNLVGIPIAAGVFLPVGLVLQ
Pufferfish     VLIR------NDLLDVVGSIDLSKKTVKRIRINFVFALIYNLVGIPVAAGVFLPVGLVLQ
Zebrafish      VLIR------NDLLDVVGSIDLSKKTVKRIRINFVFALMYNLVGIPIAAGVFMPVGLVLQ
Seasquirt      VLIK------SDLMDVVAAIDLSQHVIRRIRYNFVFACVYNLIGIPLAAGAFYTLGVVLE
FruitFly       VLMR------NDLLDVVACLDLSRCTVRRIRYNFFFASMYNLLGIPLASGLFAPYGFTLL
Yeast          VILCGNDLNTNSLRGLANAIDISLKTFKRIKLNLFWALCYNIFMIPIAMGVLIPWGITLP
                                                 TMD7

Human          PWMGSAAMAASSVSVVLSSLFLKLYRKPTYE-----SYELPARSQIGQKS---PSEISVH 1437
Chicken        PWMGSAAMAASSVSVVLSSLLLKMYQKPSSE-----KLEFRARGQMKQKS---PSEISVH
Seabream       PWMGSAAMALSSVSVVLSSLLLKCYTKPTAE-----KLEARLGNNRRQGS---LSDVSVH
Pufferfish     PWMGSAAMAMSSVSVVLSSLLLKCYTKPSAE-----QLEAKLGHIRRQKS---LSEISVH
Zebrafish      PWMGSAAMALSSVSVVLSSLLLKCYTKPTVE-----KLKRRLGDVRTHGS---LSDVSVH
Seasquirt      PWMGSAAMALSSVSVVMSSLFLKTYKKPSLT-----RFELKLGSSRRGGS---SSDVSVS
FruitFly       PWMASVAMAASSVSVVCSSLLLKMYRKPTAKTLRTAEYEAQLAAERASGSEDELDKLSLH
Yeast          PMLAGLAMAFSSVSVVLSSLMLKKWTPPDIES----------------------------
                        TMD8

Human          VGIDDTSRNS------PKLGLLDRIVNYSRASINSLLSDK-RSLNSVVTSEPDKHSLLV 1489
Chicken        IGIDETGTGT------RKLSLMDRIINYSRASINSLFSDK-RSVNSIVLNEPDKHSLLV
Seabream       IGMGEMRRPS------PKLSLLDRIVNYSRASINSLRSDK-HSLNSLVLSEPDKHSLLV
Pufferfish     IGMGTARRPS------PRLSLLDRIVNYSRASINSLRSDK-HSLNSLALSEPDKHSLLV
Zebrafish      IGMGELRRPS------PKLSLLDRFVNYSRASINSLRSDK-HSMNSMALSEPDKHSLLV
Seasquirt      IGCGSCRKPS------PKLTLVDYVRRVSRVSVNQTASDQHAINAKSLSEQCRNSLQSV
FruitFly       RGLDDLPEKGRMPFKRSSTSLISRIFMHDYGNITSPDAKYGEGLLDPEEQYDGRTKIVR
Yeast          HGISDFKSKF----------------------SIGNFWSRLFSTRAIAGEQDIESQAGLM
                                                                      **
                                                Protein relocalization to the TGN

Human          GDFREDD**DTAL**--- 1500
Chicken        GDFGEDDDTTL--- 1494
Seabream       GESLCEEEFC---- 1526
Pufferfish     GEPLCEEELC---- 1513
Zebrafish      GDDHCDNEI----- 1469
Seasquirt      NNRTLKRFEMLK
FruitFly       SRFHANDSTELQKL 1219
Yeast          SNEEVL---------1004
                      ****
               Protein retention at the basolateral membrane
```

Figure 2-15 ATP7A sequence alignment. Residues identical in all proteins are shaded. The position of the metal binding domain (MBD) is indicated. Trans-membrane domains (TMD) are underlined. Functionally important domains in human ATP7A are indicated with stars. The residues at the P and the N-domain (located between TMD6 and TMD7) required for ATP binding activity are highlighted in green and blue respectively and the residues predicted to be involved in Cu coordination within the membrane are highlighted in yellow. The residues boxed have been shown by mutagenesis to be necessary for activity.

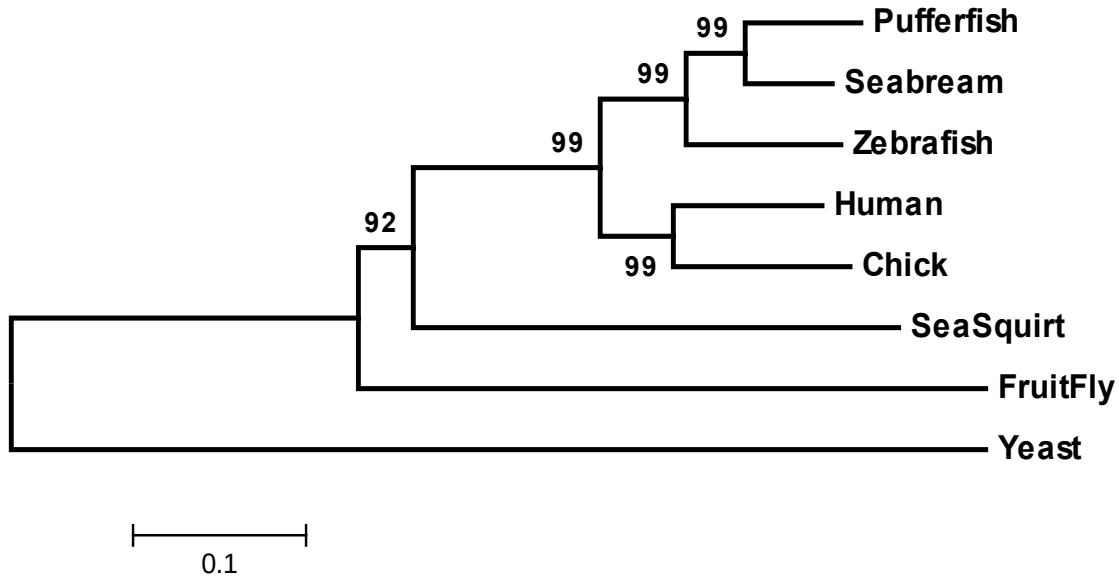

Figure 2-16 ATP7A phylogeny tree. Human (NM_000052), chick (*Gallus galllus*, XM_420307), fly (*Drosophila melanogaster*, AE014298), sea squirt (*Ciona intestinalis,* Ensembl-Chr14q ENSCING00000007245), yeast (*Saccharomyces cerevisiae*, L35270), zebra fish (*Danio rerio*, NP_001036185), puffer fish (*Tetraodon negroviridis* Ensembl-Chr1 scaf14573) and the sea bream ATP7A 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.

2.3.4 Sea bream ATP7B cDNA

The first fragment of ATP7B synthesised was the 3'end which was obtained by two rounds of RACE nested PCR using the degenerate primers 3'ATP7B-F2 and 3'ATP7B-F1 and yield a fragment of 742 bp. This fragment showed 91 and 86 % identity

to the zebrafish and human ATP7B respectively. Then based on this fragment, sea bream specific reverse primers (ATP7B-R5 and ATP7B-R4) combined with degenerate primers (ATP7B-F1 and ATP7B-F3) were used to generate a central fragment of 2270 bp which overlapped 100 % with the 3'end fragment. This fragment showed 83 and 77 % identity to zebrafish and human ATP7B. Finally the 5'end was obtained by two rounds of RACE nested PCR using sea bream specific primers 5'ATP7B-R1 and 5'ATP7B-R2 which yielded a fragment of 2160 bp which overlapped 100% identity with the central fragment sequence and showed 82 and 73 % identity with zebrafish and human ATP7B respectively. The final assembling of the cDNA fragments allowed the construction of the full length cDNA for sea bream ATP7B (SaATP7B) which resulted in an open reading frame of 1327 amino acids and 5' and 3' untranslated regions of 55 bp and 250 bp respectively (Figure 2-10). To confirm the SaATP7B sequence, primers (ATP7BFull-F1, ATP7BFull-F2 and ATP7BFull-R1, ATP7BFull-R2) were designed to amplify the entire sea bream ATP7B ORF. All primers used are specified in Table 2-1. The sequence obtained (SaATP7B) was 100 % identical to the assembled sea bream sequence and showed 75 % identity with the human sequence and 83 % with the zebrafish sequence.

The deduced amino acid sequence of ATP7B conserved all the domains which functionally characterize Cu-ATPases, including all amino acids shown to be important for protein activity by mutational analysis studies (Solioz and Vulpe 1996b; Lutsenko et al., 2007a). Furthermore, the saATP7B hydrophobicity plot, obtained by the online software (www.vivo.colostate.edu/molkit/hydropathy/index.html), confirmed the hydrophobicity of the 8 TMDs.

To verify if fish species contain multiple genes for which only single mammalian examples exist, the sea bream cDNA sequence was used to search for similar sequences in

the zebrafish, puffer fish, medaka and stickleback genomes. In each species a single ATP7B gene was identified. Furthermore, phylogenetic analysis, comparing fish ATP7A and ATP7B sequences with other vertebrate's ATP7A and ATP7B sequences confirmed the identity of the two isoforms of Cu ATPases synthesised in sea bream (Figure 2-18). Moreover, comparison of the fish sequences with ATP7B from other vertebrates indicated that the fish genes were monophyletic and that the sea bream ATP7B was most closely related to the puffer fish protein (Figure 2-18).

```
Human           ------------------------------------------------------------
Chicken         ------------------------------------------------------------
Frog            ------------------------------------------------------------
Seabream        ----------------------------------------MFSPKSPKSLSRYVSGPGPA
Stickleback     ----------------------------------------MFSPKSPKKPPKRVP---ES
Pufferfish      ----------------------------------------MLAAHRSRSSAGPGGVRGPQ
Zebrafish       -----------------------------------MNKLSPFSNLAKFISKSPDNGPV

Human           ----MPEQERQITAREGASRKILSKLSLPT--RAWEPAMKKSFAFDNVGYEGGLDGLG-P 53
Chicken         --------------MEPSARMIVVKMYLKTVPKAW---MRQNFAFDNMGYEESFEAMPSP
Frog            ----------------SARLIVVLWTDSPPVSVWKEAKKPSCAFDNRGYEGSPDDLCSL
Seabream        AGEHICMVECVCEPDCTC—TEHGARHCAARDVKKNG-LDAEKHGLDNLAYE---------
Stickleback     SAERIRMVDCACTPDCTCGPSECPGGGLCARDPQENGLRVDQRGLDNLAYE---------
Pufferfish      PGEDICMVDCA-----RAAPAPAPGTTVSPGPVLRSAGPDYIQGFDNLAYE---------
Zebrafish       GGEQLCMKDCKPLCHCDPELC--------ENGSQQEGWIPTKHAFDNFGYE—-PDGL---

Human           SSQVATSTVRILGMTCQSCVKSIEDRISNLKGIISMKVSLEQGSATVKYVPSVVCLQQVC 113
Chicken         SSQERTVAISIVGMTCQSCVQSVEGRMSKVKGVVSIKVSLELNNAVVKYLQSEISPEQIC
Frog            PDDVGSVVVAIQGMTCQSCVQSIEGRISKVSGVVGINVCLEQNNAIVNYLQTEITPHKIC
Seabream        ------------------------------------------------------------
Stickleback     ------------------------------------------------------------
Pufferfish      ------------------------------------------------------------
Zebrafish       ------------------------------------------------------------
                         *******
                        MBD 1
Human           HQIGDMGFEASIAEGKAASWPSRSLPAQEAVVKLRVEGMTCQSCVSSIEGKVRKLQGVVR 173
Chicken         QEIEDMGFDASIAEERLTPVSVNLPCSREAVIKLRIEGMTCQSCVTSIEGKIKKLHGVAK
Frog            EEIEDMGFDASLSEQSGMPSSVKSSYYGDNVIKIRVEGMTCQSCVNTIEGKIGKIQGVQK
Seabream        ---------------YRSQSELYPPSTASCRATFKLLGLTPEHQVHAIQSRVSGLNGVLT
Stickleback     ---------------YGSQRELRPPPKTASTATFKLLGLGAGHQARAVEGGVCGLDGVLA
Pufferfish      ---------------RGSQSELRPLAQPASRATFRLPGLGPEPSAEAVGSKFSGLAGVLS
Zebrafish       -----------------KHNLVHQLPSEEGMVKIQVEGMTCQSCVRSIEEQIGRLEGVIG
                                                 *******
                                                MBD 2
Human           VKVSLSNQEAVITYQPYLIQPEDLRDHVNDMGFEAAIKSKVAPLSLGPIDIERLQSTNPK 233
Chicken         IKVSLSNQEAVIAYHPYIIQPEELRSHISNLGYDCTVKNKSAPLKLGVLDVRNLQSADPR
Frog            IKVSLTGQEAVITYQSHIIQAEDLRKYIEDMGFEASIKNKPDPTKLGTIDIERLQNSIAE
Seabream        ASLSSASSLAKVDYDTSVITTKEIVLELQAIGFNV----------------------
Stickleback     ASLSSPSGLAKVHYDAAAVTTKDIALQLQRLGLDA----------------------
Pufferfish      -VFCPSKHLVRVDYDASVLTERDLVLAVQNRGLDV----------------------
Zebrafish       VQVSLSDKEAILRFNPAKVTPEDMRKRIEDMGFDALILALQGQIQP-------------
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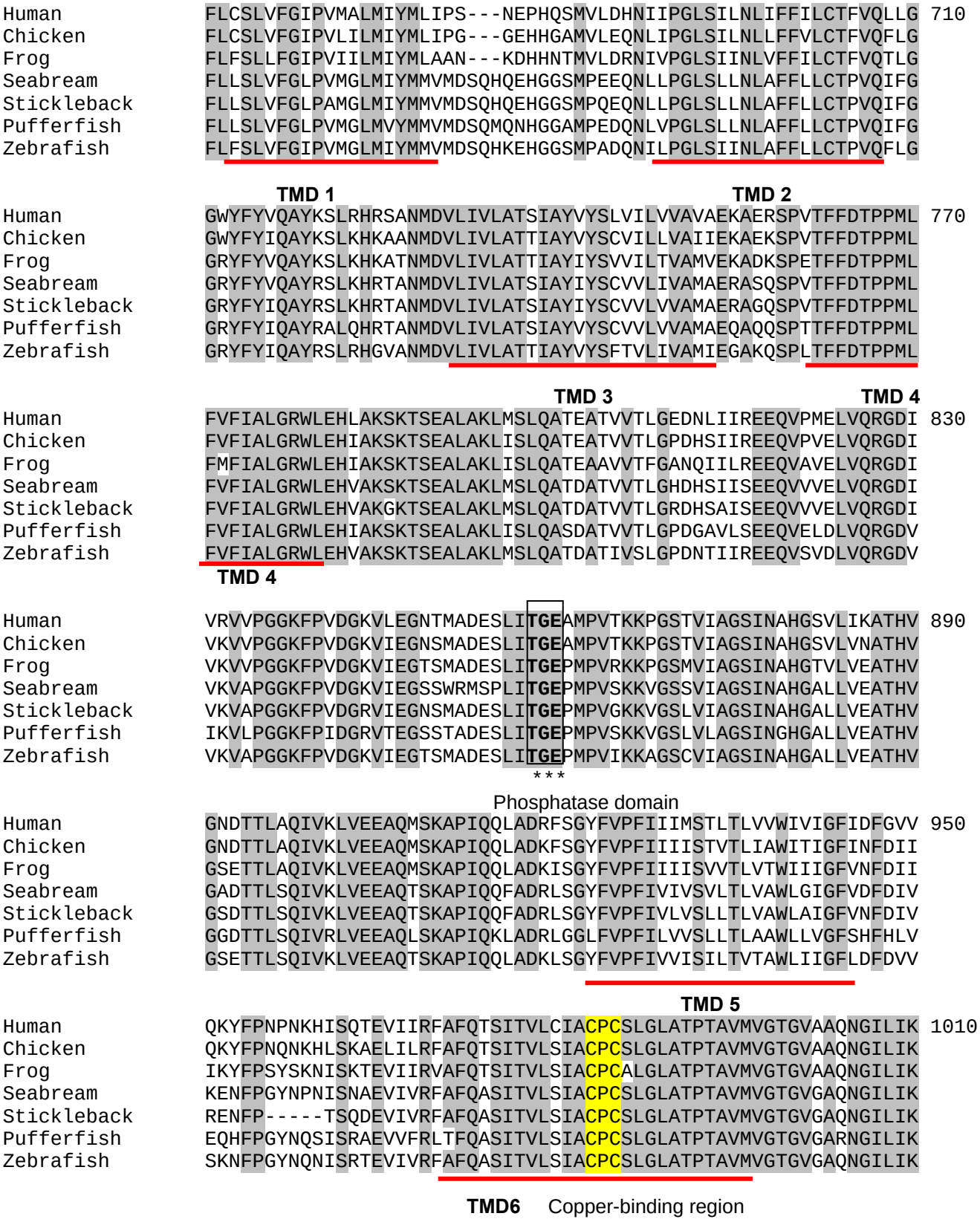
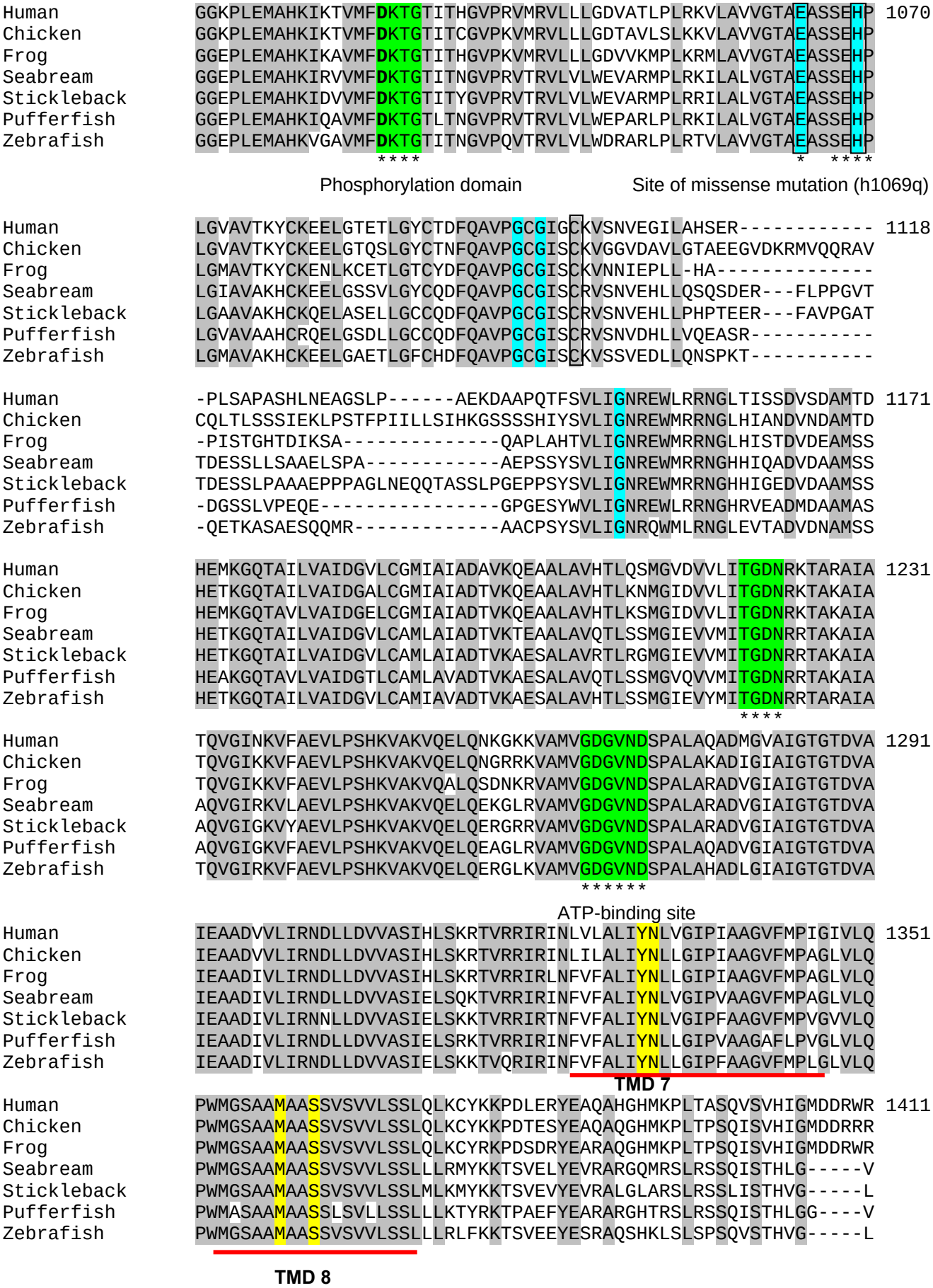
Human           RPLSSANQNFNNSETLGHQGSHVVTLQLRIDGMHCKSCVLNIEENIGQLLGVQSIQVSLE 293
Chicken         ETPVSLGKEVLHP--LVANKSSTAAVTVHIEGMHCKSCVRNIEGNISSLPGIQSIEASLE
Frog            N--------------HSGHTNSNTVTLGIDGMHCKSCVHNIEGYVSGLAGIQSIRVSLK
Seabream        ----------------------ESAVRIGVNGMHCQSCVQSIEGHIGPLSGVSHIQVSLQ
Stickleback     ----------------------ESAARVRVDGMRCQSCVQSIEGRIGARPGVSHIRVSLQ
Pufferfish      ----------------------ESVFWLRVEGAHSQPSIQTIQEQLGSLAGVSDVRGSLQ
Zebrafish       ------------------LSTDWSEVTLGVEGMHCGSCVKNITETLSGMLGVNSVFVSLE
                                         *******
                                                MBD 3
Human           NKTAQVKYDPSCTSPVALQRAIEALPPGNFKVSLPDGAEGSGTDHRSSSSHSPGSPPRNQ 353
Chicken         HKCAVVQYSPNLITLSALQQAIESLPPGNFKVCLPNSSEAN--NQASPSPALVCDLFREP
Frog            NKNAVVCLSQGSTSLLSLKESIENLPPGKFKVTLPVGVEKG----QSLARNSTHSSHRDQ
Seabream        DAAALIVYQPLLVTQEELKDKIEDMGFE----------------------ATLLTADQGD
Stickleback     DAAALIVYRPLLVSRQELRGAIEEMGFG----------------------ASLRDA----
Pufferfish      ECAVMVTYRPLLVTQQALKEHIRDLGFSSWSLAD---------------AALSCWQEVS
Zebrafish       KGSVDLRFDPSLLTLETVKGFLEEIPPGNFRVSI---------------PGWSSRLN-S

Human           VQGTCSTTLIAIAGMTCASCVHSIEGMISQLEGVQQISVSLAEGTATVLYNPAVISPEEL 413
Chicken         LKDTVCTAVVRIDGMTCNSCVQSIEGTMSQRQGVQHVAVSLADKTGTIHYDPANTNGEEL
Frog            SMG-GNIAIISIGGMTCQSCVSSIENMISQRKGVLHILVSLDEGNGNIFYNPCETNAEEL
Seabream        VFNSTQTVTIWIVGMTCNSCVQSIEGRISQATGVRSIAVSLKEEKGTITFDPSLTQPEQL
Stickleback     ---SIATVTIRIAGMTCNSCVQSVEGRISQVTGVQSIAVSLEEGKGTVTFDPGLTEPELL
Pufferfish      SDWSAHSVTLCIAGMTCSSCSSSIQERISQMGGVKSIAVSLSDGTATVTFDPRLTEAELL
Zebrafish       ASTPTQSVTIGIEGMTCNSCVQAIEGMMSQRAGVCSIKVYLQEKKGIVTFDSTVTCPEEL
                          *******
                                MBD 4
Human           RAAIEDMGFEASVVSESCSTNPLGNHSAGNSMVQTTDGTPTSLQEVAPHTGRLPANHAPD 473
Chicken         RAAIEEMGFDASLLTGNSVLSSVGSTSKG-------------LEVGTMSFKWQMPLPLD
Frog            RAAIEDMGFHSTLVSDNSPSISCSEYNSKEE--ENKQTPPKATRQISGSRDYILDVLPKK
Seabream        RAAIEDMGFEASLEEPPKSIQGQEKSRPVFS-----------GLSDLLDLKSQNKAGVSN
Stickleback     RAAVDDMGFEASLEGWQTFAFG--------------------------IMEQKAPVNS
Pufferfish      QAAIEEMGFEASVQVELSGVFFFSSECAN---------------GRLSPNRTRRTTVEN
Zebrafish       RAEIEDMGFEAWLNQD-SEICEVSSVSQMPS-----------GLKHLPSQRHPSKPSPSP

Human           ILAKSPQSTRAVAPQKCFLQIKGMTCASCVSNIERNLQKEAGVLSVLVALMAGKAEIKYD 533
Chicken         PHLDEPNQPSGATAKKCFLQITGMTCASCVSTIERNLQKEDGIISVLVALMAGKAEIKYK
Frog            SHPDFANEKYDTAPEKCFLQITGMTCISCVSNIERNLKKKDGIVSVLVALMSGKAEVKFY
Seabream        GTLSHKTTGSEVKVQKCFICVTGMTCASCVANIERNLLKHKGIIMVLVSLMAGKAEVKYD
Stickleback     GAGSQATPGSPTKAQKCFICVTGMTCASCVANVERNLHKHRGILSVLVSLMAGKAEVKYD
Pufferfish      GVGPQVTRRPEVRTQRCFIAVTGMTCASCVGNIERKLRSHGGITAVFVSLMAAKAEVTYD
Zebrafish       ITKENADGTGERELRKCFVHVTGMTCASCVSNIERNLVKHEGIKSVLVALMAGKAEVKYD
                                  *******
                                       MBD 5
Human           PEVIQPLEIAQFIQDLGFEAAVMEDYAGSDGNIELTITGMTCASCVHNIESKLTRTNGIT 593
Chicken         PEFIQPLEIAQLIQNLGFEATVIEDHSEAEGNVELLITGMTCASCVHNIESKLMRTNGIF
Frog            PDRIEPLEIAQLVEDLGFGASVMEDYTASDGNVELIITGMTCASCVHNIESRLMRTPGIL
Seabream        SEVLNAAAVTQLIEDLGFGAKLIEDNAVAHGKLDLAITGMTCASCVHNIESKLNTTKGIL
Stickleback     AEVLDAAAVTRLIEDLGFGAKLIEDHAGTDGKLDLTITGMTCASCVHNIESKLASTKGII
Pufferfish      PDSIGAAGVARLIEDLGFGATVMDQAAANPGLLELRLSGMTCASCVHKIESKLRSTPGVM
Zebrafish       PGLLDPAQIVQLISHLGFGASVMEEHSVQDGVLDLSVTGMTCASCVHNIESKLLRTKGIQ
                                                *******
                                                      MBD 6
Human           YASVALATSKALVKFDPEIIGPRDIIKIIEEIGFHASLAQRNPNAHHLDHKMEIKQWKKS 653
Chicken         YASVALATCKAHIQFDPEITGPRDIIKIIEEMGFHASVSRRVPNTHNLDHKKEIQQWRKS
Frog            QASVALATCKAQVKFDPEIVGPRDIIRIIEGIGFQASLAKRDPTAHKLDHKEEIKQWRNS
Seabream        GASVALATKKAQVQFDPDVLGARDIIKIIQSLGFEASLVKS-GYKNNLDHTEEIRQWKNS
Stickleback     VASVALATKKARIQFDPEVLGARDITKIIQSLGFEASLVKA-SFRNNLDHTEEIRQWKNS
Pufferfish      AATVSLATNRAQVRYHPEAVGARDLLAIIQDLGFQAELEKT-GLKQNLDHSKEILQWRNS
Zebrafish       EASVALATNKAHVKFDSDLVGSRDIVRIIEGLGFGVSLIKNEGLNNTLDHQEEIRQWKHS
```


Human FLCSLVFGIPVMALMIYMLIPS---NEPHQSMVLDHNIIPGLSILNLIFFILCTFVQLLG 710
Chicken FLCSLVFGIPVLILMIYMLIPG---GEHHGAMVLEQNLIPGLSILNLLFFVLCTFVQFLG
Frog FLFSLLFGIPVIILMIYMLAAN---KDHHNTMVLDRNIVPGLSIINLVFFILCTFVQTLG
Seabream FLLSLVFGLPVMGLMIYMMVMDSQHQEHGGSMPEEQNLLPGLSLLNLAFFLLCTPVQIFG
Stickleback FLLSLVFGLPAMGLMIYMMVMDSQHQEHGGSMPQEQNLLPGLSLLNLAFFLLCTPVQIFG
Pufferfish FLLSLVFGLPVMGLMVYMMVMDSQMQNHGGAMPEDQNLVPGLSLLNLAFFLLCTPVQIFG
Zebrafish FLFSLVFGIPVMGLMIYMMVMDSQHKEHGGSMPADQNILPGLSIINLAFFLLCTPVQFLG
TMD 1
TMD 2
Human GWYFYVQAYKSLRHRSANMDVLIVLATSIAYVYSLVILVVAVAEKAERSPVTFFDTPPML 770
Chicken GWYFYIQAYKSLKHKAANMDVLIVLATTIAYVYSCVILLVAIIEKAEKSPVTFFDTPPML
Frog GRYFYVQAYKSLKHKATNMDVLIVLATTIAYIYSVVILTVAMVEKADKSPETFFDTPPML
Seabream GRYFYVQAYRSLKHRTANMDVLIVLATSIAYIYSCVVLIVAMAERASQSPVTFFDTPPML
Stickleback GRYFYIQAYRSLKHRTANMDVLIVLATSIAYIYSCVVLVVAMAERAGQSPVTFFDTPPML
Pufferfish GRYFYIQAYRALQHRTANMDVLIVLATSIAYVYSCVVLVVAMAEQAQQSPTTFFDTPPML
Zebrafish GRYFYIQAYRSLRHGVANMDVLIVLATTIAYVYSFTVLIVAMIEGAKQSPLTFFDTPPML
TMD 3
TMD 4
Human FVFIALGRWLEHLAKSKTSEALAKLMSLQATEATVVTLGEDNLIIREEQVPMELVQRGDI 830
Chicken FVFIALGRWLEHIAKSKTSEALAKLISLQATEATVVTLGPDHSIIREEQVPVELVQRGDI
Frog FMFIALGRWLEHIAKSKTSEALAKLISLQATEAAVVTFGANQIILREEQVAVELVQRGDI
Seabream FVFIALGRWLEHVAKSKTSEALAKLMSLQATDATVVTLGHDHSIISEEQVVVELVQRGDI
Stickleback FVFIALGRWLEHVAKGKTSEALAKLMSLQATDATVVTLGRDHSAISEEQVVVELVQRGDI
Pufferfish FVFIALGRWLEHIAKSKTSEALAKLISLQASDATVVTLGPDGAVLSEEQVELDLVQRGDV
Zebrafish FVFIALGRWLEHVAKSKTSEALAKLMSLQATDATIVSLGPDNTIIREEQVSVDLVQRGDV
TMD 4
Human VRVVPGGKFPVDGKVLEGNTMADESLITGEAMPVTKKPGSTVIAGSINAHGSVLIKATHV 890
Chicken VKVVPGGKFPVDGKVIEGNSMADESLITGEAMPVTKKPGSTVIAGSINAHGSVLVNATHV
Frog VKVVPGGKFPVDGKVIEGTSMADESLITGEPMPVRKKPGSMVIAGSINAHGTVLVEATHV
Seabream VKVAPGGKFPVDGKVIEGSSWRMSPLITGEPMPVSKKVGSSVIAGSINAHGALLVEATHV
Stickleback VKVAPGGKFPVDGRVIEGNSMADESLITGEPMPVGKKVGSLVIAGSINAHGALLVEATHV
Pufferfish IKVLPGGKFPIDGRVTEGSSTADESLITGEPMPVSKKVGSLVLAGSINGHGALLVEATHV
Zebrafish VKVAPGGKFPVDGKVIEGTSMADESLITGEPMPVIKKAGSCVIAGSINAHGALLVEATHV
* * *
Phosphatase domain
Human GNDTTLAQIVKLVEEAQMSKAPIQQLADRFSGYFVPFIIIMSTLTLVVWIVIGFIDFGVV 950
Chicken GNDTTLAQIVKLVEEAQMSKAPIQQLADKFSGYFVPFIIIISTVTLIAWITIGFINFDII
Frog GSETTLAQIVKLVEEAQMSKAPIQQLADKISGYFVPFIIIISVVTLVTWIIIGFVNFDII
Seabream GADTTLSQIVKLVEEAQTSKAPIQQFADRLSGYFVPFIVIVSVLTLVAWLGIGFVDFDIV
Stickleback GSDTTLSQIVKLVEEAQTSKAPIQQFADRLSGYFVPFIVLVSLLTLVAWLAIGFVNFDIV
Pufferfish GGDTTLSQIVRLVEEAQLSKAPIQKLADRLGGLFVPFILVVSLLTLAAWLLVGFSHFHLV
Zebrafish GSETTLSQIVKLVEEAQTSKAPIQQLADKLSGYFVPFIVVISILTVTAWLIIGFLDFDVV
TMD 5
Human QKYFPNPNKHISQTEVIIRFAFQTSITVLCIACPCSLGLATPTAVMVGTGVAAQNGILIK 1010
Chicken QKYFPNQNKHLSKAELILRFAFQTSITVLSIACPCSLGLATPTAVMVGTGVAAQNGILIK
Frog IKYFPSYSKNISKTEVIIRVAFQTSITVLSIACPCALGLATPTAVMVGTGVAAQNGILIK
Seabream KENFPGYNPNISNAEVIVRFAFQASITVLSIACPCSLGLATPTAVMVGTGVGAQNGILIK
Stickleback RENFP-----TSQDEVIVRFAFQASITVLSIACPCSLGLATPTAVMVGTGVGAQNGILIK
Pufferfish EQHFPGYNQSISRAEVVFRLTFQASITVLSIACPCSLGLATPTAVMVGTGVGARNGILIK
Zebrafish SKNFPGYNQNISRTEVIVRFAFQASITVLSIACPCSLGLATPTAVMVGTGVGAQNGILIK
TMD6
Copper-binding region


Human GGKPLEMAHKIKTVMFDKTGTITHGVPRVMRVLLLGDVATLPLRKVLAVVGTAEASSEHP 1070
Chicken GGKPLEMAHKIKTVMFDKTGTITCGVPKVMRVLLLGDTAVLSLKKVLAVVGTAEASSEHP
Frog GGEPLEMAHKIKAVMFDKTGTITHGVPKVMRVLLLGDVVKMPLKRMLAVVGTAEASSEHP
Seabream GGEPLEMAHKIRVVMFDKTGTITNGVPRVTRVLVLWEVARMPLRKILALVGTAEASSEHP
Stickleback GGEPLEMAHKIDVVMFDKTGTITYGVPRVTRVLVLWEVARMPLRRILALVGTAEASSEHP
Pufferfish GGEPLEMAHKIQAVMFDKTGTLTNGVPRVTRVLVLWEPARLPLRKILALVGTAEASSEHP
Zebrafish GGEPLEMAHKVGAVMFDKTGTITNGVPQVTRVLVLWDRARLPLRTVLAVVGTAEASSEHP
Phosphorylation domain
Site of missense mutation (h1069q)
Human LGVAVTKYCKEELGTETLGYCTDFQAVPGCGIGCKVSNVEGILAHSER---------- 1118
Chicken LGVAVTKYCKEELGTQSLGYCTNFQAVPGCGISCKVGGVDAVLGTAEEGVDKRMVQQRAV
Frog LGMAVTKYCKENLKCETLGTCYDFQAVPGCGISCKVNNIEPLL-HA----------
Seabream LGIAVAKHCKEELGSSVLGYCQDFQAVPGCGISCRVSNVEHLLQSQSDER---FLPPGVT
Stickleback LGAAVAKHCKQELASELLGCCQDFQAVPGCGISCRVSNVEHLLPHPTEER---FAVPGAT
Pufferfish LGVAVAAHCRQELGSDLLGCCQDFQAVPGCGISCRVSNVDHLLVQEASR----------
Zebrafish LGMAVAKHCKEELGAETLGFCHDFQAVPGCGISCKVSSVEDLLQNSPKT----------
Human -PLSAPASHLNEAGSLP------AEKDAAPQTFSVLIGNREWLRRNGLTISSDVSDAMTD 1171
Chicken CQLTLSSSIEKLPSTFPIILLSIHKGSSSSHIYSVLIGNREWMRRNGLHIANDVNDAMTD
Frog -PISTGHTDIKSA----------QAPLAHTVLIGNREWMRRNGLHISTDVDEAMSS
Seabream TDESSLLSAAELSPA----------AEPSSYSVLIGNREWMRRNGHHIQADVDAAMSS
Stickleback TDESSLPAAAEPPPAGLNEQQTASSLPGEPPSYSVLIGNREWMRRNGHHIGEDVDAAMSS
Pufferfish -DGSSLVPEQE----------GPGESYWVLIGNREWLRRNGHRVEADMDAAMAS
Zebrafish -QETKASAESQQMR----------AACPSYSVLIGNRQWMLRNGLEVTADVDNAMSS
Human HEMKGQTAILVAIDGVLCGMIAIADAVKQEAALAVHTLQSMGVDVVLITGDNRKTARAIA 1231
Chicken HETKGQTAILVAIDGALCGMIAIADTVKQEAALAVHTLKNMGIDVVLITGDNRKTAKAIA
Frog HEMKGQTAVLVAIDGELCGMIAIADTVKQEAALAVHTLKSMGIDVVLITGDNRKTAKAIA
Seabream HETKGQTAILVAIDGVLCAMLAIADTVKTEAALAVQTLSSMGIEVVMITGDNRRTAKAIA
Stickleback HETKGQTAILVAIDGVLCAMLAIADTVKAESALAVRTLRGMGIEVVMITGDNRRTAKAIA
Pufferfish HEAKGQTAVLVAIDGTLCAMLAVADTVKAESALAVQTLSSMGVQVVMITGDNRRTAKAIA
Zebrafish HETKGQTAILVAIDGVLCAMIAVADTVKAESALAVHTLSSMGIEVYMITGDNRRTARAIA
Human TQVGINKVFAEVLPSHKVAKVQELQNKGKKVAMVGDGVNDSPALAQADMGVAIGTGTDVA 1291
Chicken TQVGIKKVFAEVLPSHKVAKVQELQNGRRKVAMVGDGVNDSPALAKADIGIAIGTGTDVA
Frog TQVGIKKVFAEVLPSHKVAKVQALQSDNKRVAMVGDGVNDSPALARADVGIAIGTGTDVA
Seabream AQVGIRKVLAEVLPSHKVAKVQELQEKGLRVAMVGDGVNDSPALARADVGIAIGTGTDVA
Stickleback AQVGIGKVYAEVLPSHKVAKVQELQERGRRVAMVGDGVNDSPALARADVGIAIGTGTDVA
Pufferfish AQVGIGKVFAEVLPSHKVAKVQELQEAGLRVAMVGDGVNDSPALAQADVGIAIGTGTDVA
Zebrafish TQVGIRKVFAEVLPSHKVAKVQELQERGLKVAMVGDGVNDSPALAHADLGIAIGTGTDVA
ATP-binding site
Human IEAADVVLIRNDLLDVVASIHLSKRTVRRIRINLVLALIYNLVGIPIAAGVFMPIGIVLQ 1351
Chicken IEAADVVLIRNDLLDVVASIHLSKRTVRRIRINLILALIYNLLGIPIAAGVFMPAGLVLQ
Frog IEAADIVLIRNDLLDVVASIHLSKRTVRRIRLNFVFALIYNLLGIPIAAGVFMPAGLVLQ
Seabream IEAADIVLIRNDLLDVVASIELSQKTVRRIRINFVFALIYNLVGIPVAAGVFMPAGLVLQ
Stickleback IEAADIVLIRNNLLDVVASIELSKKTVRRIRTNFVFALIYNLVGIPFAAGVFMPVGVVLQ
Pufferfish IEAADIVLIRNDLLDVVASIELSRKTVRRIRINFVFALIYNLLGIPVAAGAFLPVGLVLQ
Zebrafish IEAADIVLIRNDLLDVVASIELSKKTVQRIRINFVFALIYNLLGIPFAAGVFMPLGLVLQ
TMD 7
Human PWMGSAAMAASSVSVVLSSLQLKCYKKPDLERYEAQAHGHMKPLTASQVSVHIGMDDRWR 1411
Chicken PWMGSAAMAASSVSVVLSSLQLKCYKKPDTESYEAQAQGHMKPLTPSQISVHIGMDDRRR
Frog PWMGSAAMAASSVSVVLSSLQLKCYRKPDSDRYEARAQGHMKPLTPSQISVHIGMDDRWR
Seabream PWMGSAAMAASSVSVVLSSLLLRMYKKTSVELYEVRARGQMRSLRSSQISTHLG-----V
Stickleback PWMGSAAMAASSVSVVLSSLMLKMYKKTSVEVYEVRALGLARSLRSSLISTHVG-----L
Pufferfish PWMASAAMAASSLSVLLSSLLLKTYRKTPAEFYEARARGHTRSLRSSQISTHLGG----V
Zebrafish PWMGSAAMAASSVSVVLSSLLLRLFKKTSVEEYESRAQSHKLSLSPSQVSTHVG-----L
TMD 8

```
Human           DSPRATPWDQVSYVSQVSLSSLTSDKPSRHSAAADDDGDKWSLLLNGRDEEQYI- 1465
Chicken         DSFRSAPWDQIS---QVSLSSLTSDKLPRHNGFFEEEGDKWSLLMNGGDEEQYI- 1432
Frog            DLPKTKAWDQIS---YISQVSRASQKPKRHGSLVEQQ-DKWSLLINETHEDQMI- 1406
Seabream        NVQRRSPVLPREQPGQSAAVTPALSGQGLSINSVQEQQDRCSLLDHQTAEDSNV- 1327
Stickleback     DADPAVQVP-------------------TTNSVQ-GRGRHSRLDHQAADDLNVL 1237
Pufferfish      DSVQPCPALRGQRRDKPAVATPS-----STRAPPSEQQDRRSLLEGWSADE---- 1291
Zebrafish       ESRRCSPLSDRKRRSRSAS----------GSSSFISGSFHPSTTQPYNTSGRSI- 1342
                                                        **
```

Figure 2-17 ATP7B sequence alignment. Residues identical in all proteins are shaded. The positions of the metal binding domains (MBDs) are indicated. Trans-membrane domains (TMD) are underlined. Functionally important domains in human ATP7A are indicated with stars. The residues at the P and the N-domain (located between TMD6 and TMD7) required for ATP binding activity are highlighted in green and blue respectively and the residues predicted to be involved in Cu coordination within the membrane are highlighted in yellow. The residues boxed have been shown by mutagenesis to be necessary for activity.

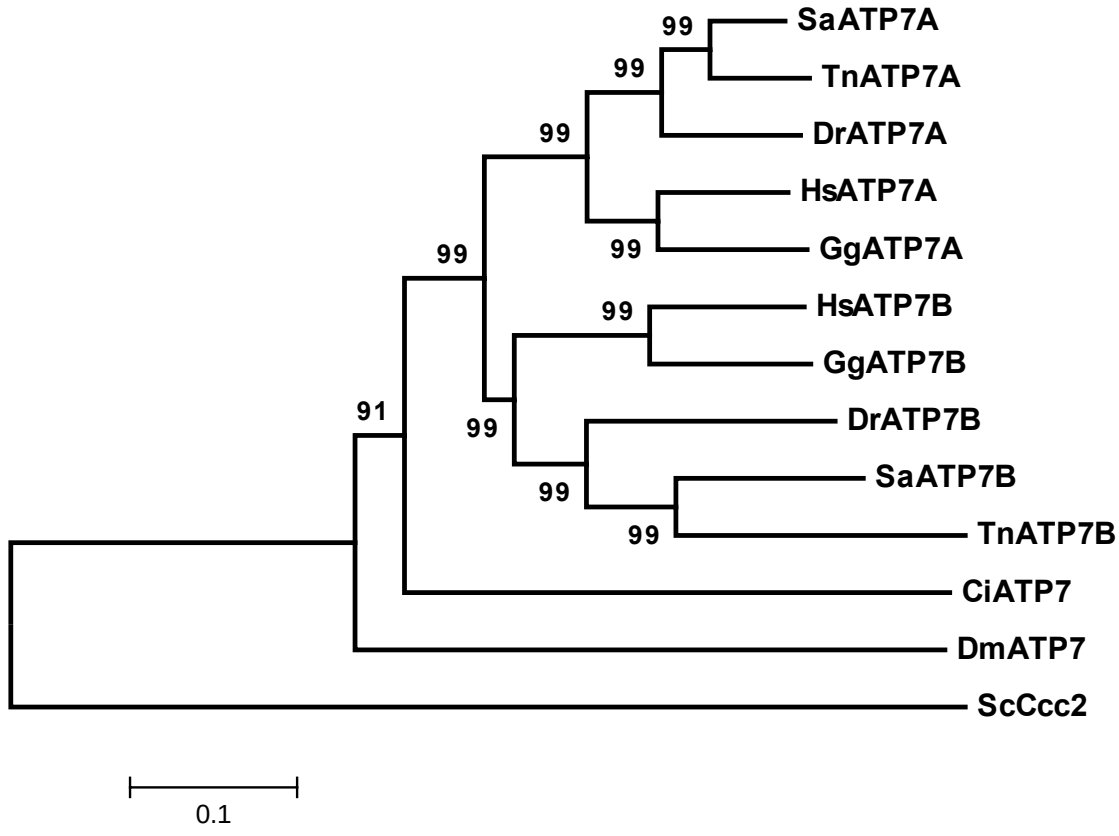


Figure 2-18 Cu-ATPases phylogeny tree. Human ATP7A (NM_000052) and ATP7B (NM_000053) chick ATP7A (*Gallus galllus*, XM_420307) and ATP7B (XM_417073), fruit fly ATP7 (*Drosophila melanogaster*, AE014298), yeast Ccc2 (*Saccharomyces cerevisiae*, L35270), sea squirt (*Ciona intestinalis,* Ensembl-Chr14q ENSCING00000007245), zebra fish ATP7A

(*Danio rerio*, Ensemble Chr14 ENSDARG00000003699, NP_001036185) and ATP7B (Ensemble Chr6 ID: ENSDART00000030246) puffer fish ATP7A (*Tetraodon negroviridis* Ensemble Chr1 GSTENG00017010001**)** and ATP7B (Ensemble Chr3 GSTENG00020077001) the sea bream (*Sparus aurata*) ATP7A and ATP7B 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.

2.3.5 MT and oxidative stress genes cloning in sea bream

2.3.5.1 Metallothionein

The first round of PCR produced a fragment of 274 bp which contained the full coding ORF consisting of 60 amino acids and showed 100 % identity to sea bream MT and 78 % to human MT.

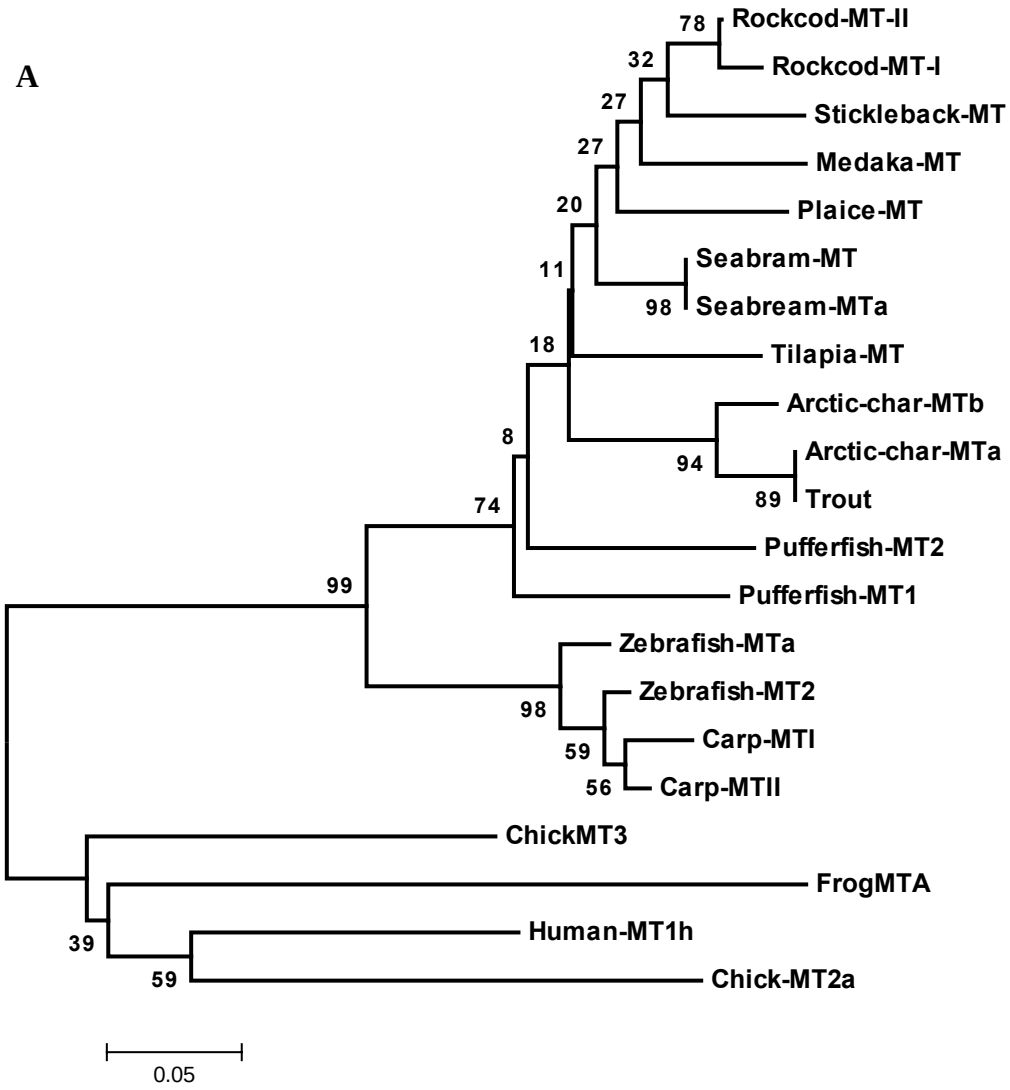
The alignment of the deduced amino acid sequence of fish MTs with MT proteins from, frog, chick and human, shows strong sequence conservation. Of note is the position of the cysteine, which asides from one position (highlighted in green in Figure 2-19) is always conserved.

To verify if fish species contain multiple MT genes, the sea bream cDNA sequence was used to search for similar sequences in the zebrafish, puffer fish, medaka and stickleback genomes. In stickleback and medaka a single MT gene was identified. In puffer fish and zebrafish two distinct genes were found. Furthermore after "BLASTing" the sea bream MT sequence on the entire (NCBI) database more fish sequences were found. In addition, a sea bream MT sequence, named MTa (X97276), has been submitted by Kille and Olsson (unpublished result) which would suggest the existence of a second isoform of sea bream MT. However, this result has not been reported yet and the protein sequence of MTa is 100 % identical to the MT cloned in this study (U58774), while the

DNA sequence had 3 different nucleotides. Further studies are required to prove the existence of 2 MT isoforms in sea bream, but given the similarity between MTa and the sequence reported here, it is likely that they represent allelic variations of a single locus. The phylogenetic comparison, using the protein alignment of all of the fish sequences with other vertebrates, indicates that the fish genes were monophyletic and that the sea bream MT was most closely related to the plaice, medaka, stickleback and rockcod (Figure 2-20 A). Moreover, considering the high level of protein conservation as suggested by Binz and Kägi (1999), the nucleotide sequence was used to generate a more reliable phylogenetic tree of the fish MT sequences. Due to the missing data of the 5'UTR in some sequences and because the high variability of this region can alter the reliability of phylogenetic analysis (Egg et al., 2000) only the coding region and the 3' non-coding regions were considered in the analysis (Figure 2-20 B).

```
Carp-MT-II          MDP--CDCAKTGTCNCGATCKCTNCQCTTCKKS-CCSCCPSGCSKCASGCVCKG-NSCGSSCCQ 60
Carp-MT-I           MDP--CDCAKTGTCNCGATCKCTNCQCKTCKKS-CCPCCPSGCSKCASGCVCKG-NSCGSSCCQ 60
Zebrafish-MT2       MDP--CECAKTGTCNCGATCKCTNCQCTTCKKS-CCSCCPSGCSKCASGCVCKG-NSCGSSCCQ 60
Zebrafish-MTa       MDP--CECAKTGACNCGATCKCTNCQCTTCKKS-CCSCCPSGCSKCASGCVCKG-NSCGTSCCQ 61
Artic-char-MTa      MDP--CECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVCKG-KTCDTSCCQ 61
Artic-char-MTb      MDP--CECSKTGSCNCGGSCKCSNCACTSCKKS-CCPCCPSDCSKCASGCVCKG-KTCDTSCCQ 60
Trout               MDP--CECSKTGSCNCGGSCKCSNCACTSCKKASCCDCCPSGCSKCASGCVCKG-KTCDTSCCQ 60
Rockcod-MT-II       MDP--CECSKSGTCNCGGSCTCTNCSCTSCKKS-CCPCCPSGCTKCASGCVCKG-KTCDTSCCQ 60
Rockcod-MT-I        MDP--CECSKSGTCNCGGSCTCTNCSCKSCKKS-CCPCCPSGCTKCASGCVCKG-KTCDTSCCQ 60
Plaice              MDP--CECSKTGTCNCGGSCTCKNCSCTTCNKS-CCPCCPSGCPKCASGCVCKG-KTCDTSCCQ 60
Seabream            MDP--CECSKTGTCNCGGSCTCTNCSCTSCKKS-CCSCCPAGCSKCASGCVCKG-KTCDTSCCQ 60
Stickleback-MT      MDP--CDCSKSGTCNCGGSCSCTNCSCTTCKKS-CCPCCPTGCTKCASGCVCKG-KTCDTSCCQ 60
Medaka-MT           MDP--CDCSKTGKCNCGGSCTCTNCSCTSCKKS-CCACCPSGCTKCASGCVCKG-KTCDTTCCQ 60
Pufferfish-MT1      MDP--CDCAKSGSCTCGGSCACTNCACTTCKKS-CCPCCPSGCSKCASGCVCKG-KTCDTSCCQ 60
Pufferfish-MT2      MDP--CDCAKTGNCKCGGSCTCKDCSCTDCKKS-CCSCCPSGCSKCASGCVCKG-KTCDTSCCQ 60
Tilapia             MDP--CECAKTGTCNCGGSCSCTKCSCKSCKKS-CCDCCPSGCSKCASGCVCKG-KTCDTSCCQ 60
chickMT-2a          MDPQDCTCAAGDSCSCAGSCKCKNCRCRSCRKS-CCSCCPAGCNNCAKGCVCKEPASSKCSCCH 63
Frog-MTA            MDPQDCKCETGASCSCGTTCSCSNCKCTSCKKS-CCSCCPAECSKCSQGCHCEK-GSKKCSCCN 62
Human-MT1h          MDP-NCSCEAGGSCACAGSCKCKKCKCTSCKKS-CCSCCPLGCAKCAQGCICKG-ASEKCSCCA 61
```

Figure 2-19 MT sequence alignment. Residues identical in all proteins are highlighted in grey; conserved cysteines are highlighted in yellow or in green when the position was not conserved.


A
78
Rockcod-MT-II
32
Rockcod-MT-I
27
Stickleback-MT
27
Medaka-MT
20
Plaice-MT
11
Seabram-MT
98
Seabream-MTa
18
Tilapia-MT
Arctic-char-MTb
8
94
Arctic-char-MTa
89
Trout
74
Pufferfish-MT2
99
Pufferfish-MT1
Zebrafish-MTa
98
Zebrafish-MT2
59
Carp-MTI
56
Carp-MTII
ChickMT3
FrogMTA
39
Human-MT1h
59
Chick-MT2a
0.05

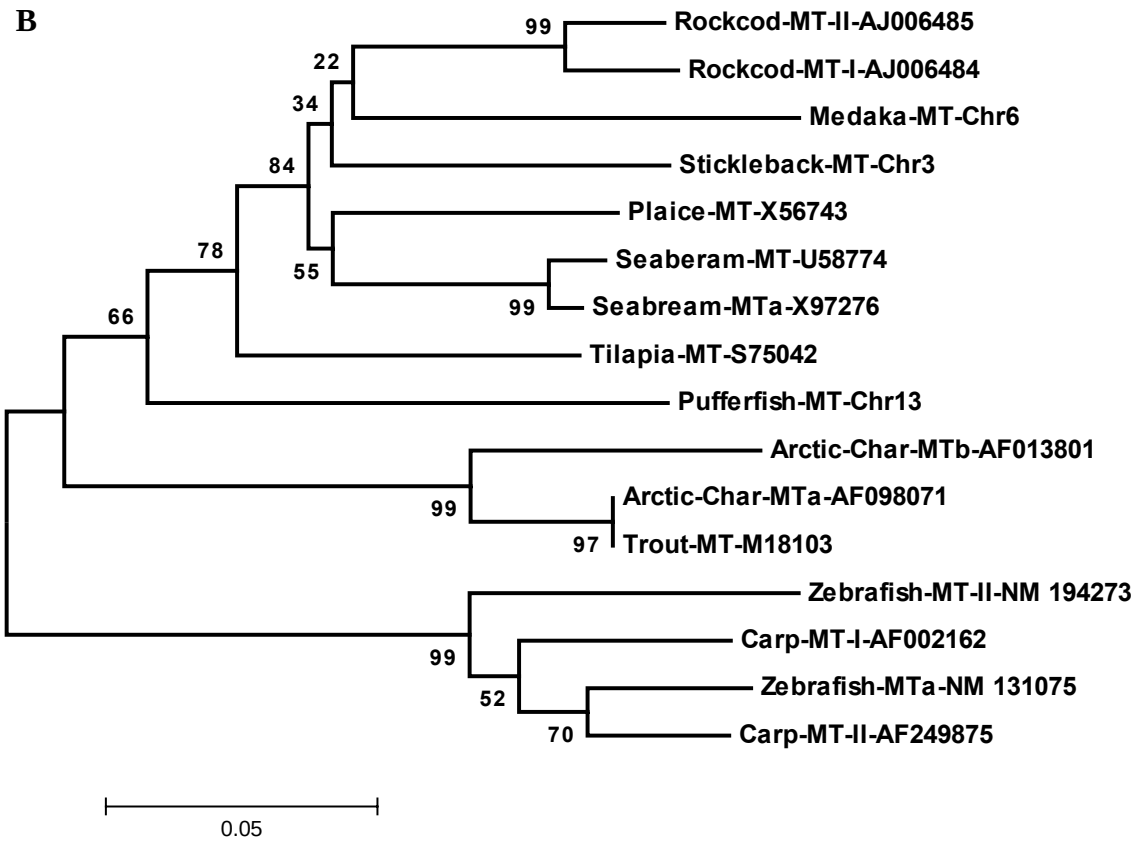


Figure 2-20 MT phylogenetic tree. Sea bream (*Sparus aurata*), medaka (*Oryzias latipes*), stickleback (*Gasterosteus aculeatus*), zebrafish (*Danio rerio*), puffer fish (*Tetraodon nigroviridis*), tilapia (*Oreochromis mossambicus*), plaice (*Pleuronectes platessa*), arctic char (*Salvelinus alpinus*), yellowbelly rockcod (*Notothenia coriiceps),* common carp (*Cyprinus carpio*), trout (*Oncorhynchus mykiss*), along with human (NM_005951), frog (*Xenopus laevis*)(NM_001087573) and chick (*Gallus gallus*) (NM_205275) were used to generate the phylogenetic tree using ClustalW. Phylogenetic tree A was generated using protein sequences and phylogenetic tree B using the the cDNA coding reagion plus the 3`end. Numbers (bootstrap values) represent the percentage of times the associated branch topology was returned after 1000 iterations of tree generation. The Genebank accession numbers or the ENSEMBL peptide ID for the protein sequence of each fish species is provided.

2.3.5.2 CuZn-SOD

The first round of PCR produced a fragment of 202 bp which contained about 45 % of the coding ORF and showed 95 % identity to the closely related black-porgy

(*Acanthopagrus schlegelii*) CuZn-SOD and 71 % to the mouse (*Mus musculus*) CuZn-SOD.

The alignment of the deduced amino acid sequences of fish CuZn-SOD with CuZn-SOD proteins from, frog, chick, mouse and human, shows strong sequence conservation. Of note, is the position of the histidines and aspartate involved in Cu and Zn coordination (Hough and Hasnain 1999)(Figure 2-21).

Only one copy of the CuZn-SOD gene was found in the genome of zebrafish, medaka, puffer fish and stickleback.

```
Human            -MATKAVCVLKGDGPVQGIINFEQKES--NGPVKVWGSIKGLTEGLHGFHVHEFGDNTAG 57
Mouse            -MAMKAVCVLKGDGPVQGTIHFEQKAS--GEPVVLSGQITGLTEGQHGFHVHQYGDNTQG 57
Trout            -MAMKAVCVLKGTGEVTGTVFFEQEGA--DGPVKLIGEISGLAPGEHGFHVHAYGDNTNG 57
Salmon           -MALKAVCVLKGTGEVTGTVFFEQEGD--GAPVKLTGEIAGLTPGEHGFHVHAFGDNTNG 57
Flounder         -------------------------------------IAGLAPGEHGFHVHSFGDNTNG 22
Seabream         ------------------------------------------------------------
Black-porgy      -MVLKAVCVLKGAGETTGVVHFEQESE--SAPVKLTGEIKGLTPGEHGFHVHAFGDNTNG 57
Red-seabream     -MVQKAVCVLKGAGETTGVVHFEQESE--SAPVTLKGEISGLTPDEHGFHVHAFGDNTNG 57
Perch            ----------------------------------------FTPGEHGSHVHVFGDNTNG 19
Tilapia          -MVLKAVCVLKGTGDTSGTVYFEQEND--SAPVKLTGEIKGLTPGEHGFHVHAFGDNTNG 57
Pufferfish       -MVIKAVCVLKGAGETSGTVYFEQQDE--KAPVKLTGEIKGLTAGEHGFHVHAFGDNTNG 57
Zebrafish        -MVNKAVCVLKGTGEVTGTVYFNQEGE--KKPVKVTGEITGLTPGKHGFHVHAFGDNTNG 57
Chick            MATLKAVCVMKGDAPVEGVIHFQQQGS---GPVKVTGKITGLSDGDHGFHVHEFGDNTNG 57
Frog             --MVRAVCVLAGSGDVKGVVHFQQQASIDEGPVTVEGKIYGLTDGKHGFHIHEFGDNTNG 58

Human            CTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRT 117
Mouse            CTSAGPHFNPHSKKHGGPADEERHVGDLGNVTAGKDGVANVSIEDRVISLSGEHSIIGRT 117
Trout            CMSAGPHFNPHNQTHGGPTDAVRHVGDLGNVTAGADNVAKINIQDKMLTLTGPDSIIGRT 117
Salmon           CMSAGPHFNPHNHTHGGPTDTVRHVGDLGNVTAAADSVAKINIQDEILSLAGPHSIIGRT 117
Flounder         CMSAGPHFNPHGKNHAGPTDADRHVGDLGNVTAGADNVAEINISDKMLTLNGPNSIIGRT 82
Seabream         ----------HGKNHGGPTDAERHVGDLGNVTAGADNVAKIDITDKMLTLSGPLSIIGRT 50
Black-porgy      CISAGPHLNPHGKNHGGPTDEERHVGDLGNVTAGADNVAKIDITDKMLTLTGPLSIIGRT 117
Red-seabream     CISAGPHFNPHNKNHAGPTDAERHVGDLGNVTAGADNVAKIDITDKMLTLNGPFSIIGRT 117
Perch            CISAGPHYNPHGKNHAGPNDAERHVGDLGNVTAGADNVAKIDITDKMPSLTGPYSIIGRT 79
Tilapia          CISAGPHFNPYNKNHGGPKDAERHVGDLGNVTAGADNVAKIEITDKVITLTGRDSIIGRT 117
Pufferfish       CISAGPHYNPHDKTHAGPNDENRHVGDLGNVTAGADQIAKIDITDSVINLHGKFSIIGRT 117
Zebrafish        CISAGPHFNPHDKTHGGPTDSVRHVGDLGNVTADASGVAKIEIEDAMLTLSGQHSIIGRT 117
Chick            CTSAGAHFNPEGKQHGGPKDADRHVGDLGNVTA-KGGVAEVEIEDSVISLTGPHCIIGRT 116
Frog             CISAGPHFNPESKTHGAPEDAVRHVGDLGNVTA-KDGVAEFKLTDSLISLKGNHSIIGRC 117
```

```
Human            LVVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ- 154
Mouse            MVVHEKQDDLGKGGNEESTKTGNAGSRLACGVIGIAQ- 154
Trout            MVIHEKADDLGKGGNEESLKTGNAGGRQACGVIGIAQ- 154
Salmon           MVIHEKADDLGKGDNEESRKTGNAGSRLACGVIGIAQ- 154
Flounder         MVIHEKADDLGKGGNDESLKTGNA-------------- 106
Seabream         MVIHEKVDDLGKGGNEE--------------------- 67
Black-porgy      MVIHEKTDDLGKGGNEESLKTGNAGGRLACGVIGITQ- 154
Red-seabream     MVIHEKADDLGKGGNEESLKTGNAGGRLACGVIGICQ- 154
Perch            MVIHEKADDLGKGGNEESLKTGNAGGRLACGVIGITQ- 116
Tilapia          MVIHEKVDDLGKGGNEESLKTGNAGGRLACGVIGITQ- 154
Pufferfish       MVIHEKADDLGKGGNEESLKTGNAGGRLACGVIGITQ- 154
Zebrafish        MVIHEKEDDLGKGGNEESLKTGNAGGRLACGVIGITQ- 154
Chick            MVVHAKSDDLGRGGDNESKLTGNAGPRLACGVIGIAKC 154
Frog             AVVHEKEDDLGKGGNDESLKTGNAGGRLACGVIGLCQ- 154
```

Figure 2-21 CuZn-SOD sequance alignment. Residues identical in all proteins are highlighted in grey; the histidine coordinating both Cu and Zn is highlighted in yellow and the other residues coordinating copper and zinc are highlighted in green and blue respectively (Hough and Hasnain, 1999).

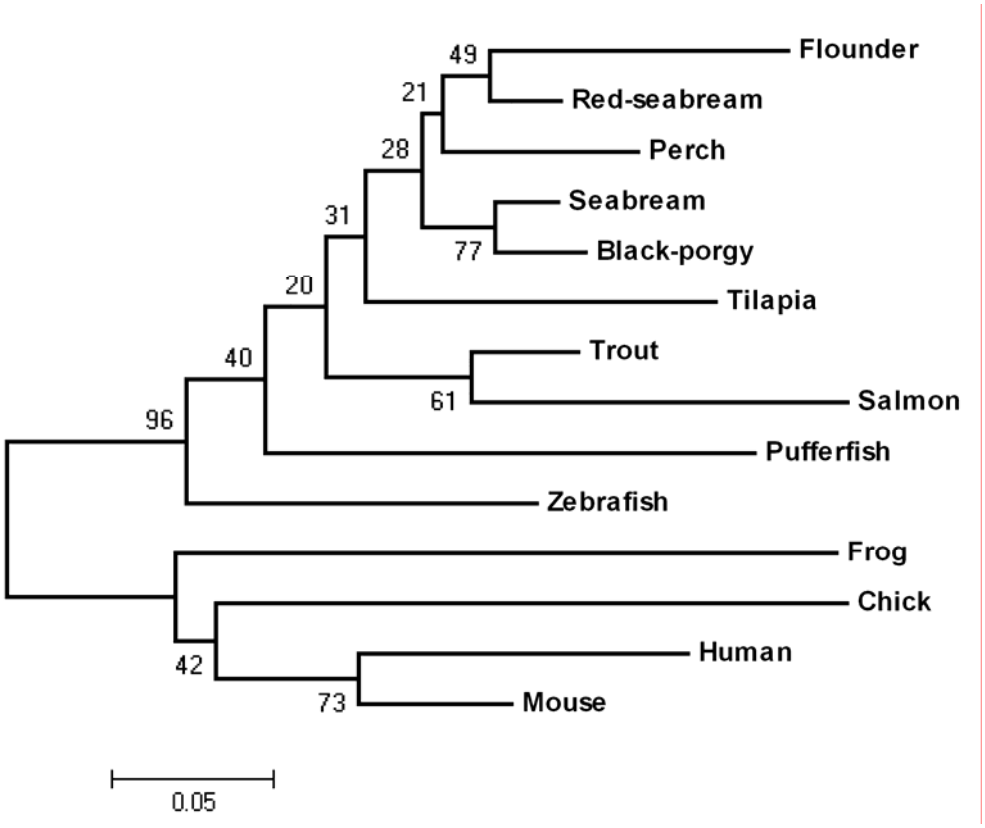


Figure 2-22 CuZn-SOD phylogenetic tree. sea bream (*Sparus aurata*) (AJ937872), black porgy (*Acanthopagrus schlegelii*) (AJ000249), red sea bream (*Pagrus major*) (AF329278), chinese perch (*Siniperca chuatsi*) (AY909486), European flounder (*Platichthys flesus*) (AJ291980), trout (*Oncorhynchus mykiss*) (AF469663), Atlantic salmon (*Salmo salar*) (AY736282), zebrafish (*Danio rerio*) (NM_131294), puffer fish (*Tetraodon nigroviridis*) (CR650645), tilapia

(*Oreochromis mossambicus*) (AY491056), along with human (NM_000454), house mouse (*Mus musculus*) (X06683), frog (*Xenopus laevis*) (ENSXETG00000007350) and chick (*Gallus gallus*) (GGU28407) 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. The Genebank accession numbers or the ENSEMBL ID for the protein sequence is provided.

2.3.5.3 Glutathione reductase

The first round of PCR produced a fragment of 1151 bp which contained about 85 % of the coding ORF and showed 92 % identity to European flounder GR and 77 % to the mouse (*Mus musculus*) GR.

The search of the fish genomes showed that only stickleback and puffer fish resulted in having two copies of the GR gene but the second copy of the puffer fish gene was not used in the alignment because it was probably assembled incorrectly.

```
Pufferfish-GR1      ---------MASDPSSTTTTRFDFLVIGGGSGGLAGARRASELGASAAVIESHKLGG-TC 50
Fugu-GR             ---------MASDPSSTDITRFDFLVIGGGSGGLAGARRASELGASAAVIESHKLGG-TC 50
Seabream-GR         ------------------------------------------------------------
Medaka-GR           ------------MASADATRFDFLVIGGGSGGLAGARKLGEKGPPFPLKGMHPIHSNNK 47
Stickleback-GR1     -----------MASADPQTTRLDFLVIGGGSGGLAGARRASELGASAAVIESHRLGG-TC 48
Stickleback-GR2     -----------MASADPQTTRLDFLVIGGGSGGLAGARRASELGASAAVIESHRLGG-TC 48
Zebrafish-GR        ------------MASGSVSRFDFLVVGGGSGGLAGARRAAELGATTAVIESHRLGG-TC 46
Mouse-GR            MASPGEPQPPAP-----DTSSFDYLVIGGGSGGLASARRAAELGARAAVVESHKLGG-TC 54
Human-GR            MACRQEPQPQGPPPAAGAVASYDYLVIGGGSGGLASARRAAELGARAAVVESHKLGG-TC 59
Frog-GR             ---MHKPVPDSHSSDGHLPRYFDYLVVGGGSGGLASARRAAELGARTAVVESSKLGG-TC 56
                                              ************************
                                                        DBM motif

Pufferfish-GR1      VNVGCVPKKVMWNAAVHAEYLHDHSDYGFEVGNVHFSWETLKAKRDAYVSHLNRIYRSNL 110
Fugu-GR             VNVGCVPKKVMWNAAVHAEYLHDHSDYGFEVGNVRFSWEALKTKRDAYISHLNRIYRNNL 110
Seabream-GR         -------KKVMWNAAVHAEYLHDHNDYGFDVGNVRFSWETLRAKRDAYIGHLNRIYRSNL 53
Medaka-GR           VNVGCVPKKVMWNAAVHAEYLHDHCDYGFETGSVRFSWEALKAKRDAYIAHLNRIYRNNL 107
Stickleback-GR1     VNVGCVPKKVMWNAAVHAEYLHDHSDYGFEVESVRFSWEALKAKRDAYISHLNRIYRNNL 108
Stickleback-GR2     VNVGCVPKKVMWNAAVHAEYLHDHSDYGFEVESVRFSWEALKAKRDAYISHLNRIYRNNL 108
Zebrafish-GR        VNVGCVPKKVMWNTSTHAEYLHDHEDYGFEGAKAHFSWQIIKHKRDAYVSRLNQIYRSNL 106
Mouse-GR            VNVGCVPKKVMWNTAVHSEFMHDHVDYGFQSCEGKFSWHVIKQKRDAYVSRLNTIYQNNL 114
Human-GR            VNVGCVPKKVMWNTAVHSEFMHDHADYGFPSCEGKFNWRVIKEKRDAYVSRLNAIYQNNL 119
Frog-GR             VNVGCVPKKIMWNAAIHSEYIHDHEDYGFETSAIKFTWKVIKEKRDAYVSRLNDIYQNNL 116
```

```
Pufferfish-GR1      DKAKIQTIQGHARFTNDPEPSVEVNGKKYTAPHILIATGGQPTVLSDTEVPG-ASLGITS 169
Fugu-GR             DKAKIQTIQGHARFTNDPEPTVEVNGKKYTAPHILIATGGQPSVLSDTEVPG-ASLGITS 169
Seabream-GR         DKAKIQTIQGHARFTSDPEPTVEVNGKKYTAPHILIATGGQPSVLCDDEVPG-ASLGITS 112
Medaka-GR           DKAKVTFIQGYARFTDDAEPTVEVNGKKYTAPHILISTGGQPSVLSDEEVPG-ASLGINS 166
Stickleback-GR1     DKAKVQNIQGHARFTNDPEPTVEVDGRKYTAPHILIATGGQPTVLSDADIPG-GNLGITS 167
Stickleback-GR2     DKAKVQNIQGHARFTNDPEPTVEVDGRKYTAPHILIATGGQPTVLSDADIPG-GNLGITS 167
Zebrafish-GR        EKGKIEFIHGYARFTDDPEPTVEVNGKKYTATHILISTGGHPSTVSEDDVPG-SSLGITS 165
Mouse-GR            TKSHIEIIHGYATFADGPRPTVEVNGKKFTAPHILIATGGVPTVPHESQIPG-ASLGITS 173
Human-GR            TKSHIEIIRGHAAFTSDPKPTIEVSGKKYTAPHILIATGGMPSTPHESQIPG-ASLGITS 178
Frog-GR             QKAQIEIIRGQANFTSDSEPTVEVNGQKYIAPHILIATGGKPSMPSDAEVPGNKSLGICI 176

Pufferfish-GR1      DGFFELETLPKRSVVVGAGYIAVEMAGILSTLGSKTSLIIRQTGVLRNFDSLISTNCTKE 229
Fugu-GR             DGFFELESLPKRSVVVGAGYIAVEMAGILSTLGSKTSLIIRQTGVLRNFDSLISTNCTKE 229
Seabream-GR         DGFFELETLPKRSVIVGAGYIAVEMAGILSTLGSKTSLVIRQTTVLRNFDSLISTNCTKE 172
Medaka-GR           DGFFELETLPKRSVIVGAGYIAVEMAGILSTLGSKTSLIIRQTGVLRNFDALISANCTKE 226
Stickleback-GR1     DGFFELETLPKRTVIVGAGYIAVEMAGILSTLGSKTSMIIRQSGVLRNFDSFLSTNCTKE 227
Stickleback-GR2     DGFFELETLPKRTVIVGAGYIAVEMAGILSTLGSKTSMIIRQSGVLRNFDSFLSTNCTKE 227
Zebrafish-GR        DGFFELESCPKRSVIVGAGYIAVEMAGILSTLGSKTSIIIRQGGVLRNFDALISSNCTKE 225
Mouse-GR            DGFFQLEDLPSRSVIVGAGYIAVEIAGILSALGSKTSLMIRHDKVLRNFDSLISSNCTEE 233
Human-GR            DGFFQLEELPGRSVIVGAGYIAVEMAGILSALGSKTSLMIRHDKVLRSFDSMISTNCTEE 238
Frog-GR             PQFFTSP--PQRSVVVGAGYIAIEIVGILSALGSKASLLIRQDKVLRTFDSMISSNCTEE 234
                              ************
                              NAD(P)H binding motif

Pufferfish-GR1      LQNSGIDLWKNSQVKSVSKTDKGLEVTIATRDPEKKNEEEKLRTIQEVDCLLWAIGRQPN 289
Fugu-GR             LQNSGIDLWKNSQVRSVCKTDKGLEVTIATRDPERKNEEEKLRTIQEVDCLLWAIGRQPN 289
Seabream-GR         MQNSGVDLWKNSQVKSVRKTDKGLEVTIATKDPEKKNEEEKIRTIQEVDCLLWAIGRQPN 232
Medaka-GR           IQNNGIDLWKNSQVKSVCKTEKGLEVTIVTKDPDKTNDE-KISVIEEVDCLLWAIGRQPN 285
Stickleback-GR1     LQNSGVDLWKNSQVTSVRKTEKGLEVTVVTKDQEKKNDEEKTSTIQEVDCLLWAIGRQPN 287
Stickleback-GR2     LQNSGVDLWKNSQVTSVRKTEKGLEVTVVTKDQE------------VDCLLWAIGRQPN 274
Zebrafish-GR        LQNNGIDLRKNTQVKSVKKNGKGLSITLVTKDPDDKDSQEKFDTINDVDCLLWAIGREPN 285
Mouse-GR            LENAGVEVLKFTQVKEVKKTSSGLELQVVTSVPGRKP---TTTMIPDVDCLLWAIGRDPN 290
Human-GR            LENAGVEVLKFSQVKEVKKTLSGLEVSMVTAVPGRLP---VMTMIPDVDCLLWAIGRVPN 295
Frog-GR             LENAGVEVWKYAQVKSVKKSATGLEINVQCSMPGRKP---TVRTIQDVDCLLWAIGRDPN 291

Pufferfish-GR1      ITGLNIGHLNVDTDEKGHIIVDEFQNTSRAGIYAVGDVCGKALLTP--VAIAAGRKLAHR 347
Fugu-GR             ITGLNIGHLNVDTDEKGHIVVDEFQNTSRAGIYAVGDVCGRALLTP--VAIAAGRKLAHR 347
Seabream-GR         SSGLNIGEMIVDTDEKGHIVVDEFQNTSRAGIYAVGDVCGKALLTP--VAIAAGRKLAHR 290
Medaka-GR           TAGLNIGAMGVDTDDRGHIIVDDFQNTTRSGIYAVGDVCGKALLTPGTVAIAAGRKLAHR 345
Stickleback-GR1     TSGLNVAAMGLEMDERGHIVVDEFQNTSRPGIYAVGDVCGKALLTP-------------- 333
Stickleback-GR2     TSGLNVAAMGLEMDERGHIVVDEFQNTSRPGIYAVGDVCGKALLTPGPVAIAAGRKLAHR 334
Zebrafish-GR        TAGLNLSQIGVKLDERGHIVVDEFQNTSRPGVYAVGDVCGRALLTP-------------- 331
Mouse-GR            SKGLNLNKVGIQTDEKGHILVDEFQNTNVKGVYAVGDVCGKALLTP--VAIAAGRKLAHR 348
Human-GR            TKDLSLNKLGIQTDDKGHIIVDEFQNTNVKGIYAVGDVCGKALLTP--VAIAAGRKLAHR 353
Frog-GR             TEDLGLENLGLELDEKGHIVVDEFQNTSRKGVYAVGDVCGRALLTP--VAIAAGRKLSHR 349
                                       ****************
                                       FAD binding motif

Pufferfish-GR1      LFEGKKDSKLDYSTIPTVVFSHPPIGTVGLTEEEAVRSHGKENVKIYKTSFTPMYHAITN 407
Fugu-GR             LFEGKKDSKLDYSTIPTVVFSHPPIGTVGLTEEEAVRSNGKENVKIYKTSFTPMYHAITN 407
Seabream-GR         LFEGKKDSKLDYSTIPTVVFSHPPIGTVGLTEDEAIKARGKENVKIYKTSFTPMYHAITS 350
Medaka-GR           LFEGKKDSKLDYSCIPTVVFSHPPIGTVGLTEEEAVKTKGKENVKIYKTSFTPMYHAITR 405
Stickleback-GR1     --------------------------------EEAIRSRGKENVKVYKTSFTPMYHAITS 361
Stickleback-GR2     LFEDKNDSKLDYSCIPTVVFSHPPIGTVGLTEEEAIRSRGKENVKVYKTSFTPMYHAITS 394
Zebrafish-GR        --------------------------------DEAVKTYGKDKVKVYTTSFTPMYYAITT 359
Mouse-GR            LFECKQDSKLDYDNIPTVVFSHPPIGTVGLTEDEAVHKYGKDNVKIYSTAFTPMYHAVTT 408
Human-GR            LFEYKEDSKLDYNNIPTVVFSHPPIGTVGLTEDEAIHKYGIENVKTYSTSFTPMYHAVTK 413
Frog-GR             LFEGQEDSKLDYDNIPTVVFSHPPIGTVGLTEEEAVTAKGRENVKVYTTSFSPMYHAVTR 409

Pufferfish-GR1      RKSQCIMKLVCVGKEEKVVGLHMQGLGCDEMLQGFSVAIKMGATKADFDKTVAIHPTSSE 467
Fugu-GR             RKSQCVMKLVCVGKEEKVVGLHMQGLGCDEMLQGFSVAIKMGATKADFDKTVAIHPTSSE 467
Seabream-GR         RKSQCIMKLVCVGKEEKVVGLHMQGLGCDEMLQ-------------------------- 383
Medaka-GR           RKSQCIMKLVCVGKEEKVVGLHMQGLGCDEMLQGFAVAIKMGATKEDFDKTVAIHPTSSE 465
Stickleback-GR1     RKSQCIMKLVCEGKEEKVVGLHMQGLGCDEMLQGFAVAIKMGATKADFDKTVAIHPTSSE 421
Stickleback-GR2     RKSQCIMKLVCEGKEEKVVGLHMQGLGCDEMLQGFAVAIKMGATKADFDKTVAIHPTSSE 454
Zebrafish-GR        RKSQCIMKLVCAGENEKVVGLHMQGFGCDEMLQGFAVAVNMGATKADFDRTIAIHPTSSE 419
Mouse-GR            RKTKCVMKMVCANKEEKVVGIHMQGIGCDEMLQGFAVAVKMGATKADFDNTVAIHPTSSE 468
Human-GR            RKTKCVMKMVCANKEEKVVGIHMQGLGCDEMLQGFAVAVKMGATKADFDNTVAIHPTSSE 473
Frog-GR             RKTKCVMKLVCVGKEEKVVGLHMQGLGCDEMLQGFSVAIKMGATKKDFDNTVAIHPTSSE 469
```

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Pufferfish-GR1        EFVTMR 473
Fugu-GR               EFVTMR 473
Seabream-GR           ------
Medaka-GR             EFVTMR 471
Stickleback-GR1       EFVTMR 427
Stickleback-GR2       EFVTMR 460
Zebrafish-GR          ELVTLR 425
Mouse-GR              ELVTLR 474
Human-GR              ELVTLR 479
Frog-GR               ELVTLR 475
```

Figure 2-23 GR sequance alignment. Residues identical in all proteins are highlighted in grey; the residues conserved in the dinucleotide-binding motif (DBM) motif are highlighted in blue, the NAD(P)H binding motif in red, FAD binding domain in purple, the motif situated at the interface between the NAD(P)H and FAD binding domains in green, the Arg292 making the polar contact with the isoalloxazine ring is boxed and in yellow are highlighted all the residues conserved but not directly involved in binding FAD (Dym and Eisenberg 2001).

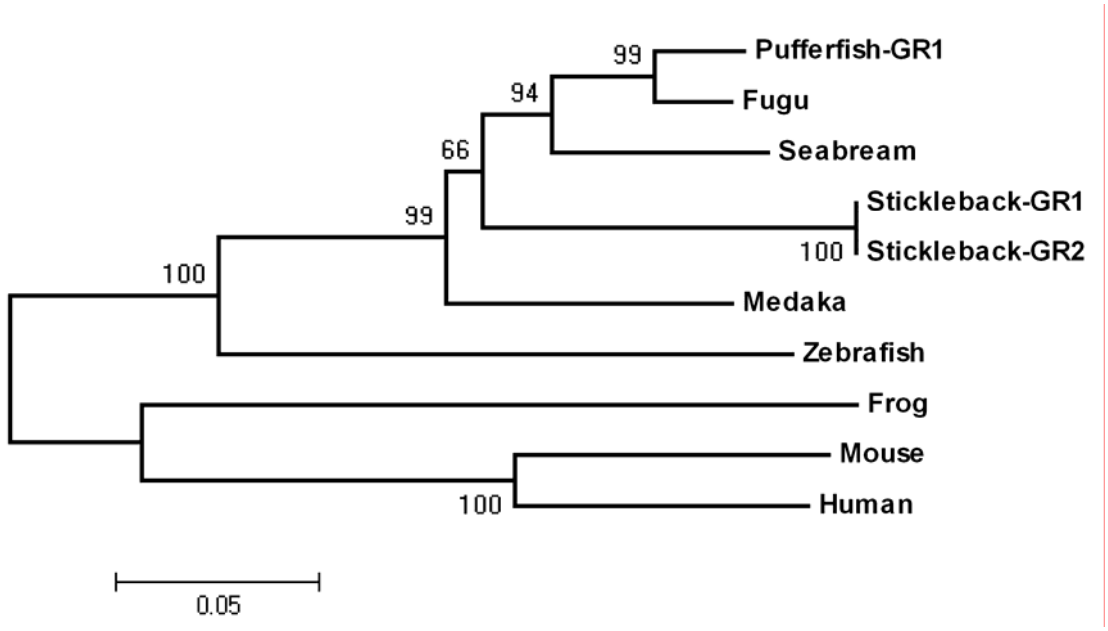


Figure 2-24 GR phylogenetic tree. Sea bream (*Sparus aurata*, AJ937873), stickleback (*Gasterostreus aculeatus*, ENSGACG00000017557 Chr-group IV), zebrafish (*Danio rerio*, NM_001020554), puffer fish (*Tetraodon nigroviridis*, GR1 GSTENT00029259001 Chr1, GR2 GSTENT00031088001 Chr12), medaka (*Oryzias latipes*, ENSORLG00000000624 Chr10) along with human (NM_000637), mouse (*Mus musculus*, NM_010344), frog (*Xenopus laevis*, ENSXETG00000011293) 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. The Genebank accession numbers or the ENSEMBL ID for the protein sequence is provided.

2.3.5.4 Reference genes

Following the first round of PCRs fragments of 690, 514 and 693 bp were produced for β-actin, EF1α and GAPDH respectively. After sequencing those PCR fragments were shown to be 100 % identical to the sequence from which the primers where designed (see 2.2.9.4).

2.4 Discussion

The isolation/synthesis of the genes of interest was achieved through the study of known homologous proteins structure first, enabling the designing of primers on conserved areas and then the obtained cDNA sequences were identified by BLAST on the NCBI gene databank and comparing the deduced amino acid sequence of the cDNAs with known homologous proteins.

Considering the conserved structural features, comparative genomes and phylogenetic analyses, it is most likely that the cDNAs described here, such as saCtr1, saAtox1, saATP7A and saATP7B all encode functional Cu transporters and that one single copy of each gene is present in the sea bream genome.

2.4.1 Ctr1

The deduced amino acid sequence of the sea bream Ctr1 (saCtr1) cDNA possesses all the domains that characterize Ctr1 in other species (Figure 2-11); the metal binding motif, the three transmembrane domains and the HCH motif. Moreover, the saCtr1 protein sequence possesses all of the amino acid residues shown to be essential for optimal Ctr1 activity (Eisses and Kaplan 2005). The metal binding motif in the sea bream sequence differs from other vertebrate sequences in consisting solely of MXM rather than the more

extensive MXXMXM. However mutant Ctr1 proteins lacking the first methionine but containing either of the second or third methionines in the MXXMXM motif retain near maximal Cu uptake activity (Puig et al., 2002). Furthermore, based on phylogenetic and comparative genome analysis it is very unlikely that sea bream contain more than one Ctr1 gene.

2.4.2 Atox1

The structure of the saAtox1, 68 amino acids and one metal binding domain (MxCxxC), was conserved in all other vertebrate sequences to which it was compared (Figure 2-13).

The mechanism of Cu transfer from Cu-Atox1 to Cu-ATPases is not completely clear, however the high level of similarity and the structurally conserved amino acids suggest that the three-coordination geometry which allow Cu transfer is the key point of Cu transfer from chaperone to Cu-ATPase (Lutsenko et al., 2007a; Pufahl et al., 1997; Lutsenko et al., 2007a).

2.4.3 ATP7A

The saATP7A deduced amino acid sequence showed a high level of conservation with the other vertebrate ATP7A (Figure 2-15). Six metal binding domains (MBDs) are completely conserved in the saATP7A N-terminal region (see 2.1.4.3). In addition, in the mammalian ATP7A C-terminus, a di-leucine (LL) 1487-8 motif has been shown to be essential for protein relocalization to the TGN from the plasma membrane. Inactivating this motif by A substitution leads to impaired endocytosis and trapping of the ATP7A protein at the plasma membrane (Petris et al., 1998; Petris and Mercer 1999). This di-

leucine motif is conserved in all vertebrate ATP7A including saATP7A (Lutsenko et al., 2007a). However, the human ATP7A C-terminus has been shown to contain a PDZ-binding motif (**P**DS-95/**D**rosophila disc large/**Z**O-1 homology) (DTAL) which interacts with a specific PDZ protein AIPP1, and is responsible for retention at the basolateral membrane. AIPP1 (**A**TPase **I**nteracting **P**DZ **P**rotein) is a newly identified protein implicated in Cu homeostasis which is composed mainly of a single PDZ domain (Stephenson et al., 2005). PDZ domains are widespread and function as protein-protein interaction modules, binding to the C-terminal 4-5 residues of target proteins and they regulate protein localization in polarized cells (Fanning and Anderson 1999). The DTAL 1497-1500 motif is present only in the human sequence. All non-mammalian vertebrate sequences analyzed contained a stretch of acidic residues, DDD 1495-7 in mammals and birds and EEE in fish. It would be interesting to test whether these differences are responsible for functional differences between mammalain ATP7A and ATP7A from other vertebrates, although all of the 15 last C-terminal amino acids of ATP7A have been shown to be essential for normal protein trafficking (Stephenson et al., 2005).

2.4.4 ATP7B

Sea bream ATP7B cDNA was isolated only after a comprehensive analysis of the areas of both ATP7A and ATP7B which were suitable for the successful designing of ATP7B-specific primers (see 2.2.8). Because the annotations on the *Ensembl* ATP7B genes in fish species were not accurate, this was only possible after predicting exons/intron boundaries and after comparing sequences extracted from *Ensembl* genomes with the known saATP7A cDNA sequence.

The sea bream ATP7B cDNA is the first to be described in fish species. All of the domains shown to characterize Cu-ATPases were conserved between saATP7B and its

mammalin homologues including all amino acids demonstrated to be essential for activity by mutational analysis (Lutsenko et al., 2007a) (Figure 2-17). It is notable that Cu-ATPases from different species vary the number of MBDs they contain. Yeast Cu-ATPase (Ccc2) has 2 MBDs, the single fruit fly (*Drosophila melanogaster*) and sea squirt (*Ciona intestinalis*) Cu-ATPase (ATP7) have 4 and 5 MBDs respectively and both mammalian ATP7A and ATP7B present 6 MBDs. Interestingly, two of the six metal binding domains present in higher vertebrate species were missing in sea bream, stickleback, Medaka and Fugu ATP7B (these last two sequences Ensemble ID: ENSORLG00000000063 and SINFRUG00000140127 are not shown in the protein alignment; Figure 2-17). Moreover zebrafish and puffer fish ATP7B showed 5 and 3 MBDs respectively. The implications of the differences in MBDs contents in fish are discussed in 6.2.

ATP7B delivers Cu to apo-ceruloplasmin and mediates excretion of excess Cu into the bile. These two distinct functions require ATP7B to be at two different subcellular compartments. Therefore the regulation of protein trafficking is of crucial importance for the functions of ATP7B (Cater et al., 2004). Indeed the protein loop between TMD6 and TMD7 that has been shown to be involved in protein trafficking (Lutsenko et al., 2007a) are highly conserved.

Chapter 3. Tissue mRNA expression levels of copper homeostasis genes in sea bream

3.1 Introduction

Excluding the zygote and its very early descendent cells which are totipotent (potentially able to express all the genes in their genome and therefore able to evolve/differentiate to any cell type), the cells present in mature tissues express a specific expression pattern that characterizes the cell type and more broadly the tissue function (Raven and Johnson 2005).

Cu homeostasis genes are expressed at different levels in all mammalian tissues; however some of them, such as Cu-ATPases, ATP7A and ATP7B, are expressed exclusively in certain tissues. Body Cu homeostasis is maintained and regulated by a communicating network of different Cu-transporters in different tissues and knowing their tissue expression profile may give an insight in to the understanding of the functional importance in whole body Cu-homeostasis (see 1.5.2).

3.1.1 Tissue selection criteria

With the exception of the reference genes which were measured in four tissues such as kidney, gill, intestine and liver, all the other genes investigated in this thesis were measured in eight different sea bream tissues including kidney, gill, intestine, liver, heart, red muscle, white muscle and brain. The selection criteria for the tissues was related to the supposed relevance of those tissues in Cu homeostasis, for instance kidney, gill, intestine and liver are involved in reabsorption, uptake and excretion of Cu in mammals and fish

(Linder 1991; Bury et al., 2003), whereas the other tissues (heart, muscle and brain) are assumed to have a requirement for Cu but no overall homeostatic role.

3.2 Materials and Methods

The experimental design including animal holding condition, fish treatments and fish sampling are described in section 4.2.

The tissue cDNAs from which the tissue expression profile of each gene was determined were synthesised from total RNA extracted from untreated fish (low Cu diet) for 15 days, following rearing on normal commercial diet (commercial diet) (see 4.2.2). Gene-specific cDNA quantification was performed by QPCR.

3.2.1 Quantitative PCR

All cDNA for quantitative reverse transcription PCR (QPCR) was synthesised using the method described in 2.2.2.

QPCR primers for target genes, Ctr1 (AJ630205), qCtr1-F and qCtr1-R, Atox1 (AJ966735), qAtox1-F and qAtox1-R, ATP7A, qATP7A-F and qATP7A-R, ATP7B, qATP7B-F and qATP7B-R, MT (U58774), qMT-F and qMT-R, CuZn-SOD (AJ937872), qSOD-F and qSOD-R and GR (AJ937873), qGR-F and qGR-R and reference genes, sea bream β-actin (X89920), qActinF and qActinR, GAPDH (DQ641630) qGAPDHF and qGAPDHR and EF1α (AF184170) qEF1αF and qEF1αR (Table 3-1) were used at 0.3 µM with one fortieth of the cDNA synthesis reaction (5µl of a 1:10 dilution) and 10 µl of SYBR-green QPCR mix (ABgene, UK) in a total volume of 20 µl. Reactions were run in a Techne Quantica thermocycler at annealing temperatures of 58°C for CTR1 and MT, 55°C for β-actin, CuZn-SOD and GR, 56°C for Atox1 and ATP7A and 60°C for GAPDH and

EF1α and 62°C for ATP7B to give PCR products of 186, 171, 171, 164, 170, 130, 175, 250, 174 and 157 bp respectively. The QPCR cycling program was for all the reaction as follow:

Enzyme activation (Taq) - 15 min 95°C 1 cylce

Denaturation 20 sec 95°C
Annealing 20 sec (temperature as indicated above)
Extension 30 sec 72°C) 45 cycles

Dissociation peak 70 - 90°C measuring every 0.5°C

3.2.1.1 QPCR validation

SYBR green exhibits greatly enhanced fluorescence, when interacting with the minor groove of double stranded DNA. In the absence of DNA, or in the presence of single stranded DNA or RNA, SYBR green shows minimal fluorescence. These features of SYBR green enable its use to detect increasing amounts of double stranded DNA, produced at the end of each cycle, during PCR (van der Velden et al., 2003). This method permits accurate quantification of PCR products, although not necessarily in a specific manner since increase in double stranded non-target DNA would also result in an increase of SYBR green fluorescence. Therefore the identity of the amplified PCR product using specific primers must be verified under specific reaction conditions.

During optimization of QPCR conditions each QPCR product was sequenced to confirm its identity. Under each of the conditions described here the sequence of the product was identical to the predicted sequence. The real-time PCR machine (Techne Quantica thermocycler) not only monitors DNA synthesis during the PCR, but also determines the melting point of the product at the end of the amplification reactions. The melting temperature of a double stranded DNA depends on its base composition and its

length. All PCR products for a particular primer pair should have the same melting temperature unless there is contamination, mispriming (aspecific amplification), primer-dimer artefacts (primers anneal to themselves and create small templates), or some other problem. Since SYBR green does not distinguish between one DNA and another, an important means of quality control during routine QPCR is to check that all samples have a similar melting temperature.

As shown in the QPCR cycling program above, the dissociation (melting point) peak was determined by increasing the temperature by 0.5˚C every 10 seconds and measuring the change in fluorescence. Fluorescence is high at low temperatures when all DNA is double stranded and at the melting point, when the two strands of DNA separate, the fluorescence rapidly decreases (Figure 3-1 A). The dissociation peak plotted by Quantica software is the first negative derivative of the fluorescence *vs* temperature (Figure 3-1 B). The dissociation peaks, confirmed in every case that the primers pairs used for QPCR were highly specific and produced one single product.

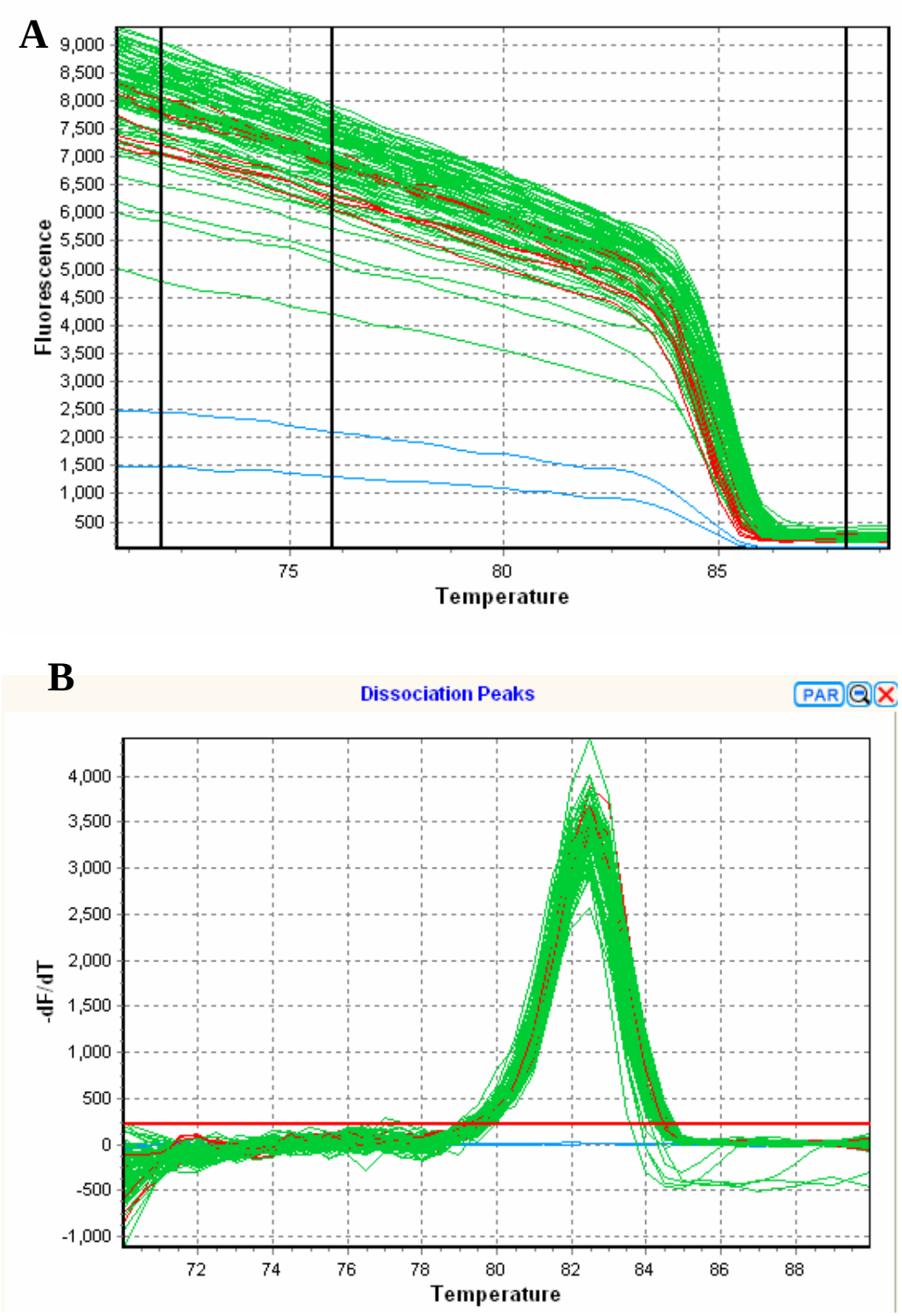


Figure 3-1 Dissociation peak shown as fluorescence vs temperature (A) and first negative derivative of fluorescence vs temperature (B). Output produced by the Quantica software. The red horizontal line is the automatic threshold which determines the area of the peak. Samples are shown in green, standards in red and non template controls in blue lines.

3.2.1.2 Gene copy number determination

Gene copy number in each reaction was automatically calculated by the Quantica software by comparison to a standard curve. Each sample set was run with a parallel set of serially diluted linearised plasmids containing a ligated fragment of the target gene (TG) sequence (Figure 3-2).

The construction of the standard curve consisted of:

1. Cloning of the TG into the plasmid vector as described in 2.2.3.

2. Cutting the plasmid, containing the ligated TG, at a single site which is not present in the insert sequence using an appropriate restriction enzyme (Figure 2-7). TGs were checked using SeqBuilder (DNASTAR, USA) to ensure they lacked a restriction site for the selected restriction enzyme. The restriction digestion was achieved as follows: 500 ng of plasmid was added to a solution containing 1 µl of 10X specific enzyme buffer (Invitrogen, UK), 2 unit of restriction enzyme (0.2 µl; Invitrogen, UK) and water in a final volume of 10 µl. The solution was then incubated at 37°C for 2 hours and then 2 µl of digest was checked by electrophoresis on a 1% agarose gel. The concentration of DNA in the digest was measured using a Nanodrop spectrophotometer.

3. Calculate the mass of a single plasmid molecule:

$$m = \left[n \right] \left(\frac{1 \cancel{\text{ mole}}}{6.023\ 10^{23} \text{ molecules (bp)}} \right) \left(\frac{660 \text{ g}}{\cancel{\text{mole}}} \right) = \left[n \right] \left(\frac{1.096\ 10^{-21} \text{ g}}{\text{bp}} \right)$$

Where: n = DNA size (bp) plasmid size + fragment of TG ligated

m = mass

Avogadros number = $6.023\ 10^{23}$ molecules / 1 mole

Average molecular weight of a double –stranded DNA molecule = 660g/mole

(http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf)

For example, Ctr1 standards have been calculated as follow:

$$m = \left(4307\ \text{bp (plasmid size + Ctr1 fragment size)} \right) \frac{1.096\ 10^{-21}\text{g}}{\text{bp}} = 4.72196\ 10^{-18}\text{g}$$

therefore 10^8 copy of the plasmid would be = $4.7219\ 10^{-9}$g = 4.72 ng

Each QPCR reaction contains 5 µl of standard or cDNA sample and the 10^8 copy stock is prepared in 1 ml:

$$\left(\frac{4.72\ \text{ng}}{5\ \mu\text{l}} \right) 200\ \mu\text{l (dilution factor)} \frac{1}{50\ \text{ng}/\mu\text{l (cut plasmid concentration)}} = 3.8\ \mu\text{l}$$

Summarizing, the 10^8 copies/reaction (5 µl) stock standard was obtained adding 3.8 µl of cut plasmid to 996.2 µl of λTE buffer. After that, serial dilutions, of the stock were then prepared using λTE buffer, from 10^7 to 10^2 copies per reaction. Twenty µl of diluted standards were then aliquoted in PCR tubes and stored at -20˚C. λTE buffer consisted of a solution 10 mM Tris pH 8.0 (Sigma, UK), 100 mM EDTA (Sigma, UK) and 50 pg/µl of λDNA (Promega, UK). TE buffer is used for general DNA storage, and the λDNA maintains the plasmid standards at a constant concentration by saturating binding sites in storage tubes, which would otherwise effectively reduce the concentration of standard DNA, especially at low concentrations.

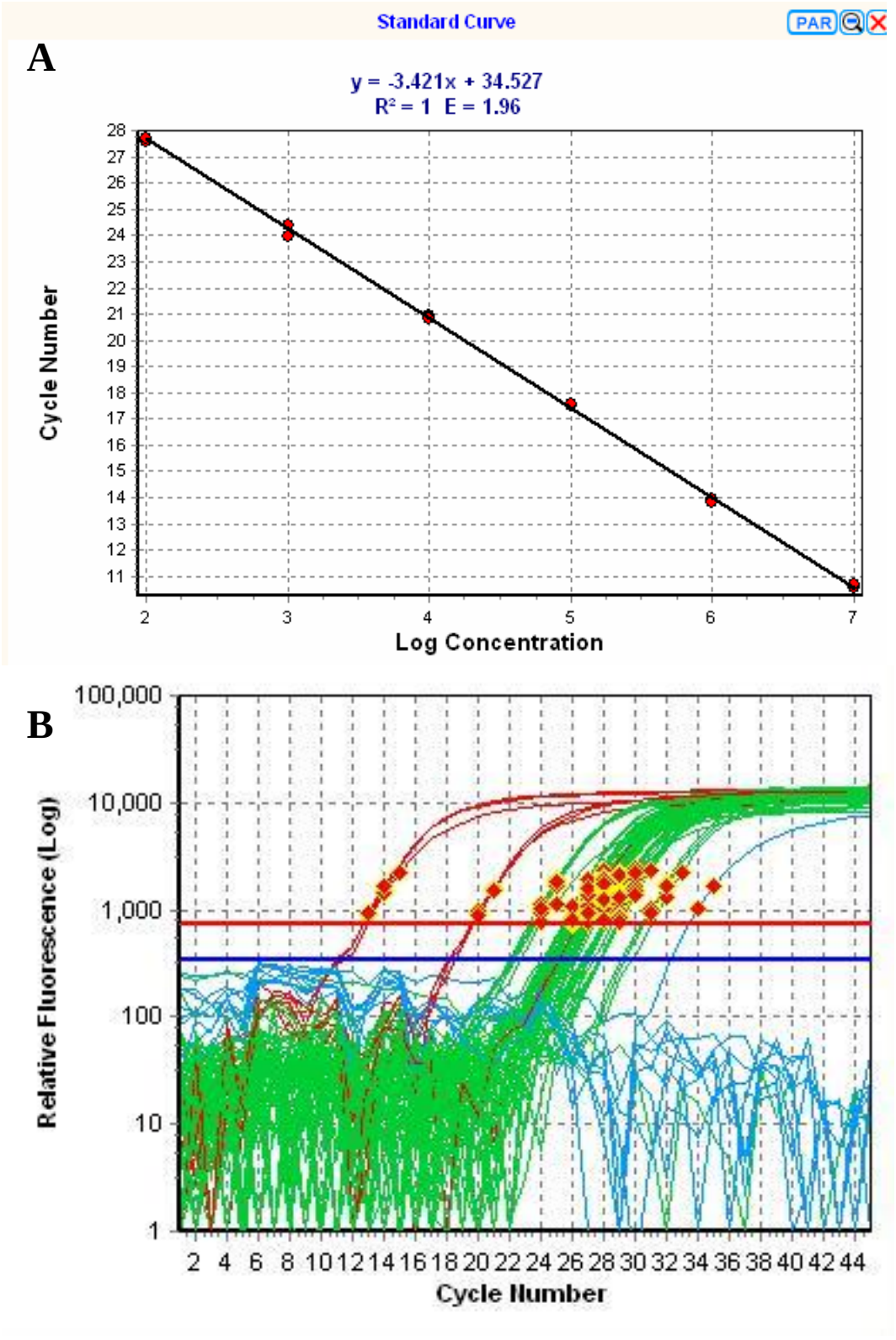


Figure 3-2 A Standard curve prepared from the serial dilution of known amount of linearised plasmid containing the ligated TG. **B** Amplification plot, the red horizontal line is the threshold of fluorescence at which the crossing point (cp) values, then converted in copy numbers by the

Quantica software through the comparison with the standards, was measured. The blue horizontal line is the noise threshold. The samples are shown in green, the standards in red and the non template control in blue.

In addition to QPCR of Cu-homeostasis genes, reference genes (GAPDH, β-actin and EF1α) were also measured. All reference gene expression varied widely between tissues, 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. However, since EF1α was the most stable gene, (see 3.3.1) the target gene expression profiles normalized by this gene have also been analysed.

Table 3-1. Primers used for QPCR.

ID	*Sequence 5`→3`*	*length*	*Tm*	*GC%*
qCtr1-F	cgggtctgctcatcaacaccc	21	63.7	61.9
qCtr1-R	tgtgcgtctccatcagcaccg	21	63.7	61.9
qAtox1-F	gtgtgagggttgttcaggagc	21	61.8	57.1
qAtox1-R	gagaacttccacgtctttgtcg	22	60.3	50
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
qMT-F	gctcctgcacctcctgcaag	20	63.4	65
qMT-R	gggtcacacacaggcgccat	20	63.4	65
qSOD-F	ccatggtaagaatcatggcgg	21	59.8	52.4
qSOD-R	cgtggatcaccatggttctg	20	59.4	55
qGR-F	caaagcgcagtgtgattgtgg	21	59.8	52.4
qGR-R	ccactccggagttttgcatttc	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).

3.2.2 Statistical analyses

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 were transformed to improve normality. Sample data were 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.

3.3 Results

3.3.1 Reference genes tissue expression profiles

Prior to analyses of the target genes, the tissue expression profiles of the three reference genes were analyzed in kidney, gill, intestine and liver by geNorm software (Vandesompele et al., 2002) and EF1α resulted in being the one least variable between tissues. Although EF1α normalization reduced the standard deviation of the raw target gene expression data (copy no/µg of total RNA), clear differences were observed in the absolute expression of EF1α between tissues, which were likely the result of genuine expression differences and not efficiency of cDNA synthesis or RNA input differences. After normalizing by ng of input RNA the expression of β-actin was observed to be 19 fold higher in the kidney than in liver. Similarly, GAPDH expression was shown to be between 8.8 and 3.6 fold higher in the kidney than in the gill, intestine and liver. Even though EF1α expression was the most stable between tissues some significant differences were found. EF1α mRNA levels were between 4.7 and 3.8 fold higher in the kidney, gill and intestine than in white and red muscle, moreover EF1α expression was 2.5 fold higher

in the gill than in the liver (Figure 3-3). Therefore, TG tissue expression profiles have been reported here as copy no per ng of input RNA, nevertheless the results normalized per EF1α showed a similar profile across tissues to the one normalised per ng of input RNA (data not shown).

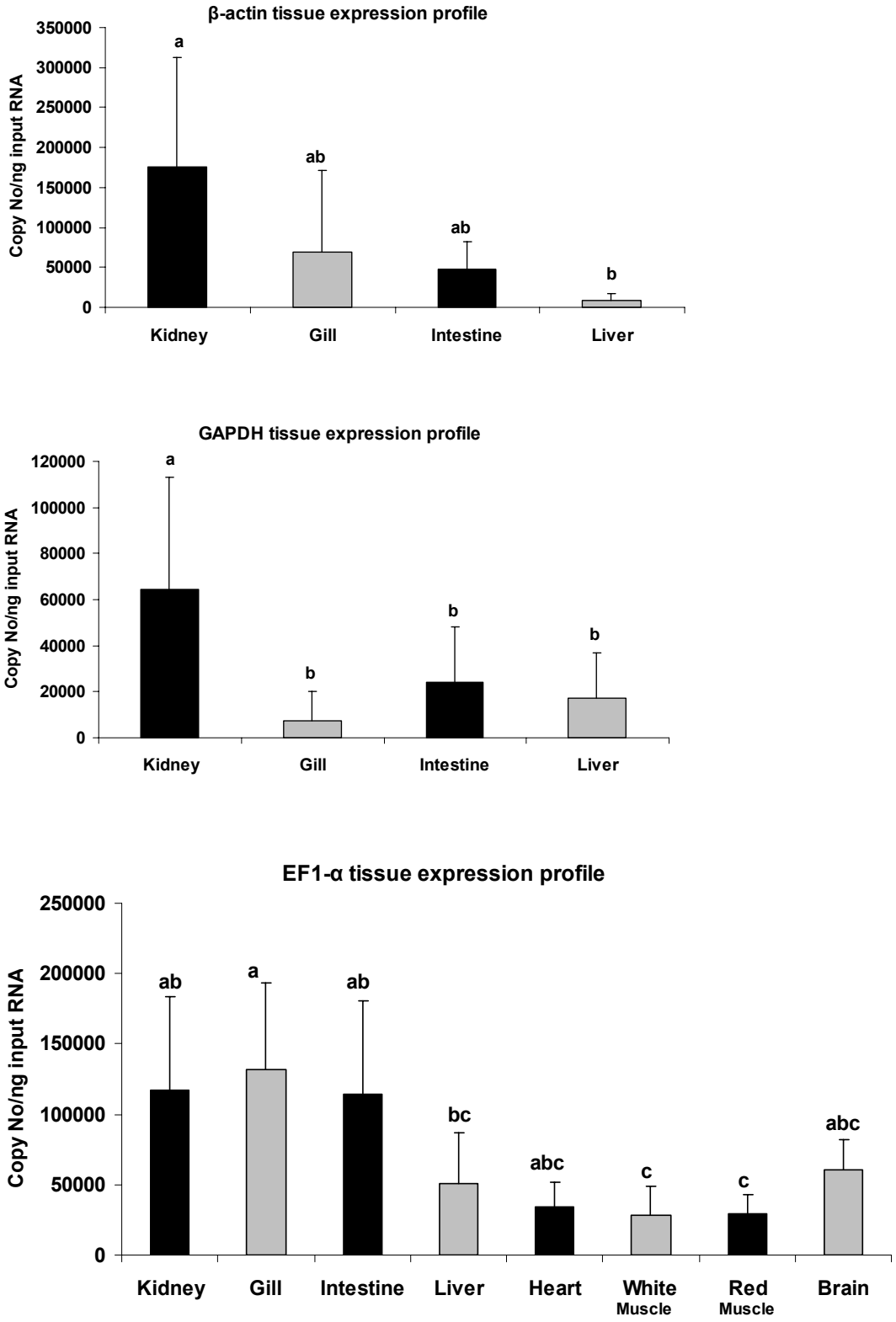


Figure 3-3 Ref. genes tissue expression profile. Ref. genes mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.3.2 SaCtr1 mRNA tissue expression profile

The tissue expression profile of SaCtr1 is shown in Figure 3-4. Of the eight tissues tested, levels of Ctr1 in small intestine were significantly higher than the others excluding the gill which was the second highest tissue in Ctr1 levels. Ctr1 mRNA levels in kidney, liver, brain, heart, white and red muscle were 6- to 10-fold lower than levels in intestine.

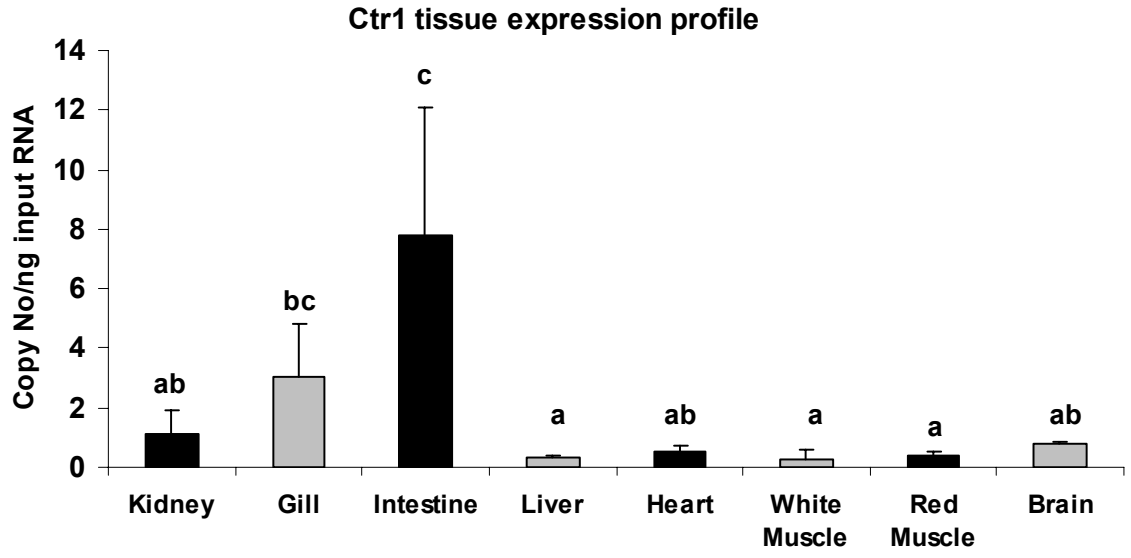


Figure 3-4 Ctr1 mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.3.3 SaAtox1 mRNA tissue expression profile

The tissue expression profile of SaAtox1 is shown in Figure 3-5. Atox1 mRNA was expressed at similar levels in the kidney and brain, levels which were between 3- (gill) and 13.4- (liver) fold higher then those in the gill, intestine, liver white and red muscle. Moreover Atox1 expression showed levels 1.8-fold higher in the kidney then in the heart.

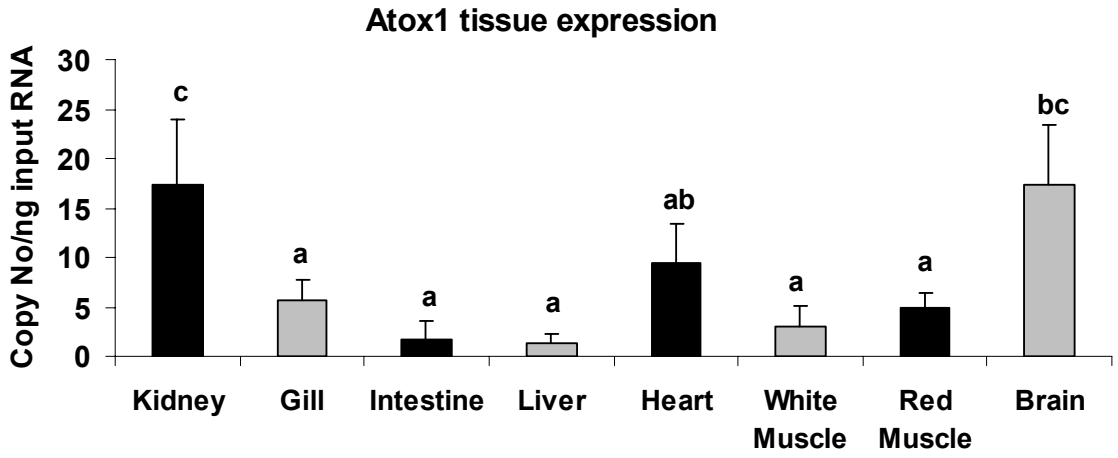


Figure 3-5 Atox1 mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.3.4 SaATP7A mRNA tissue expression profile

Compared to all other target genes saATP7A mRNA was expressed at lower levels. SaATP7A expression profile is shown in Figure 3-6. The brain was the tissue with the highest levels of expression. Kidney, gill, intestine, heart, white and red muscle were expressed at 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.

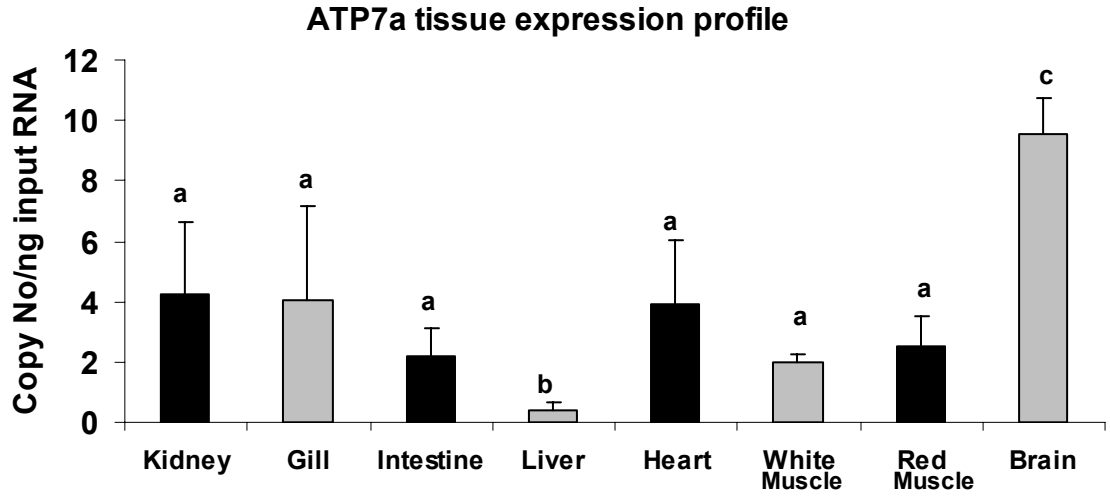


Figure 3-6 ATP7A mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.3.5 SaATP7B mRNA tissue expression profile

Unexpectedly, the highest level of ATP7B mRNA expression was in the intestine and was between 4- and 200- fold higher that the liver and the heart respectively. Following the intestine, the liver was the second highest in ATP7B expressing levels between 14- and 54- fold higher than white muscle and heart respectively. ATP7B in the kidney and brain was expressed at levels not significantly different from the liver.

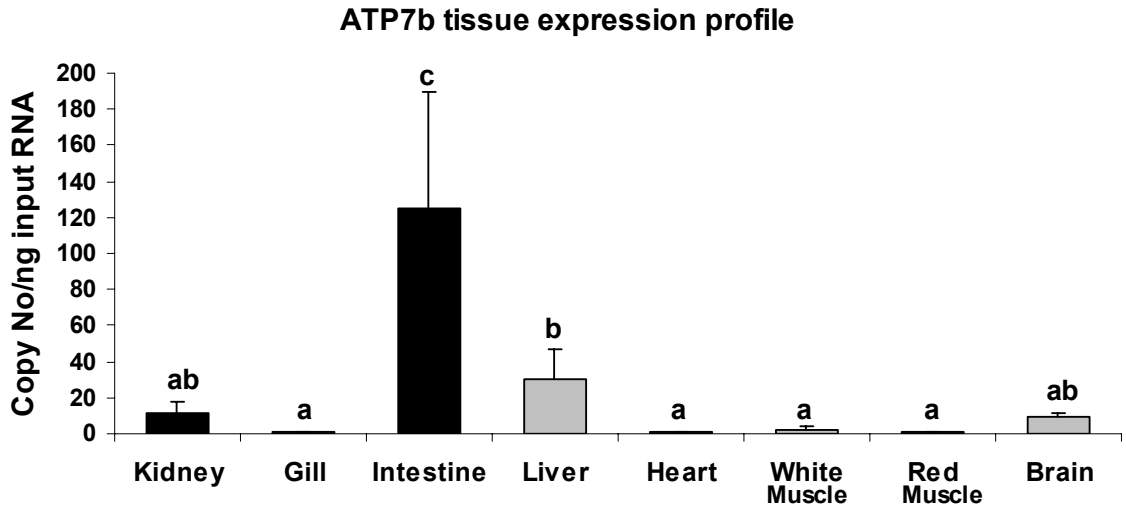


Figure 3-7 ATP7B mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.3.6 MT mRNA tissue expression profile

The kidney was the tissue which had the highest level of MT mRNA with levels 2.2- and 27.3- fold higher than brain and gill respectively. Next highest levels were observed in the brain and liver which were in turn between 12- and 5- fold higher than in the gill and white muscle respectively.

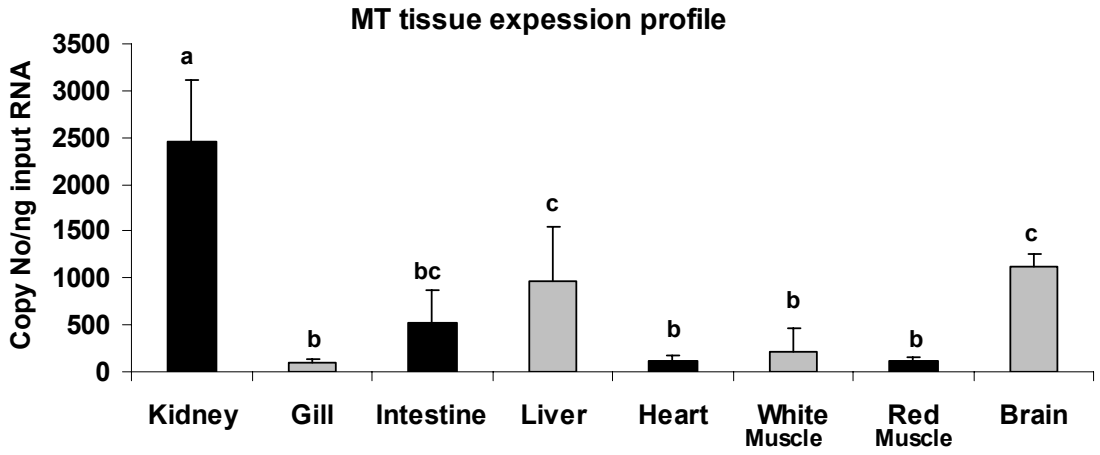


Figure 3-8 MT mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different (P<0.05; ANOVA, Tukeys test).

3.3.7 CuZn-SOD tissue expression profile

CuZn-SOD mRNA was highest in the kidney and gill, which were expressed at levels between 3.4- and 70- times higher then intestine and white muscle respectively.

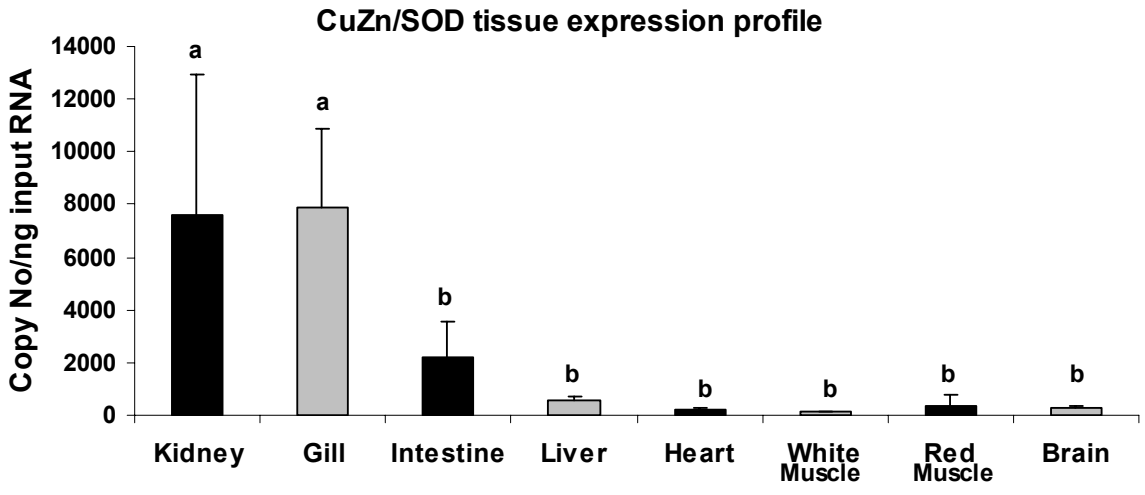


Figure 3-9 CuZn-SOD mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different (P<0.05; ANOVA, Tukeys test).

3.3.8 GR tissue expression profile

Similarly to CuZn-SOD, the other oxidative response gene measured, GR, had higher mRNA levels in kidney and gill compared to all other tissues with level of mRNA between 12- and 100- fold higher then intestine and red muscle respectively. Overall GR was expressed at levels about 200- times lower then CuZn-SOD.

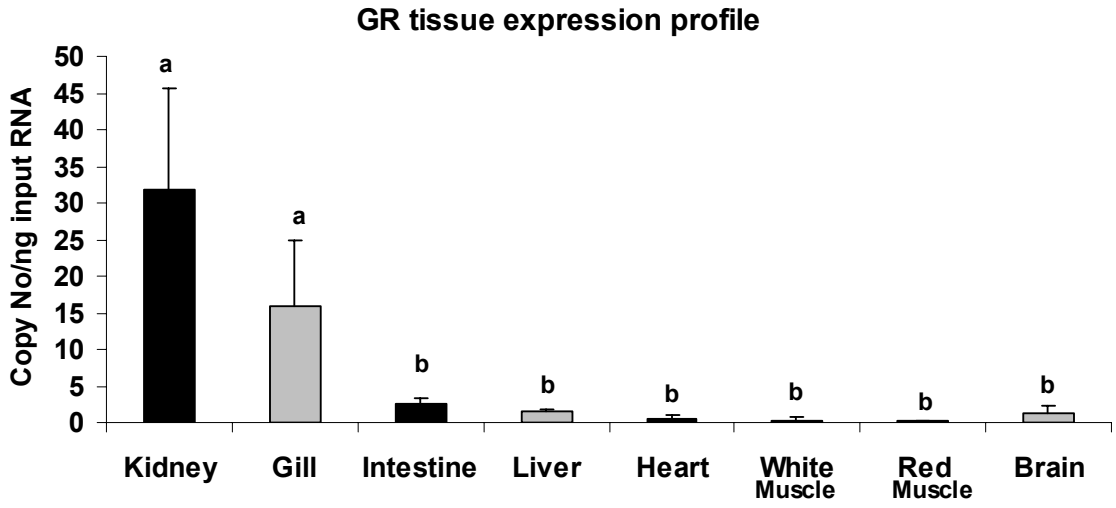


Figure 3-10 GR mRNA levels were determined by QPCR of cDNA synthesized from equal amounts of total RNA from each tissue. Values are means ± S.D. n=5. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

3.4 Discussion

As discussed in 1.5.5, body Cu homeostasis in fish and in mammals is regulated mainly by the intestine, regulating absorption and by the liver, regulating biliary excretion. The gill and kidney have also been shown to have a role in Cu homeostasis, though not as clear or to such an extent.

In mammals, Cu uptake is entirely from the diet via the intestine, however expression of Ctr1 is considerably less in intestine than in liver, brain or kidney, (Lee et

al., 2001; Kuo et al., 2001) which would infer a primary role in systemic rather than dietary uptake. Ctr1 is expressed in all tissues tested in sea bream, however, intestinal Ctr1 mRNA expression was 4 to 10 fold higher than in the other measured tissues (Figure 3-4) and Ctr1 expression during zebrafish embryogenesis is mainly in the intestinal region after 24 hours and it continues to be strongly expressed in intestine until adulthood (Mackenzie et al., 2004). Ctr1 has been clearly demonstrated to be the main uptake system of bio-available Cu in mammals (Nose et al., 2006a). In mice with intestinal Ctr1 knock out, Cu can still enter the enterocyte, but it is not available for cuproenzymes and also is not delivered to the blood portal vein resulting in systemic Cu deficency, as it accumulates in a not bioavailable form. Thus, to enter the pathway of incorporation into cupro-enzyme and to carrier proteins, Cu must be taken up by Ctr1. Since intestinal uptake of Cu predominates in fish (Kamunde et al., 2002b; Kamunde et al., 2002a; Kamunde et al., 2001) this infers that Ctr1 has a role in uptake of dietary Cu in fish. Indeed, previous studies on Cu uptake in fish intestine provide evidence for the presence of a transporter with similar kinetics (high affinity/low capacity) to that of Ctr1 (Grosell and Wood 2002; Burke and Handy 2005) and the finding that Ctr1 mRNA is most highly expressed in intestine supports this contention. Moreover, consistent with the evidence that fish can absorb Cu from the water, especially in conditions of low dietary Cu (Grosell and Wood 2002; Kamunde et al., 2002a), in sea bream gill, Ctr1 was expressed at levels similar to intestine (Figure 3-4).

ATP7A and ATP7B are essential Cu transporters exhibiting distinct distribution in mammalian tissues (Linz and Lutsenko 2007). This distribution reflects specific homeostatic roles (Figure 1-5) (see 1.5.4). In mammals, ATP7A is ubiquitously expressed during development, but very low levels are found in the liver of adult animals, whereas, ATP7B expression is more delimited with high levels of expression mainly in liver and

intestine (Kuo et al., 1997). The expression profile of ATP7A and ATP7B in sea bream is similar to the mammalian tissue expression (Figure 3-6; Figure 3-7). In sea bream, the equal distribution of ATP7A in all the tissues analysed, excluding the liver where ATP7A was expressed at lower levels, reflects its biosynthetic and homeostatic function. ATP7A is known to deliver Cu to cuproezymes, for example, peptydyl-α-monooxygenase (El Meskini et al., 2003), tyrosinase (Petris et al., 2000) and lysyl oxidase (Tchaparian et al., 2000). Interestingly sea bream brain showed higher levels of ATP7A which may be a reflection, of a higher requirement for the incorporation of Cu into cuproenzymes involved in neurological functions (e.g. peptydyl-α-monooxygenase), as shown by the prevalence of neurological dysfunction in Cu disorders (Madsen and Gitlin 2007). Importantly, there is evidence of Cu transport mediated by ATPase in fish gill (Bury et al., 1999; Campbell et al., 1999) and intestine (Bury et al., 2003; Handy et al., 2000). Therefore, the expression of an homologue gene to the human ATP7A in sea bream gill and intestine supports the hypothesis of basolateral, ATP dependent, Cu transport in these tissues by this protein as proposed by Bury et al (2003).

The high level of hepatic ATP7B is related to its known function in mammals, delivering Cu to ceruloplasmin (Terada et al., 1998) and its role in Cu biliary excretion. The high level of expression of ATP7B in the intestine, of both sea bream and mammals has not been satifactorily explained. Considering that there is not a known homeostatic function for ATP7B in the intestine, it is possible that ATP7B is involved in the delivery of Cu to a serum carrier or an enzyme. Ceruloplasmin is a serum ferroxidase which mediates the release of iron from the liver and other tissue but it does not appear to play a role in iron transport from the intestine (Wessling-Resnick 2006) (see also 1.5.5). This role is attributed to another cuproenzyme, hephaestin, an intra membrane ferroxidase. The mechanism by which Cu is incorporated into hephaestin is unknown and considering its

high level of intestinal expression, ATP7B is a possible candidate. Notably, ATP7A and ATP7B are co-expressed in some mammalian tissues including intestine, kidney and brain (Kuo et al., 1997; Linz et al., 2007; Barnes et al., 2005) and this co-expression was observed also in sea bream. In these tissues Cu-ATPases have distinct roles as shown in the mutated phenotypes of Menkes and Wilson disease, where, in some tissues, one functionally active ATPase cannot compensate for lack of the other. Indeed, Cu accumulates in the intestine of Menkes disease (ATP7A-deficient) patients even though ATP7B is co-expressed and functionally active. In the brain, however, defects in ATP7B function appear to be compensated by ATP7A (Barnes et al., 2005), but not vice versa since the lack of ATP7A function is not compensated by ATP7B (Niciu et al., 2007). Explanations include, as suggested by Linz and Lutsenko (2007), different protein turn-over rates and the observation that in response to increasing levels of Cu, ATP7A and ATP7B are trafficked to basolateral and apical membranes respectively.

Atox1 expression in sea bream was higher in the kidney, heart and brain than in other tissues (Figure 3-5). This profile is similar to that observed in rat where Atox1 mRNA levels were high in heart, brain, liver, kidney and testis which, excluding the liver and the testis (levels in sexual organs were not measured) show a very similar expression profile (Naeve et al., 1999). Moreover, it is interesting that, in sea bream, Atox1 and ATP7A expression profiles are similar with Atox1 expressed roughly at levels two times higher which giving the relation between this two proteins suggests that they may be co-regulated.

The highest level of MT in sea bream tissues was found in the kidney followed by the liver and brain. The liver and kidney are organs of accumulation of heavy metals, in marine fish, which are inducers of metallothionein (George et al., 1996a; Isani et al.,

2003). Even though for lack of tissue the metal analysis was not possible in the kidney, the liver showed higher levels of Cu and Zn compared to the gill and intestine and a relation between MT induction and metals could be made (Table 4-1). Therfore, the high MT levels in sea bream kidney suggests that this farmed sea bream may have been exposed to excessive metal levels possible form the diet (argument discussed further in chapter 4). FW fish also express MT mainly in the liver and kidney and a comparative study between three FW fish species has shown that species with a positive relationship between tissue Cu accumulation and MT induction are more resistant to metal toxicity (De Boeck et al., 2003). The high basal level of MT in sea bream brain may suggest a role in tissue protection against oxidative stress (Hidalgo et al., 2001). Increased levels of MT in the brain have been reported in other fish species in response to heavy metals (Choi et al., 2007; Gonzalez et al., 2006).

CuZn/SOD and GR show very similar expression profiles, with higher levels of expression in the kidney and gill. This result may suggest that those tissues are more exposed or susceptible to oxidative stress. A recent study showed that a river trout (Salmo trutta) population exposed chronically to heavy metals had increased levels of CuZn-SOD and GR mRNA in gill and kidney (Hansen et al., 2006). On the other hand, CuZn-SOD and GR tissue expression profile could also be function of the chemical antioxidant levels in the tissues such as tissue glutathione or vitamine E.

Chapter 4. Effects of dietborne and waterborne copper on expression of copper homeostasis genes

4.1 Introduction

Fish can absorb Cu through two routes, through the gill from the water and through the intestine from the diet (see 1.5.5). There is physiological evidence that indicates different toxicological and homeostatic responses to waterborne or dietborne Cu (see 1.4.2; 1.4.3; 1.5.5). However, molecular studies which support this physiologic evidence are very few and exclusively focused on biomarkers of Cu accumulation and toxicity (De Boeck et al., 2004; Isani et al., 2003; Bury et al., 2003). In multicellular organisms, Cu homeostasis pathways operate in the context of different cell types and tissues, each of whose macromolecular composition and metabolic functions are unique (Mendelsohn et al., 2006; Lutsenko et al., 2007a). This means that the requirement for the essential cofactor Cu and its homeostatic intracellular regulation is cell/tissue specific, and reflects the complexity of the Cu homeostasis mechanisms.

A variety of Cu transporter genes have previously been described in yeast and mammals (see 1.5). The cloning of these genes from sea bream, described in chapter 2, and the measurement of their tissue expression, has enabled the investigation of homeostatic and toxicological responses to waterborne or dietborne Cu from a molecular point of view. Furthermore, the metal concentration of Cu and Zn was measured in selected tissues to evaluate metal accumulation under different routes of exposure.

The main questions I wanted to answer through this experiment were:

- Are Cu homeostasis genes affected by dietary or waterborne Cu exposure in sea bream tissues?
- In which tissues is Cu accumulated under waterborne or dietborne Cu exposure?
- Can Cu homeostasis gene expression explain tissue metal distribution?

Farmed fish are fed diets supplemented with relatively high levels of Cu and Zn which have been empirically determined to maximise growth rate through the production cycle (Subcommittee on Fish Nutrition 1993). Although attractive from a meat production viewpoint, little is known of the effects of such high Cu and Zn levels on fish health or on the environment surrounding farms (see 1.4.1). Fish and molluscs can absorb and accumulate dissolved minerals from the surrounding water (Grosell and Wood 2002; Serra et al., 1999) and Cu and Zn pollution have been demonstrated to be particularly deleterious on early life stages, which can have large impact on ecosystems (Johnson et al., 2007; Guy et al., 2006). In order to prepare fish diets containing optimal levels of Cu and other trace elements, further studies are required to improve both fish health and the environmental impact of aquaculture in the marine environment. Study of the molecular components of metal homeostasis provides an understanding of the mechanisms governing metal uptake, interaction and optimal requirements.

4.2 Materials and Methods

4.2.1 Animal holding conditions

Juvenile gilthead sea bream (*Sparus aurata*), average 40 g mass, were obtained from farm stock (Valle Ca' Zuliani, Rovigo, Italy). One hundred fish were acclimatised in

a 1500 L aerated, flow through polyethylene tank. Water temperature was 19.0 ± 0.5 °C and a salinity of 33.6 ± 2.6 ‰. Experimental tanks were supplied with seawater pumped ashore from the Adriatic Sea. The trial was performed at the Marine Research Centre of Cesenatico, Bologna University, Italy.

Before and during acclimatization, fish were fed a pre-experimental normal commercial pelleted diet (Skretting, Italy) at 2% of body mass per day which contained 12.6 ± 0.2 mg Cu Kg^{-1} and 160.0 ± 2.0 mg Zn Kg^{-1} (Table 4-1). Metal concentration was analyzed by atomic absorption spectrophotometry (AAS) to determine actual Cu and Zn concentrations.

4.2.2 Fish treatments

After 15 days of acclimatisation, fish were divided into 3 tanks of 150 L with 20 fish in each. The mineral premix, which contained Cu, Zn and other trace ingredients, was omitted from the experimental diets. In tank 1, fish were fed an experimental diet (low Cu control) consisting of a pelleted diet prepared using 60 % (percentage of dry mass) of fish meal, 20 % of wheat middlings, 10 % of soya meal, and 10 % of fish oil and which contained 7.7 ± 0.3 mg Kg^{-1} Cu and 60.1 ± 4.1 mg Kg^{-1} Zn (Table 4-1). Bulk ingredients to make the fish pellet were provided by Skretting and mixed with water. Mixing and extrusion were carried out with a commercial pasta maker and extruded diets were air dried and then chopped into pellets. In tank 2 fish were fed an experimental diet with high Cu (high Cu diet) that was produced with the same method described above but replacing water with an aqueous solution of $CuSO_4$ to generate a final nominal dietary concentration of 130 mg Kg^{-1} Cu (6.17 mM). This sub-lethal level of dietary Cu was based upon a preliminary experiment where 120 mg Kg^{-1} Cu was insufficient to elicit induction of MT (Carpenè unpublished data). Diets were then analyzed by AAS to determine actual Cu

concentrations (Table 4-1). Fish were fed at 1.5 % of body mass twice daily. In tank 1 and 2 the background Cu concentration was < 0.005 mg L^{-1}. In tank 3 fish were exposed to Cu via water. Copper sulphate ($CuSO_4 \bullet 5H_2O$) was added to the tank to give a nominal Cu concentration of 0.3 mg L^{-1} (1.2 μM). In a static renewal protocol 87 % of the water was replaced with freshly prepared sea water containing 0.3 mg L^{-1} Cu sulphate, previously equilibrated in a testing chamber, every 24 hours for 30 days in waterborne treatment and without Cu sulphate in control and diet tanks (tanks 1 and 2). This concentration of Cu was chosen based on the evidence of previous studies which indicated that this level of Cu was about 25 % of the 96 hours LC50 for the closely related *Sparus sarba* (96h LC50 between 1.03 and 1.24 mg L^{-1}; (Wong et al., 1999). In addition, this concentration of Cu has been shown to have a biological effect since MT was induced in the liver of sea bream at concentrations between 0.1 and 0.5 mg L^{-1} (Isani et al., 2003). Fish in waterborne exposure were also fed the low Cu control diet at 1.5% of body mass twice daily. Copper concentration in the water was verified by AAS (see 4.2.4). Fish were weighed before and after sampling and there were no differences in mean masses in animals subjected to Cu treatments compared to controls at 15 d and 30 d (Table 4-1).

4.2.3 Fish sampling

After an acclimatization period of 15 days at 0 days (day 0) prior to treatment and after 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. There were no mortalities in the tanks. Samples for RNA were immediately homogenised in TriReagent RNA extraction buffer (Sigma, UK) using a rotating probe homogeniser (see 2.2.1). At day 0 and 30, RNA was extracted from 4 tissues: kidney, gill, intestine and liver while at day 15 in the control low Cu group (tank

1), RNA was extracted from 8 tissues: the 4 mentioned above plus heart, white muscle, red muscle and brain from which the genes tissue expression profiles, described in chapter 3, were obtained.

4.2.4 Metal determination

Copper and Zn were determined in gills, intestine and liver tissues and also in the fish pellet and water. All polyethylene disposables were washed with 1M HCl. The samples (400-500 mg of wet weight) were placed in individual acid-washed Teflon jars and were digested in a microwave oven (Model 1200, Milestone, Italy) for 5 min at 250 W, 5 min at 400 W, 5 min at 500 W, and one min at 600 W in 2 ml 65 % HNO_3 and 0.5 ml 30 % H_2O_2. Cooled samples were transferred into 10 ml polyethylene volumetric flasks and directly aspired into the flame of an atomic absorption spectrophotometer (Model IL 11, Instrumentation Laboratory USA) equipped with a deuterium lamp background correction. When samples wet weight were lower the amount of digesting and diluting solutions were reduced accordingly. All samples were run in batches that included blanks, initial calibration standards and standard reference materials (CRM 278: lyophilized mussels); there was no evidence of any contamination in these blanks, moreover all values of reference materials were within certified limits given by the Community Bureau of Reference – BCR (Brussels). Recovery ranged from 94 % to 104 %. The detection limit (LOD) was established by analysing the blank solutions. LODs were 0.12 μg Cu g^{-1} and 0.08 μg Zn g^{-1}.

Sea water Cu concentrations in tanks were determined by a Varian Atomic Absorption Spectrophotometer Mod. AA20plus equipped with a graphite furnace Mod. GTA96plus. Water analysis was performed after 50 times dilution of sea water to avoid salt interference. Using this method the detection limit for Cu was 5 μg L^{-1} in seawater.

4.2.5 Target genes normalization

QPCR material and methods are described in 3.2.1. Gene copy number in each reaction was automatically calculated by the Quantica software by comparison to a standard curve as described in 3.2.1.2. 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). Within tissue the stability of individual reference genes varied and geNorm was used to select the two most stable genes for a particular tissue, which were then used to normalize target gene expression level.

4.3 Results

4.3.1 Growth and metal levels in tissues

Growth was not significantly affected by inclusion of Cu in the diet or in water after 15 days or after 30 days (Table 4-1). Cu and Zn levels in fish tissue and diets are provided in Table 4-1. Cu levels in the water of the low Cu control and dietary Cu-treatment tanks were < 0.005 mg L^{-1} throughout the experiment. At the beginning of the waterborne exposure Cu concentration was 0.283 ± 0.004 mg L^{-1} and at the end 0.294 ± 0.013 mg L^{-1}.

There were no significant changes in tissue Cu levels in fish fed the comercial diet containing 12.6 ± 0.2 mg Cu Kg^{-1} dry diet and the low Cu control diet containing 7.7 ± 0.3 mg Cu Kg^{-1} dry diet. After exposure to the high Cu diet which contained 135.5 ± 4.4 mg

Cu Kg^{-1} dry diet an accumulation of Cu was observed in the liver (1.6 fold greater) and intestine (1.5 fold greater) after 30 d treatment.

After waterborne Cu exposure (0.3 mg Cu L^{-1}), Cu levels in the gills were higher at both 15 and 30 day timepoints, whilst hepatic Cu was higher at 15 d and had returned to control levels at 30 d. Zn levels in the liver and intestine from control fish sampled at 0, 15 and 30 d did not differ. Zn levels were reduced in the gills of control fish sampled at 30 days, as well as those fish exposed to enhanced dietary and waterborne Cu for 30 d, as compared to fish fed the commercial diet. In the liver of fish exposed to waterborne Cu for 30 d, Zn levels were lower compared to those in all other treatments. Due to a lack of tissue, Cu levels were not determined in kidney.

Table 4-1. Fish weights (g), copper and zinc tissue and diet concentrations (mg kg^{-1} dry mass) at 0, 15 and 30 days after exposure to dietary or waterborne (mg L^{-1}) copper.

	Day 0 Commercial diet	Day 15 – low Cu diet	Day 15 – high Cu diet	Day 15 – low Cu diet + Cu in water	Day 30 – low Cu diet	Day 30 – high Cu diet	Day 30 – low Cu diet + Cu in water
Fish weights	46.7 ± 5.9	51.1 ± 8.2	48.9 ± 9.1	51.6 ± 6.6	55.1 ± 6.6	52.3 ± 9.8	55.1 ± 7.5
Water [Cu]	< 0.005	< 0.005	< 0.005	**0.294 ± 0.013***	< 0.005	< 0.005	**0.294 ± 0.013***
Diet [Cu]	12.6 ± 0.2	7.7 ± 0.3	**135.5 ± 4.5***	7.7 ± 0.3	7.7 ± 0.3	**135.5 ± 4.5***	7.7 ± 0.3
Diet [Zn]	160.0 ± 2.0	60.1 ± 4.1	67.6 ± 2.3	60.1 ± 4.1	60.1 ± 4.1	67.6 ± 2.3	60.1 ± 4.1
Gills [Cu]	1.45 ± 0.14	1.47 ± 0.21	1.40 ± 0.29	**2.51 ± 0.29***	1.7 ± 0.1	1.4 ± 0.2	**2.2 ± 0.2***
Gills [Zn]	27.91 ± 1.69	33.46 ± 3.22	30.66 ± 10.74	33.32 ± 4.35	**19.4 ± 2.4***	**19.1 ± 7.3***	**20.1 ± 1.1***
Liver [Cu]	5.38 ± 2.02	8.72 ± 1.81	8.95 ± 1.36	**10.43 ± 1.69***	8.5 ± 2.3	**13.3 ± 4.2***	8.5 ± 2.6
Liver [Zn]	38.25 ± 11.2	61.09 ± 29.13	54.78 ± 18.32	44.31 ± 18.62	52.1 ± 13.8	40.0 ± 17.04	**20.4 ± 7.8***
Intestine [Cu]	2.83 ± 0.64	3.21 ± 0.37	3.29 ± 0.83	3.07 ± 1.06	2.6 ±0.9	**3.9 ± 0.8***	2.9 ± 0.5
Intestine [Zn]	19.52 ± 5.2	22.53 ± 0.52	31.37 ± 9.85	26.42 ± 15.32	24.4 ± 4.2	31.3 ± 13.6	21.8 ± 6.1

Values are means ± S.D. n = 5, asterisks indicate a significant (ANOVA, $P < 0.05$) difference between experimental groups. Copper and zinc content in tissue and water was determined by AAS.

4.3.2 Effect of excess dietary and waterborne copper exposure on gene expression

Results are presented in Figure 4-1. Changing from the pre-experimental normal commercial diet to the reduced mineral mix (low Cu control) diet had no effect on intestinal, gill or kidney Ctr1 mRNA levels after 15 or 30 d. Switching from commercial diet to low Cu diet resulted in an increase of Ctr1 mRNA in the liver of control fish. After adding Cu to the diet (high Cu diet; 135 ± 4.4 mg Kg^{-1} Cu) Ctr1 mRNA levels were affected differently in different tissues. Intestinal Ctr1 mRNA was 3.9 fold lower at 15 d, however, at 30 d, no significant decrease was detected (ANOVA P = 0.0593) whilst Ctr1 levels were not affected in gill or kidney. In fish fed Cu-enhanced diets (high Cu diet), liver Ctr1 mRNA decreased relative to low Cu diet fed controls and returned to the levels measured in pre-experimental control fish at day 0, which had been maintained on the commercial diet. After exposing fish on low Cu diet to waterborne Cu (0.3 mg L^{-1} Cu) for 30 days, intestinal (at 30 d) and renal Ctr1 mRNA was higher than in controls on commercial diet or on low Cu diet alone. Hepatic Ctr1 mRNA did not change after waterborne Cu exposure relative to controls on low Cu diet (Figure 4-1A).

ATP7A was expressed at similar levels in kidney, gill and intestine of control fish. Hepatic levels of ATP7A were significantly lower (Figure 3-6). Switching from the commercial diet to the low Cu diet resulted in a dramatic reduction of intestinal (11 fold) and hepatic (3 fold) ATP7A mRNA levels after 15 or 30 d. Exposing fish to the high Cu diet resulted in a further reduction of intestinal ATP7A at both 15 and 30 d, moreover ATP7A expression was significantly reduced in kidney and in the gill (15 d). Waterborne Cu exposure in fish fed the low Cu diet resulted in an increase of intestinal ATP7A restoring levels to those observed in fish fed the commercial diet. Waterborne Cu also resulted in an increase (up to 4 fold) of hepatic ATP7A when compared to low Cu

controls. In contrast, ATP7A expression decreased in the gill (15 d) and kidney compared to low Cu controls (Figure 4-1A).

ATP7B was mainly expressed in the intestine and liver, however some expression was shown by the kidney and brain (Figure 3-7). Changing from the commercial diet to the low Cu diet had no effect on intestinal ATP7B expression levels but had different effects in kidney and liver. In the kidney of low Cu diet fed fish at 15 d an increase in ATP7B mRNA was shown when compared to commercial diet controls, while in the liver a decrease was shown at 15 and 30 d. Dietary Cu exposure (high Cu diet), at 15 d, resulted in an increase in hepatic ATP7B when compared to low Cu diet controls. Dietary Cu resulted also in an increase of intestinal ATP7B mRNA levels compared to commercial diet controls. At 30 d no effects of dietary Cu were observed on ATP7B expression levels in any of the tissues analyzed. Waterborne Cu in fish fed the low Cu diet resulted in an induction of intestinal and hepatic ATP7B at 15 d when compared to commercial diet and low Cu diet controls, and hepatic ATP7B induction persisted up to 30 d (Figure 4-1B).

Changing the commercial diet to the low Cu diet resulted in a reduction of intestinal and hepatic Atox1 expression levels at 15 d and in a further reduction at 30 d. Conversely, in the gill and kidney at 30 d, Atox1 showed higher expression levels than commercial diet controls. Dietary Cu (high Cu diet) had no effect on Atox1 expression if compared to low Cu diet controls in the intestine. Expression levels were lower than commercial diet controls though. In the liver dietary Cu resulted in a further reduction of Atox1 expression at 15 d. Waterborne Cu had a very similar effect to dietborne Cu exposure on Atox1 expression in all tissues and time points except in the kidney (30 d) where Atox1 was resulted repressed compared to low Cu diet controls (Figure 4-1B).

Intestinal, gill and liver MT mRNA levels were lower in low Cu diet fed control fish (15 and 30 d) compared to commercial diet pre-experimental controls. In intestine and liver, after dietary Cu (high Cu diet), MT levels also decreased when compared to commercial diet controls. There was no change in renal MT mRNA after switching control diets. Dietary Cu had no effect on MT mRNA relative to levels in low Cu control in any of the tissues tested. In contrast, waterborne exposure to Cu dramatically increased MT mRNA in liver relative to levels in fish fed the commercial, low Cu or high Cu diet. Smaller but significant increases in MT mRNA were also measured in kidney (30 d) and in gill (15 d) after exposure to waterborne Cu relative to low Cu diet controls. Waterborne Cu had no effect on intestinal MT mRNA relative to fish fed the low Cu diet (Figure 4-1C).

Changing from commercial to low Cu diet had no effect on GR in gill, kidney or liver. Intestinal GR mRNA levels were decreased in experimental control fish after switching from commercial to low Cu diet, similar to effects observed on MT. Inclusion of Cu in both diet and water led to further reductions in intestinal GR mRNA. Dietary Cu had no effect on GR in gill or liver, but did lead to decreases in the kidney after 30 days. Waterborne Cu increased GR mRNA in gill and liver but had no apparent effect in kidney (Figure 4-1C).

Changing from the commercial diet to the low Cu diet did not show any difference in the mRNA levels of CuZn-SOD in any of the tissue measured. Dietborne Cu (high Cu diet) resulted in a reduction of intestinal CuZn-SOD expression levels at 15 d compared to commercial diet and low Cu diet controls. In the gill of fish fed excess Cu (high Cu diet) CuZn-SOD mRNA levels were lower than low Cu diet controls at 30 d. In contrast in the kidney (at 30 d) and liver (at 15 d) expression levels were higher than commercial diet controls. Waterborne Cu exposure resulted in intestinal CuZn-SOD mRNA levels lower

than commercial diet and low Cu diet controls at 15 d while at 30 d CuZn-SOD expression levels were higher than low Cu diet controls. In the gill CuZn-SOD expression decreased compared to commercial diet and low Cu diet controls. Conversely in the kidney at 30 d CuZn-SOD levels were higher than commercial diet controls. No effect was observed in the liver of waterborne exposed fish on CuZn-SOD expression compared to controls (Figure 4-1C).

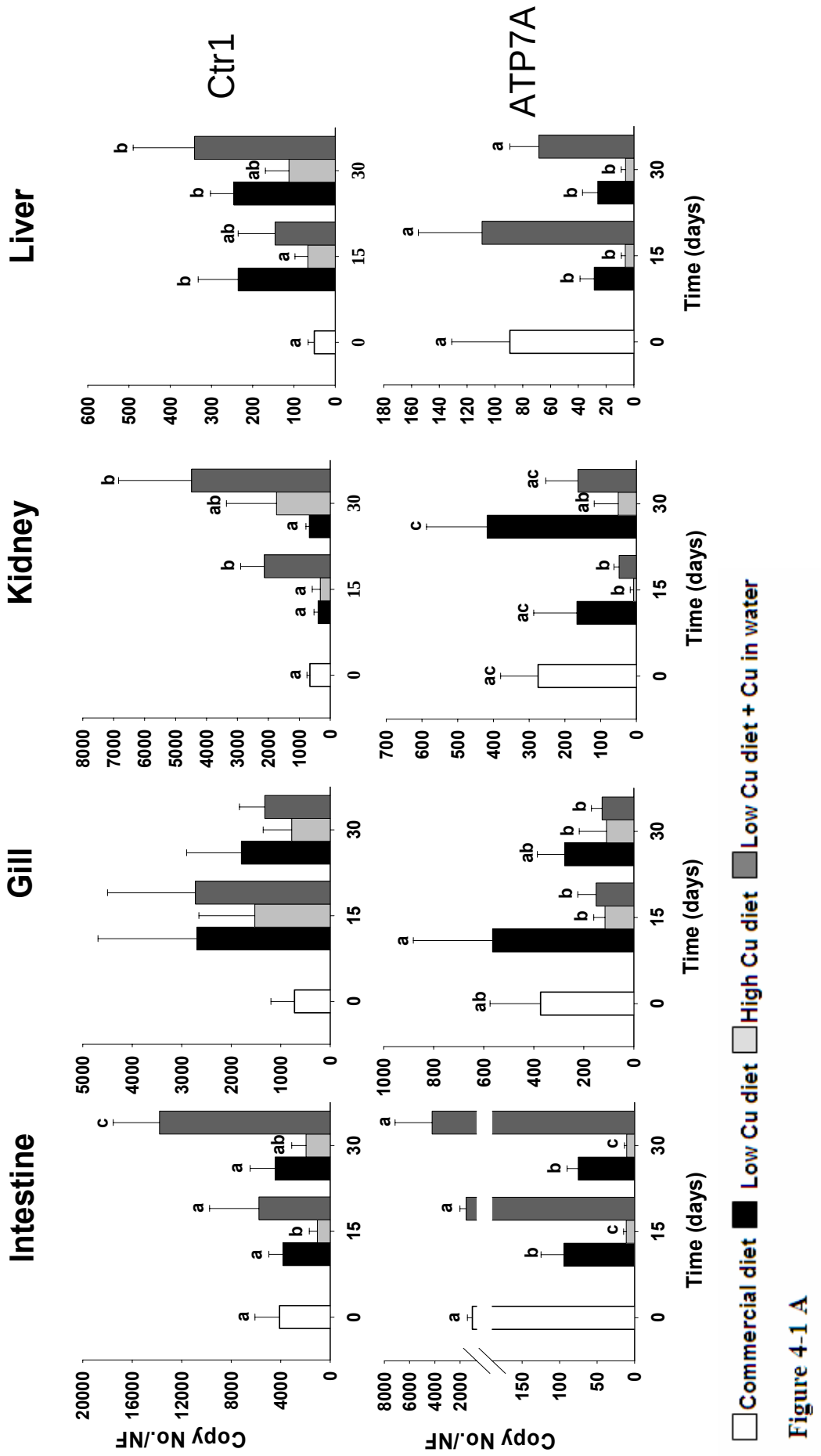

Liver
Kidney
Gill
Intestine
Ctr1
ATP7A
Copy No./NF
Time (days)
Commercial diet
Low Cu diet
High Cu diet
Low Cu diet + Cu in water

Figure 4-1 A

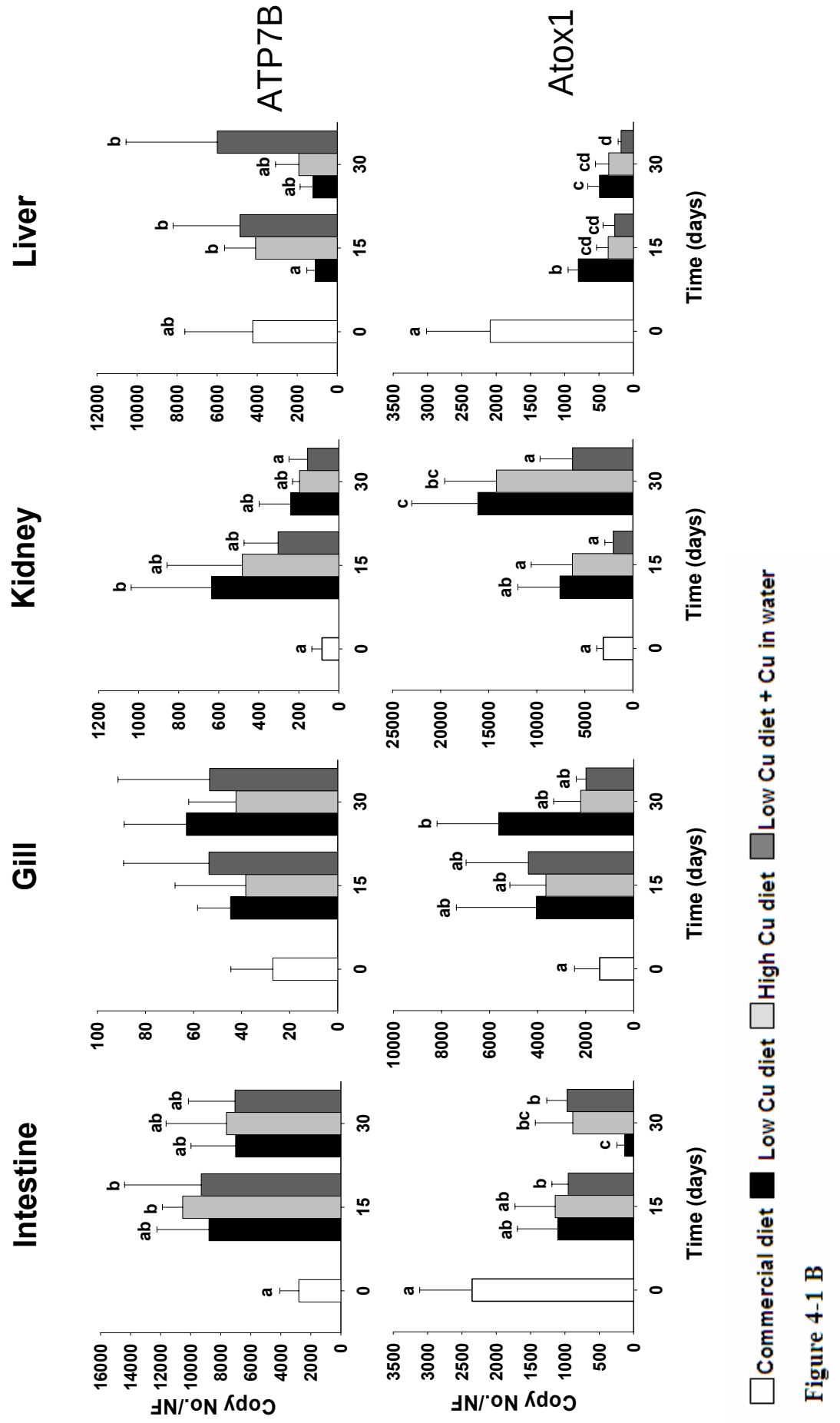

Liver
Kidney
Gill
Intestine
ATP7B
Atox1
Copy No./NF
Time (days)
Commercial diet
Low Cu diet
High Cu diet
Low Cu diet + Cu in water

Figure 4-1 B

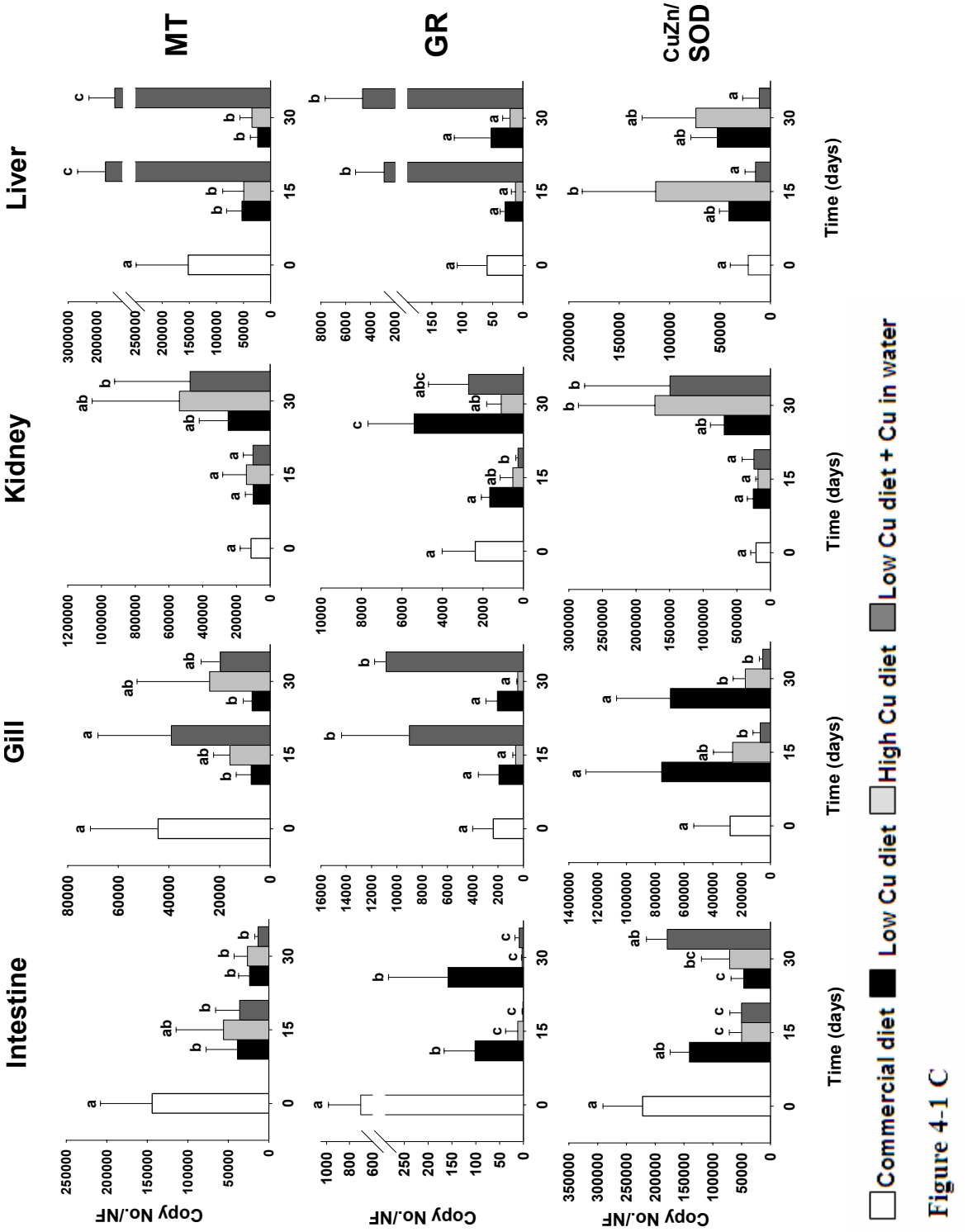


Figure 4-1 Tissue- and copper-specific regulation of sea bream Ctr1 and ATP7A (A); ATP7B and Atox1 (B); MT, GR and CuZn-SOD (C). Messenger RNA levels were measured in fish after 15 and 30 d fed an low Cu diet (7.7 ± 0.3 mg Kg^{-1} Cu), or a diet containing excess Cu (130 mg Kg^{-1}, high Cu diet), or after exposure to excess waterborne Cu (0.3 mg L^{-1}, low Cu diet + Cu in water). Fish at day 0 had been maintained on a normal commercial diet (12.6 ± 0.2 mg Kg^{-1} Cu). Ctr1,

ATP7A, ATP7B, Atox1, 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 or GAPDH reference genes. Values are means ± S.D. N=5. Bars bearing different lettering are significantly different ($P < 0.05$; ANOVA, Tukeys test).

4.4 Discussion

4.4.1 Effect of dietary Cu on gene expression

In this study, the effect of excess Cu on gene expression during two possible routes of intake, diet and water was measured. In order to generate control diets containing low Cu, the mineral mix containing Cu, Zn and other trace minerals was omitted resulting in an experimental control diet with Cu and Zn levels lower than the pre-experimental commercial diet which was used until the start of the experiment (day 0) (Table 4-1). Thus it is possible that effects on gene expression during exposure to high Cu may have been the result of lower levels of minerals other than Cu. Switching from commercial to experimental diet can often generate changes in the mineral content of fish and is common problem (Kamunde et al., 2002b; Clearwater et al., 2002). However, the low mineral experimental diet (low Cu diet) did not cause reductions in the concentration of Cu and Zn in the tissues of control fish at 15 d or on growth rates, despite clear effects on gene expression. There were reductions in Zn in gill in all treatments after 30 days, although these reductions did not change the pattern of gene expression compared to 15 days, indicating that the reduction in dietary Zn was not a major factor in causing the differences in gene expression between fish fed the experimental diets (Figure 4-1). Furthermore, even though the level of Zn in the experimental control (60 mg Zn Kg^{-1}) diet was lower than that of the pre-experimental diet (160 mg Zn Kg^{-1}), this level would not be considered deficient since the dietary Zn requirement for various fish species is known to range

between 15 and 30 mg Kg^{-1} (NRC 1993). Nevertheless, it is clear from the results that the omission of mineral premix did affect gene expression in sea bream tissues, based on the differences seen between fish at day 0, reared on the commercial diet, and fish at days 15 and 30 fed the experimental low Cu diet. The most pronounced effects were reductions in MT expression in intestine, gill and liver and reductions in GR in the intestine. These reductions were not subsequently reversed by inclusion of high Cu in the diet, in fact intestinal GR mRNA levels were further reduced by the inclusion of high dietary Cu. Therefore these effects on GR and MT are most likely to be caused by reductions in constituents of the mineral premix, other than Cu (Figure 4-1C). The most likely candidate constituent is Zn, as it was reduced from 160 to 60 mg kg^{-1} in the control diets, whilst Cu was reduced to a much lesser extent. MT is known to mainly respond to Zn status (Coyle et al., 2002) and a linear correlation was shown between Zn and MT levels in European plaice (*Pleuronectes platessa)* (George and Olsson 1994). In addition GR and CuZn-SOD have been shown to respond to exposure to heavy metals (Hansen et al., 2006). However at these conditions, CuZn-SOD was not induced showing possibly a different mechanism of regulation. Thus, the relatively high level of MT and GR mRNA observed under normal commercial dietary conditions indicates that Zn levels may be excessive in this diet.

Cu absorption occurs mainly 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, while 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 suggests that Cu absorption involves 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; Linder and Hazeghazam 1996).

Ctr1 has been demonstrated to be essential for systemic Cu uptake (Lee et al., 2001). Intestinal-specific Ctr1 knock-out in mice results in an overall Cu deficiency and causes accumulation of Cu in the enterocyte, in a non bioavailable form (Nose et al., 2006a). Zimnicka et al. (2007) has shown that Ctr1 functions at the basolateral membrane providing bloodborne Cu to the enterocyte like in all other tissues. Thus, although Ctr1 is clearly an essential component of intestinal and tissue Cu uptake, an alternative intestinal apical carrier must exist (Nose et al., 2006a). The protein responsible for apical Cu uptake remains unknown (this issue will be discussed further in the general discussion). Taken together this suggests that during apical uptake intestinal Ctr1 functions to facilitate transfer of Cu through intracellular vesicles, enabling Cu to enter a basolateral secretory pathway via ATP7A. Ctr1 also has a general basolateral uptake role in all cells including in intestine (Figure 6-2). The effects of switching control diets on Ctr1 mRNA levels were distinct from effects on MT or GR. No effects on Ctr1 were observed in any control diets in intestine, gill and kidney, indicating that the omission of the mineral premix is not a factor in considering the effects of excess Cu on this gene in these tissues. However, after dietary exposure to excess Cu, sea bream exhibited up to a 4-fold decrease in intestinal Ctr1 mRNA with no associated increase in tissue Cu levels at 15 d (Figure 4-1A). This decrease may be a protective response to excess dietary Cu, resulting in decreased Cu-uptake. In the liver, changing from commercial diet to low Cu diet resulted in an increase in Ctr1 mRNA (4.6 fold) and adding Cu to the diet (high Cu diet) reduced Ctr1 levels back to levels as in commercial diet controls. The response to low Cu diet may indicate that the hepatocyte requires fine tuning of intracellular Cu and Ctr1 regulation may be a crucial component of hepatic Cu homeostasis. Increased Ctr1 protein expression was detected in

the liver of intestinal knock-out mice which showed systemic Cu deficiency (Nose et al., 2006a).

Previous studies of Cu uptake in fish have concentrated on the physiology of Cu distribution and have mainly considered the uptake and effects of Cu in freshwater. Similar studies on marine species are few in number and there may be large differences in physiology between salt and freshwater fish. For example, in freshwater (low Na^+) it is well established that Cu uptake can occur through the ENaC sodium transporters of gill, although in conditions of high Na^+ concentrations, such as the seawater used here, these transporters are thought unlikely to play a significant part in either gills or gut (Handy et al., 2002). Moreover, Cu-uptake exhibits kinetics in isolated gut cells of rainbow trout is consistent with the presence of Ctr1-like activity (Burke and Handy 2005) and rainbow trout acclimatised to Cu in the diet showed increased Cu tolerance to both waterborne and subsequent dietary exposure (Miller et al., 1993). Thus, making use of the available data, the results presented here are consistent with a role for a high affinity carrier such as Ctr1 in regulating excess and normal Cu uptake.

At the basolateral membrane, ATP7A has been demonstrated to pump Cu from the enterocyte to the portal vein in mammals (Voskoboinik and Camakaris 2002) and this mechanism is thought to be the limiting step in intestinal Cu absorption. However, a role for ATP7A in limiting Cu transport in conditions of excess dietary Cu has not yet been demonstrated. Under high Cu conditions ATP7A was initially shown to localize to the basolateral membrane (Petris et al., 1996; Greenough et al., 2004) while recent studies have shown a predominantly intracellular localization of ATP7A with some relocalization in vesicles near but not at the basolateral membrane. Low proportions of ATP7A (8-10%) were localized at the basolateral membrane when Cu levels were elevated while the main

proportion of the protein was dispersed to the cell periphery (Nyasae et al., 2007) (Figure 6-2). In fish, there is evidence for basolateral regulation of Cu distribution from the intestine to the other tissues. In perfused catfish intestine and trout with increasing luminal Cu concentration, Cu uptake efficiency declined while Cu accumulated in the intestine (Handy et al., 2000; Clearwater et al., 2000). It has been proposed that this mechanism is regulated, at the basolateral membrane, by a Cu P-type ATPase and by a Cu/anion symporter (Handy et al., 2000). In addition, Cu transport at the gill has been shown to be ATP dependent and was suggested to be mediated by an homologous protein to ATP7A (Campbell et al., 1999). The results shown here support this evidence as sea bream express ATP7A at the intestine and gill. Moreover, following dietary Cu (high Cu diet) exposure a reduction up to 9 fold was shown by intestinal ATP7A mRNA when compared to low Cu diet control ATP7A mRNA levels. This effect may also explain the accumulation of intestinal Cu at 30 d. Dietary Cu resulted also in effects in other organs as reduction of gill (15 d) and kidney ATP7A expression. Moreover hepatic levels of ATP7B were increased (3-fold higher) at 15 d. These effects on Cu ATPases are difficult to interpret because of the high level of intestinal and hepatic ATP7A and ATP7B in control fish fed the commercial diet, compared to fish on the experimental control diet (low Cu diet). It is possible that such effects may be due to high levels of Zn or other metals in the commercial diet which may compete for uptake with Cu. Nevertheless the effect of dietborne Cu (high Cu diet) compared to low Cu diet controls on Cu ATPases is consistent with their homeostatic roles (Lutsenko et al., 2007a) reducing intestinal absorption and kidney reabsorption through ATP7A reduction and increasing hepatic excretion through induction of hepatic ATP7B.

Intestinal Atox1 was dramatically reduced at 30 d in low Cu diet controls compared to all other controls and exposed fish groups. In contrast, in the kidney of low

Cu diet controls Atox1 showed higher mRNA levels than Diet1 controls and Diet2 control at 15 d effect which mirrors the increase in ATP7A in the same fish group (Figure 4-1A-B). It has been hypothesised that Cu chaperones play a role in the hierarchy of which cuproenzyme receive Cu in conditions of deficiency for example CCS (chaperone for CuZn-SOD) is up regulated by Cu deficiency (Bertinato et al., 2003; Mendelsohn et al., 2006). However, Atox1 did not respond to Cu deficiency in mammals (Hamza et al., 1999). In sea bream, high levels of Atox1 in the kidney may be a marker of low Cu status, and increase in Atox1 and ATP7A may facilitate reabsorption of the available Cu to maintain an optimal blood Cu homeostasis. On the other hand decreased levels of intestinal Atox1 may be part of the response to low dietary Cu. Cloning and analysis of the homologous gene to mammalian CCS would be required to evaluate if a hierarchy to facilitate the delivery of Cu to specific cuproenzyme exists in fish.

Consistent with other marine and fresh water species (Grosell et al., 2004a; Hoyle et al., 2007) sea bream were tolerant to dietborne Cu showing no reduction in growth rate or mortality. Moreover, even though Cu was accumulated in the intestine and liver at 30 d none of the molecular markers of oxidative stress were induced at this time point, suggesting that this accumulated Cu was not toxic because it was bound to MT or glutathione or compartmentalized in vesicles.

4.4.2 Effect of waterborne Cu on gene expression

Waterborne Cu exposure showed very different effects on gene expression than dietary exposure. Even though a direct measurement of blood Cu concentration was not performed an interpretation of tissue Cu levels suggests that bloodborne Cu levels increased faster through waterborne exposure, as after 15 d the liver showed Cu accumulation which returned to control levels at 30 d. In contrast, after dietborne

exposure, Cu was accumulated only after 30 d in liver and intestine. Despite this, there was a dramatic induction of hepatic GR (up to 55-fold) and MT (up to 53-fold) at both timepoints after waterborne Cu exposure. Large increases of GR were also observed in the gill of fish exposed to waterborne Cu. These effects are most likely a response to excess Cu exposure since the observed increases were associated with Cu accumulation in the gill (15 and 30 d) and liver (15 d) and were not observed in low mineral mix control diets (low Cu diet) (Figure 4-1C). Remarkably, and quite distinct from dietary exposure, waterborne Cu exposure also resulted in an increase in Ctr1 mRNA in sea bream intestine (30 d) and in kidney (15 and 30d) (Figure 4-1A). In freshwater rainbow trout, renal excretion of excess Cu in fish is negligible compared to hepatobiliary routes (Grosell et al., 1998) and although we did not measure Cu levels in the kidney, marine fish, in contrast to freshwater species, can accumulate Cu in the kidney following brachial exposure, although at considerably lower levels than liver (Grosell et al., 2003; Grosell et al., 1997). Furthermore rainbow trout exhibit a rapid turnover of Cu in the kidney following brachial exposure (Grosell et al., 1997) indicating that, although accumulation may be low, the kidney is an important site of Cu transport and distribution in freshwater fish. Therefore, the increase in Ctr1 mRNA in kidney is consistent with the observed low renal excretion of Cu and with re-absorption by kidney which in turn would reduce dangerous high levels of Cu in the blood. In conditions of Cu scarcity, renal Ctr1-dependent re-absorption may be an essential mechanism for maintaining optimal whole body Cu concentrations. In conditions of excess Cu it could be hypothesised that renal excretion of Cu in plasma filtrates would be an efficient way to remove Cu. This is not the case and it may be that excess Cu is reabsorbed in the kidney, via increased Ctr1, as protection from renal toxicity which would otherwise be induced by an increased concentration of Cu in the tubules, an effect which would be particularly acute in marine fish which discharge a highly

concentrated urine (Beyenbach 2004). 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 mice with specific intestinal Ctr1 knock out the kidney showed a less dramatic decrease in Cu content compared to the other tissues (Nose et al., 2006a). In Wilson disease mice models, in contrast to the liver, renal ATP7A plays a major role in the protection of the renal tissue against Cu overload exporting Cu via the basolateral membrane (Linz et al., 2007). In sea bream in contrast to Ctr1, renal ATP7A was repressed by waterborne or dietborne Cu ruling the kidney as an organ of metal detoxification through accumulation in response to Cu.

In the gill the existence of a P-type ATPase (ATP7A-like) was suggested by Campbell et al., (1999). In addition, Bury et al., (1999) identified a saturatable and ATP dependent silver (known to mimic Cu) transporter, at the gill basolateral membrane. This finding supports the hypothesis of a branchial basolateral Cu extrusion mediated by a ATP7A-like protein which in sea bream gill was down regulated by waterborne Cu acting as a defensive homeostatic mechanism against Cu entry into the blood stream which may also explain the accumulation of branchial Cu.

Intestinal gene expression in response to waterborne Cu is however more difficult to interpret. The first consideration to be made is that marine fish drink and waterborne Cu has been shown to increase the drinking rate (Grosell et al., 2004a). Thus both the gill and the gut may be involved in Cu absorption by this route of exposure. While the gill shows clear homeostatic response to waterborne Cu (ATP7A repression) and a oxidative stress response (GR induction), the intestine shows an apparently anti-homeostatic response which suggests increased absorption such as induction of Ctr1 (30 d, 3 fold) and ATP7A

(15 d 17 fold and 30 d 43 fold) not associated with marker of metal toxicity (MT) or oxidative stress (GR and CuZn-SOD) (Figure 4-1C). These effects may be part of an adaptation mechanism to waterborne Cu which will be discussed further in the general discussion. The response by the liver on the other hand is clearer. As mentioned above, the main route of excretion of Cu is performed by the liver into the bile. This metabolic function in mammals has been demonstrated to involve the trafficking of ATP7B to a compartment near the canalicular membrane (Schaefer et al., 1999). ATP7B expression is induced in sea bream by waterborne Cu and this homeostatic mechanism might explain the reduction of hepatic Cu accumulated at 15 d. Moreover, in mammals, the majority of Cu which is delivered from the intestine to the blood is delivered to the liver and less to the kidney and other tissues (Bissig et al., 2005; Linder et al., 1998), inferring the liver as a principal organ of metabolism and detoxification of Cu.

The hepatic toxic response following waterborne Cu suggests that waterborne Cu is delivered to the liver in a different form (more toxic) than dietborne Cu which may be due to differences in the absorption mechanisms such as the involvement of different serum carrier proteins. Overall these results show that the route of exposure is critical to maintenance of Cu homeostasis and the manifestation of toxic effects.

Chapter 5. The *Sparus aurata* fibroblast 1 (SAF1) cell as a model for copper metabolism

5.1 Introduction

In vitro studies using mammalian cell lines have provided fundamental knowledge of Cu metabolism in mammals, insects and plants (see 1.5). The availability of partially differentiated "tissue-like" cell lines, such as intestinal (human colonic adenocarcinoma cells, Caco2, human intestinal epithelial cells, T84), renal (human embryonic kidney, HEK239), ovarian (chinese hamster ovary, CHO) and placental (human, placenta choriocarcinoma, BeWo), has enabled the effects of Cu to be accurately modelled *in vitro*. Cell culture systems have assumed a crucial importance in the understanding of broader tissue and systemic Cu homeostasis mechanisms (Zimnicka et al., 2007; Petris et al., 2003; Harris et al., 1998) since the effects of physiological signalling (viz. hormonal effects) can be controlled and understood. Indeed, hormonal regulation of Cu homeostasis has been demonstrated in different cell-based studies (Andrews 1990; Hardman et al., 2006; Hardman et al., 2007). The identification of a suitable piscine *in vitro* system is of crucial importance to improve our understanding of Cu homeostasis in fish. In fish, perfused tissues such as perfused intestine and perfused head have been shown to be informative systems for the study of Cu homeostasis (Handy et al., 2002; Campbell et al., 1999) (see 1.5.5). However, the use of cultured cell lines would enable a faster, less expensive and more flexible experimental procedure (Segner 1998).

The use of fish cell cultures have been established to be a useful tool for environmental toxicology (George 1996; Segner et al., 2001). Their use is of particular

value to study the toxic mechanism of pollutants from which biomarkers can be discovered and tested. However, toxicity assays of pollutants, including Cu, with cultured fish cells tend to be less or equally sensitive than *in vivo* bioassay with fish (Babich and Borenfreund 1991; Magwood and George 1996). Therefore, cellular models may have little use in routine environmental monitoring (Segner et al., 2001; Smith et al., 2001). Nevertheless, cell line studies have had an essential role in the explanation of the mechanisms of response to heavy metals' toxicity, such as MT induction, in fish (George 1996; Olson 1996) and Cu homeostasis, such as protein trafficking and gene regulation, in mammals and insects (Guo et al., 2006; Petris and Mercer 1999; Selvaraj et al., 2005).

In chapter 4, Cu transporter mRNA levels were shown to be affected by Cu excess depending upon the route of exposure. Thus, markers of metal toxicity and oxidative stress such as MT and GR observed in the liver after waterborne Cu exposure were not apparent after dietary exposure. These effects suggested that Cu might be presented to the liver complexed to a different protein or ligand. In addition, ATP7A was induced by waterborne Cu in the liver and intestine indicating a possible homeostatic or detoxification mechanism. With this in mind, to investigate Cu homeostasis further in sea bream the identification of an *in vitro* model was considered, thus exposing cells to growth medium supplemented with 10 % fetal bovine serum and added Cu sulphate, may simulate the situation under waterborne Cu exposure, since in both cases Cu might be presented to the cell as a non-specifically bound protein-complex (e.g. bound to albumin rather than bound to a specific entero-hepatic transporter).

SAF1 (***S****parus* ***a****urata* **f**ibroblast) (Bejar et al., 1997) cells, are an established, stable and easy to grow fibroblastic cell line. Moreover, fibroblasts synthesise lysyl oxidase, a cupro-enzyme required for collagen-elastin cross-linking in connective tissue

(Rucker et al., 1998). Indeed, the lack of functional ATP7A in Menkes disease patients is associated with severe connective tissue defects which are likely to be due to disrupted delivery of Cu to lysyl oxidase in the secretory pathway (Kemppainen et al., 1996; Grange et al., 2005). Therefore, active Cu homeostasis machinery must be present in fibroblast type cells making them a suitable cellular model for this study. However, continuous cell cultures often lose functional, metabolic and structural properties, and therefore some kind of functional characterization is required to evaluate the suitability of a continuous cell culture for a determinate study. Evaluating gene expression under normal and sub-toxic Cu (25 μM, see 5.2.3) can indicate if SAF1 cells are actively expressing mRNAs involved in Cu homeostasis, and therefore indicate if it is an adequate system for this kind of investigation. Moreover, the transcriptional response of SAF1 under excess Cu dissolved into the medium can be compared to observed transcriptional patterns *in-vivo*, to indicate the suitability of this *in-vitro* system for the investigation of the mechanism of response to waterborne Cu in fish.

The aim of the work described in this chapter was to determine the response of the transcriptome in SAF1 cells exposed to sub-toxic levels of Cu, Cdand Zn. In addition to the measurement of Cu homeostasis genes by QPCR, a medium density cDNA microarray was used. The microarray technology enabled a broader overview of the transcriptional response of SAF1 cells to Cu. Furthermore, this powerful tool is of particular interest because it can lead to the discovery of new pathways involved in the response to excess Cu.

5.2 Materials and Methods

5.2.1 SAF1 cell culture

The continuous cell line SAF1 was provided by Bejar and colleagues and then cells were routinely propagated and maintained in the Virology Unit of the Institute of Aquaculture, University of Stirling. SAF1 cells were grown in the complete growth medium Leibovitz's L-15 with GlutaMAX™-l supplemented with sterile kanamycin (100 µg/ml), penicillin G (50 U/ml), streptomycin (50 µg/ml) and 10% of foetal bovine serum (FBS). All components were purchased from Gibco®/Invitrogen, UK. Cell cultures were monitored daily, using an inverted microscope (IMT-2, Olympus), for evidence of contamination and changes in pH. When a medium decoloration occured, due to increased catabolites and decreasing pH, one half of the volume was replaced with fresh medium. Once the monolayer cell growth reached confluence (4-6 days) cells were ready for sub-culturing.

5.2.1.1 Sub-culture procedure

- Decant and discard the spent medium from the parent flask.
- The cell monolayer was washed twice with phosphate buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Invitrogen, UK), (5 ml for 25cm^2 flasks and 10 ml for 75 cm^2 flasks).
- 0.05% trypsin/0.02% EDTA solution (Invitrogen, UK) was added to the flask to minimally cover the whole monolayer. The flask is tilted to spread the solution (1 ml for 25 cm^2 and 3 ml for 75 cm^2) and allow the enzyme to

work for 1-2 min. Then excess of trypsin/ EDTA was removed and cells were left a further 1-2 min in the residual solution.

- Once the cells were dislodged, 6 or 9 ml of supplemented medium for 25 and 75 cm^2 respectively were added and cells re-suspended by gently pipetting up and down. At this point cells were counted or directly redistributed in daughter flasks (generally 1:3) adding further medium up to 8 ml and 18 ml for 25 cm^2 and 75 cm^2 flasks respectively.

- Previously counted cells were stained with Trypan Blue dye (solution 0.4%, Sigma, UK). Trypan Blue dye is an exclusion dye, viable cells remaining unstained, and enables an estimate of overall culture viability. Counting was carried out using a standard Neubauer haemocytometer (0.1 mm) and an inverted microscope (IMT-2, Olympus).

5.2.2 Cytotoxicity assays

To determine the lethal effects of toxicant on cells, measures of cell death and cell viability have found widespread acceptance as these assays can be rapidly performed in 96 well culture micro-plates and quantified spectrophotometrically (Borenfreund and Puerner 1985; Clothier et al., 2006). Cell death can be estimated by determining the total protein per well using a dye binding assay such as the **kenacid blue** (KB) (Clothier et al., 2006). This assay relies on the principle that in cell cultures growing as an attached monolayer, dead cells usually detach from the culture substratum, whereas viable cells remain attached. As the amount of protein per well is a linear function of the number of attached cells, the percentage of viable cells can be deduced by quantifying the amount of total protein per well. Cell viability can be determined colorimetrically or fluorometrically by

the ability of cells to take up or metabolize vital dyes such as **neutral red** (NR) (Borenfreund and Puerner 1985). Neutral red (2-methyl-3-amino-7-dimethylaminophenanzine) is a weakly cationic dye that accumulates in lysosomes of living cells. Cellular uptake of neutral red is believed to occur by passive diffusion across the cell membrane, only living cells have functional proton pumps and they are thus able to accumulate and retain the dye, thus the neutral red staining intensity is directly related to the number of viable cells (Babich and Borenfreund 1993). As no literature was available on Cu, Cdor other heavy metals cytotoxicity on SAF1 cells, both kenacid blue and neutral red assay were performed on SAF1 cells exposed to a range of concentrations of Cu and cadmium.

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. Cu sulphate pentahydrate ($CuSO_4$, Sigma, UK) and Cdchloride 2.5 hydrate ($CdCl_2$, Sigma, UK) were dissolved in milliQ water at a concentration of 100 mM and then filtered with 0.45 µm filter units (MILLEX®-HV, MILLIPORE, UK). Metals were added to the cell cultures 24 hours (h) after seeding as 10 times concentrated water solutions dissolved in growth medium, to give final concentrations of 0.01 mM to 2.5 mM. Exposition time was 24 or 48 h.

5.2.2.1 Kenacid Blue cytotoxicity assay

After exposure, cells were washed three times with 200 µl of PBS. Cells were then fixed with 3% glutaraldehyde (Agar scientific, UK) in PBS for 15 min. Afterwards the fixative was removed and 200 µl of stain solution added. The cells were gently agitated for 30 min. The staining solution, consisting of Kenacid Blue (BDH, UK) dissolved in 70% ethanol, was filtered with 0.45 µm filter units (MILLEX®-HV, MILLIPORE, UK)

and then mixed with glacial acetic acid (Fisher scientific, UK) in a proportion of 9:1. The staining solution was then removed and 200 µl of destaining solution (1% glacial acetic acid, 50% ethanol and 49% milliQ water) added and agitated for 5 min. The destaining solution was removed and 200 µl of desorbing solution (1M potassium acetate in 70% ethanol) added before reading the 96 well plates using a micro-plate reader (Labsystems, Multiskan EX, USA) at 540 nm. Blank values were subtracted from samples values to give the final absorbance value. Each time point/levels of exposure were represented in four biological replicates (different wells).

5.2.2.2 Neutral red cytotoxicity assay

Neutral red (Sigma, UK) was dissolved in growth medium to a final concentration of 40 µg/ml. The solution was pre-incubated over night at 37 °C and then centrifuged at 1500 g for 10 min to precipitate un-dissolved dye. Pre-exposed cells were washed three times with 200 µl of PBS then 200 µl of NR solution was added. The cells were then incubated 3h at 25 °C. Afterwards NR solution was removed and cells were exposed to fixing solution (1% $CaCl_2$, 0.5% formaldehyde in milliQ water) for 1-2 min followed by two washing steps. The washing solution consisted of 1% acetic acid, 50% ethanol in milliQ water. After the second wash plates were incubated for 10 min and then read using a micro-plate reader (Labsystems, Multiskan EX, USA) at 492 nm. Blanks values were subtracted from samples values to give the final absorbance value. Each time point/levels of exposure were represented in four biological replicates (different wells).

5.2.3 Metal exposure

SAF1 cells were plated in 6 well-plates at a density of 0.5×10^6 cells/well since this resulted in optimal growth after seeding (80-90% confluency after 24 h). 24h after seeding

cells were exposed to 25 μM Cu or 10 μM Cd or 100 μM Zn and samples were taken at 0, 3 and 24 h. Each time point/metal exposure was represented in four biological replicates (different wells). Samples for microarray analysis (6 wells) were taken at 4 h from controls and Cu treated cells (25 μM). Sub toxic levels were calculated using cytotoxicity assays (Figure 5-2) and using previous literature. Denizeau and Marion (1989) set a sub-toxic level for Cu at 20 μM for trout hepatocytes. SAF1 exposed to 25μM Cu after 24 h showed 95 % viability by NR assay. SAF1 exposed to 10 μM Cd for 24 h showed 73% cells viability. This level of exposure was previously used and shown to induce MT (Kling and Olsson 2000). Zinc cytotoxicity was not performed on SAF1 cells. However, previous studies on *epithelioma papulosum cyprini* (EPC) cells grown on the same medium used in this experiment (Lebovitz supplemented with 10% FBS) showed an extremely high EC_{50} (~ 1500 μM $ZnCl_2$) (Muylle et al., 2006). Moreover, 100 μM Zn resulted to have the highest MT promoter induction and therefore was chosen as the level of exposure in this experiment (Mayer et al., 2003).

5.2.4 Striped sea bream (*Lithognathus marmoratus*) liver cDNA microarray

The medium density cDNA microarray was constructed from an enriched liver cDNA library in the laboratory of Dr M. Tom (IOLR, Haifa Israel). The 4608 cDNAs which were used to construct the array were isolated from hepatic mRNA populations of striped sea bream (*Lithognathus marmoratus*) pre-exposed to a series of toxic compounds one of which was cadmium chloride. The 4608 cDNAs represented 1119 distinct contigs whose sequences were submitted to the GEO NCBI database (GPL5351) (Auslander et al., 2008). In addition, all contigs were processed with *BLAST2GO* software (TBLASTX, BLASTX and BLASTN) and 1101 contigs were annotated with a Uniprot ID of which 563 contigs were annotated with GO terms. This analysis was kindly provided by Dr. M.

Tom. The distribution of Gene ontology of this microarray generated importing GO terms into Genespring GX version 7.3.1 (Agilent Technologies) is reported in Figure 5-1.

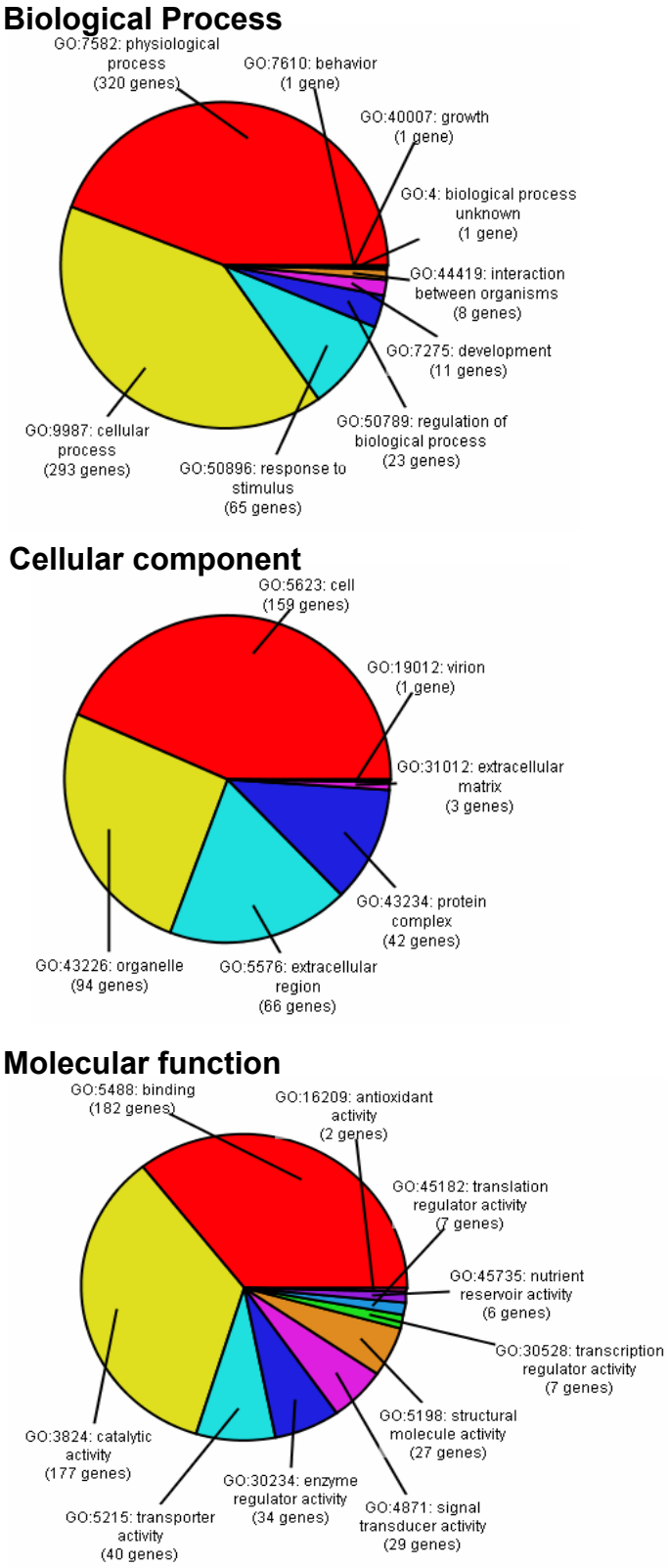


Figure 5-1 *Lithognathus marmoratus* Microarray gene ontology generated by Genespring.

The relative efficiency of cross-species hybridization in bony fish was estimated using a combination of a theoretically calculated hybridization efficiency (using a thermodynamic equation which integrates sequence identity, nucleotide composition and buffer concentration) and experimentally measured efficiency. Striped sea bream and gilthead sea bream were shown to have the highest cross hybridization score of any pair of species (Cohen et al., 2007). The calculated hybridization efficiency between sequences from these two species was 80.5% and demonstrates the validity of using this array as a tool to measure gene expression in *Sparus aurata* (Cohen et al., 2007).

5.2.5 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 the well in 0.5 ml of PBS, transferred into a sterile Eppendorf tube and centrifuged at 3000 *g* for 10 min. Afterwards, the PBS was removed and 0.5 ml of TRI Reagent® RNA extraction buffer (Sigma, UK) per well was added. Cell homogenization was performed by pipetting up and down and vortexing for 5-10 second. From this point on the method of total RNA extraction has been described in 2.2.1. Four biological replicates (different wells) were taken for QPCR. For microarray analysis RNA was extracted from 6 wells then combined to generate 3 pools of RNA each from two wells. RNA quality was checked as described in 2.2.1.1 and cDNA was synthesised as described in 2.2.2 using 1 µg of total RNA.

5.2.5.1 RNA amplification for microarray analysis and labelling

Five hundred nanograms of total RNA from each pooled control and treated RNA sample (see 5.2.7.1) was amplified using the Amino Allyl MessageAmp™ II aRNA Amplification kit (Ambion, UK). This kit amplifies messenger RNA about one hundred

fold. In addition, during the amplification process the modified nucleotide, 5-(3-aminoallyl)-UTP (aaUTP) is incorporated into the aRNA during in vitro transcription (see aRNA amplification kit manual, Ambion). The aaUTP contains a reactive primary amino group on the C5 position of uracil that can be chemically coupled to N-hydroxysuccinimidyl ester-derivatized reactive dyes, such as Cy™3 and Cy™5 dyes. Once purified from unincorporated NTPs, salts, enzymes and inorganic phosphate (using columns provided with the Ambion kit), the aRNA was labelled with Cy™3 (test samples) or Cy™5 dyes (pooled control). Dye solutions were prepared fresh, by resuspending 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 $NaHCO_3$, 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, the labelled aRNA (10 µl total volume) was purified thought a spin column (illustra AutoSeq™ G-50, GE Healthcare, UK). Dye incorporation and labelled aRNA quality was assessed by separating 0.5 µl of the sample on a 1% agarose gel and visualising fluorescent products on a Typhoon scanner (GE Healthcare, UK). Labelled aRNA samples were simultaneously quantified both for aRNA and for dye incorporation by nanodrop spectrophotometry. Samples were stored at -70°C until required.

5.2.6 Quantitative PCR

QPCR of Cu homeostasis and stress/oxidative response genes was performed as described in 3.2.1. Statistical analysis was performed as described in 3.2.2. In addition, to

enable the comparison with the microarray QPCR validation results, significantly changed gene were also analyzed by REST software (Pfaffl et al., 2002).

5.2.6.1 QPCR for Microarray Selected Genes

QPCR validation was performed on cDNA samples from SAF1 cells exposed to the same conditions as samples analyzed by microarray. Chemistry (SYBR green master mix, ABgene, UK) and equipment (Techne Quantica, UK) were the same as used previously. Selected genes that were significantly changed by microarray analysis were determined using the relative expression method. Differently from the absolute quantification method used in Chapter 3 and Chapter 4, the relative expression method is faster and cheaper since the cloning of the genes measured is not required. However, it does not allow the comparison of absolute expression levels, since it only calculates the relative expression of a particular gene in a treated sample compared to the controls. From the gene list of significantly changed genes produced by microarray analysis, 9 genes were selected using both statistical significance (p-values) and fold change ratio. Each primer set for QPCR was designed using PrimerSelect 6.1 (DNASTAR, USA) on striped sea bream (Lm) sequences submitted by Auslander et al. (2008) in to the GEO ncbi database (GPL5351). Each striped sea bream sequence was processed by TBLASTX and BLASTN searching for gilthead sea bream homologous sequences and when available primers where designed on these sequences. Primers were used at 300 nM final concentration. Primer annealing temperatures were optimized by running a temperature gradient PCR using a pool of SAF1 cDNAs. The annealing temperature which gave the strongest single PCR product at the highest annealing temperature was used for QPCR. Optimized annealing temperature of selected genes were 62˚C for cytochrome *c* oxidase (UniProt ID: Q94TF4) and gastrula specific embryonic protein 1 (Q7T0L1), 60˚C for C1q-like adipose

specific protein (Q8JI26) and peroxiredoxin 6 (Q6PBK9), 57˚C for heme-oxygenase (O73688), ankyrin-3 (Q12955), TRAP-α (P45433) and TRAP-γ (Q7ZUR5) and 55˚C for Rac-GTPase activating protein (Q6P3H8). The QPCR cycling program was for all the reactions as follows:

Enzyme activation (Taq) - 15 min 95°C 1 cycle

Denaturation 20 sec 95°C
Annealing 20 sec (as indicated above)
Extension 30 sec 72°C 45 cycles

Dissociation peak 70 - 90°C measuring every 0.5°C

The QPCR quality and identity was verified by dissociation peak analysis, agar gel electrophoresis and sequence analysis. Moreover, QPCR efficiency of each primer set was calculated by Quantica software performing the reaction on a set of serially diluted pooled SAF1 cDNA, previously measured by Nanodrop. Each target gene was analyzed by REST software (Pfaffl et al., 2002) and β-actin and elongation factor 1α (same primers used in Chapter 4) were used as reference genes as determined by most stable genes in control and Cu exposed samples by geNorm analysis (Vandesompele et al., 2002). According to Vandesompele et al., (2002), good reference genes should have M values lower than 1.5, combination of EF1α and β-actin was 1.434 while EF1α and β-actin or GAPDH singularly showed M values of 1.728, 2.031 and 2.326 respectively. Statistically significant differences between control untreated samples and treated samples were evaluated in group means by randomization test using REST software. Five thousand random allocations were applied and differences were considered to be significant at $P \leq 0.05$.

Table 5-1 Primers used for microarray validation.

ID	*Sequence 5`→3`*	*lenght*	*Tm*	*GC%*
saCytc-F	ttcgctattatggctggctttgtc	24	61.0	45.8
saCytc-R	agggtgtatgcatcggggtagtc	23	64.2	56.5
saGast-F	gctgagaaggccaagactgc	20	61.4	60
saGast-R	agaggtggacgtgaaggtaagagg	24	64.4	54.2
lmC1q-F1	cacgcaattgagggagcaggtttc	24	64.4	54.2
lmC1q-R1	gcggcgacagaggcagagatttat	24	64.4	54.2
lm-Perox-F1	gtggcggggtagaggatggaca	22	65.8	63.6
lm-Perox-R1	tcagtagtgcggccgacaaggat	23	64.2	56.5
lm-Ank3-F	cagaggtggaggtgagggtaaga	23	64.2	56.5
lm-Ank3-R	gtgagacggaaaacagagacaaag	24	61.0	45.8
lm-racGTP-F	caaaaacaaagggtgggtctg	21	57.9	47.6
lm-racGTP-R	gctccgctgctgctaagtt	19	58.8	57.9
lm-HemeOx-F	tggaggaagagctrgacaggaac	23	62.0	52.2
lm-HemeOx-R	gctgttcatcckgctgckrt	20	59.4	55.0
lm-Trap-α-F	aattgctgctgcttttcctg	20	55.2	45.0
lm-Trap-α-R	atcggcgtcatcttcgtctcct	22	62.0	54.5
lm-Trap-γ-F	gaggcgacgatgacgagaac	20	61.4	60.0
lm-Trap-γ-R	cccgcaaggagaaagacgag	20	61.4	60.0

Tm= 69.3+0.41*GC%-(650/length).

5.2.7 Microarray hybridization and analysis

5.2.7.1 Experimental design

Microarray analysis was performed on RNA from SAF1 cells after exposure to 25 µM Cu for 4 hours. Three replicate controls and 3 replicate treated samples were each co-hybridized with a pooled reference sample. The pooled reference sample was composed of equal amounts of total RNA from each one of the 6 samples. This reference sample was re-quantified and quality assessed (Nanodrop and agarose gel separation). The experiment was replicated with a dye-swap procedure which first labelled samples with Cy5 and reference with Cy3 and then samples with Cy3 and reference with Cy5 minimizing scanning artefacts and differences in dye-binding.

5.2.7.2 Hybridization protocol and analysis

All 12 microarray hybridisations were carried out at the same time. A pre-hybridisation procedure was followed to block / minimise non-specific hybridisation. For this the 12 slides were loaded into a EasyDip™ Slide Staining system (Canemco, Canada) ensuring that the label was at the bottom of the holder. Slides were briefly washed in milliQ water three times for 30 seconds, then were immediately transferred into pre-hybridization solution (5 × SSC, 0.2% SDS and 1.5% BSA, Sigma, UK) and incubated at 50˚C for 2 hours. After pre-hybridisation slides were immediately washed in warm filtered water for 30 seconds (× 3). Then slides were dried (while in a dry EasyDip container) by centrifugation (500 × g for 5 min) and finally loaded onto the Lucidea Semi-automatic hybridisation system (GE Healthcare, UK).

Appropriate Cy3 and Cy5 labelled samples which consisted of the entire labelled aRNA eluted from the illustra AutoSeq™ G-50 spin-column purification step (about 7-9 µl, 150-250 ng cDNA, 16-30 pmol of each dye-labelled sample) 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 that was already equilibrated at 60˚C. The hybridization buffer consisted of 170 µl 0.5 × Ultrahyb solution (i.e. UltraHyb solution, Ambion, UK; mixed with an equal volume of 4× SSC, pH 7.0) together with 20 µl poly(A) (10 mg/ml, Sigma, UK) and 10 µl herring sperm DNA (10 mg/ml, Sigma, UK). Following addition of the labelled target aRNA, samples were kept at 60°C in the dark and loaded (c. 180 µl) onto the Lucidea system as soon as practical. The 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. Then the slides were unloaded from the Lucidea hybridiser into the EasyDip™ Slide Staining

system and 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 the vast majority of cDNA spots were 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 transformation and normalisation procedures being implemented. 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: 1) all linear intensity values less than 0.01 were set to 0.01, to remove any potential negative values; 2) appropriate dye swap transformation was made to correctly assign signal and control channels 3) a "per spot, divide by control channel" transformation applied and finally 4) a per chip normalization to positive control genes was performed. The latter was undertaken as the array was constructed from clones derived from an SSH cDNA library which is expected to be biased towards genes differentially expressed in pollutant exposed fish and the chip contained a relatively small number of contigs (1119). Therefore, on the basis of geNorm analysis of QPCR results of SAF1 controls and Cu exposed, EF1α and β-actin were used as control genes (reference genes) since they showed the most stable average expression level (M). 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. This gave a final list of 3810 genes which were available for statistical analysis. ANOVA (t-test) was performed between controls and Cu exposed groups and statistical significance was set to $p < 0.05$. Gene enrichment analysis was performed using the GO ontology browser tool which calculated, using a hyper-geometric distribution model, the probability that a particular biological process or cellular component term was enriched in the input GO list (all GO annotated genes) compared with the output GO list (all GO annotations of the genes altered by Cu). In addition, to be able to use the UniProt annotation, and therefore perform a more comprehensive analysis, input (all UniProt ID) and output (UniProt ID altered by Cu) were analyzed using DAVID 2008 functional annotation tool (Dennis et al., 2003). In DAVID annotation system, the Fisher Exact statistic is adopted to measure the gene-enrichment of the output list compared to the input list of annotated terms. Gene annotation information of striped sea bream microarray is described in (Auslander et al., 2008).

5.3 Results

5.3.1 Cytotoxicity assays

The cytotoxicity assays results are shown in Figure 5-2. The NR assay was a more sensitive assay showing toxic effects at lower metal concentrations. SAF1 cells started showing toxic effects after 24 h of exposure at 100 μM Cu, as cell viability dropped to 80% (NR) and total protein to 89% (KB). Thus 24 h exposure to 25 μM Cu was chosen as 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 as 24 h of exposure to 10 μM Cd, the smallest Cd concentration tested, showed only 73% cell

viability (NR). On the other hand, kenacid blue assay showed a different toxicity profile for Cd exposure with levels of total proteins higher than cell viability levels. 48 h Cu exposure showed a similar cytotoxicity profile to 24 h Cu. 48 h of exposure to Cd resulted in high toxicity even at the lowest concentration (10 μM) showing a cell viability of 19%.

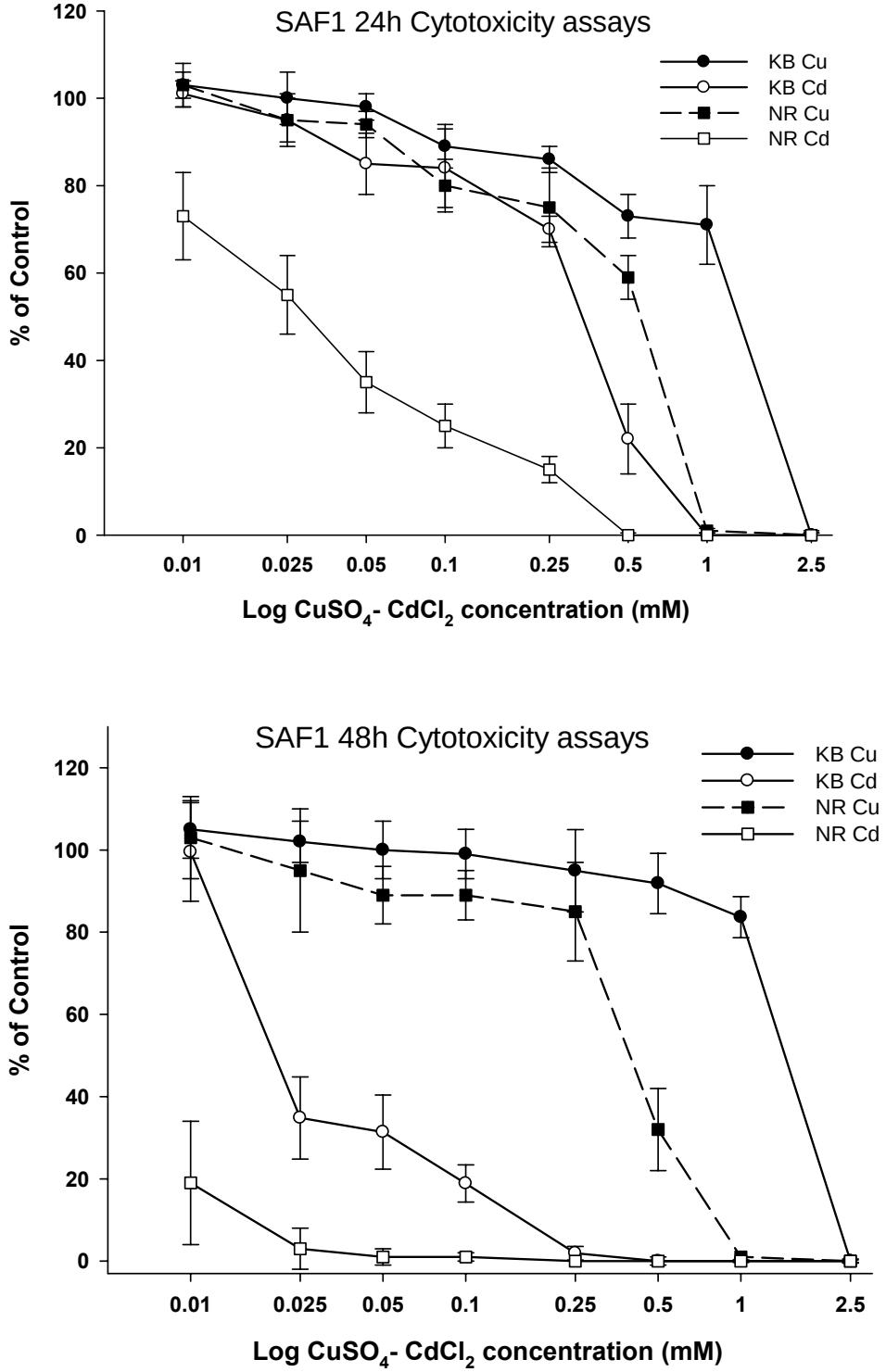


Figure 5-2 Cytotoxicity of $CuSO_4$ and $CdCl_2$ in L15 medium. SAF1 cells were exposed for 24h and 48h to a range of $CuSO_4$ and $CdCl_2$ concentrations (0.01-2.5 mM). Viability was assessed by NR uptake and total protein by KB staining. Y-axis shows % absorbance at 540 nm for KB and 492 nm for NR compared to controls (0 mM condition). Each data point represents the mean ± S.D. (n= 4).

5.3.2 Effect of Cu, Zn and Cd exposure on SAF1 gene expression

Excluding ATP7B, whose level of expression was close to detection limits all other genes measured were expressed at levels similar to those observed in sea bream tissues. Figure 5-3 reports gene expression levels expressed as absolute copy number per ng of input total RNA and can be compared with tissue expression profiles shown in chapter 3.

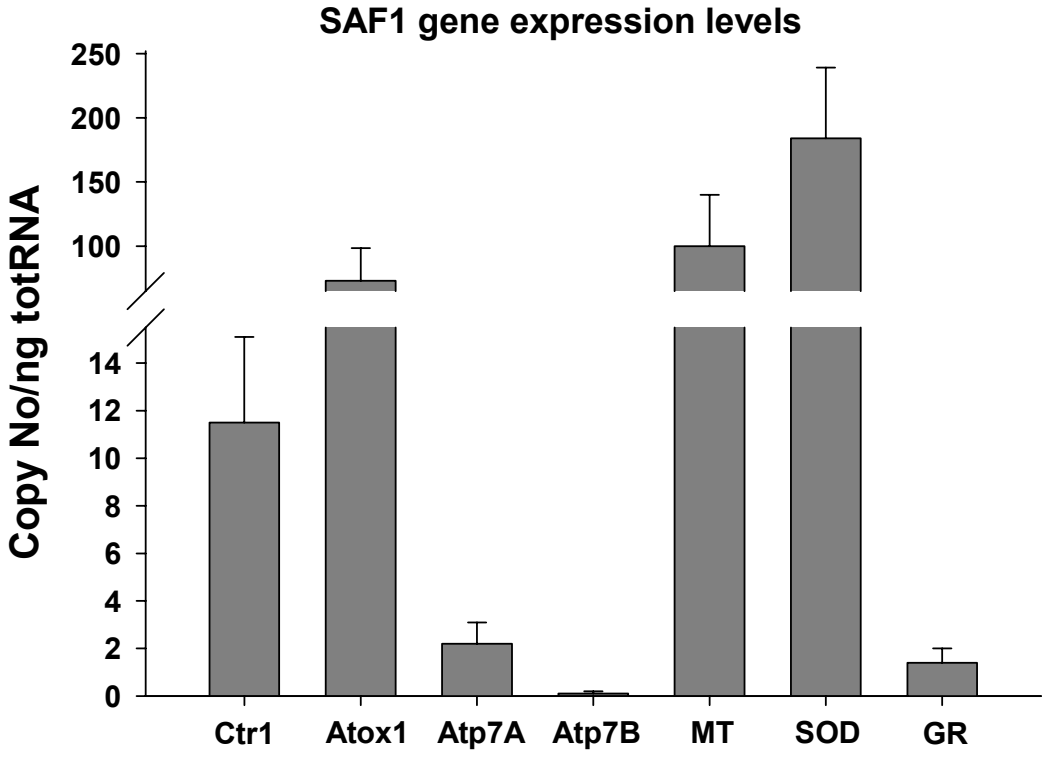


Figure 5-3 Gene expression levels in control SAF1 cells. Values are mean ± SD n = 4.

Figure 5-4 shows the normalized transcription levels of these selected genes in SAF1 cells exposed to 25 μM Cu, 100 μM Zn and 10 μM Cd.

After 3 hours of exposure to metals mRNA levels of none of Cu transporters (Ctr1, ATP7A and Atox1) changed when compared to controls in any of the metal-treated SAF1 cells. On the other hand, 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 3h showed increases in levels of GR of 2.6 and 5.5 fold respectively. Following 24h exposure, ATP7A was induced (3.1 fold) only by Cu whilst Ctr1 and Atox1 mRNA levels were not affected by Cu, Zn or Cd exposure. All heavy metal treated cells showed an induction of MT mRNA between 15 and 300 fold, with Cd exposed cells showing the highest MT levels and Cu the lowest. Moreover, in Cu and Cd exposed cells the marker of oxidative stress GR was induced. CuZn/SOD mRNA levels were unaffected by these levels of Cu, Zn or Cd in SAF1 cells.

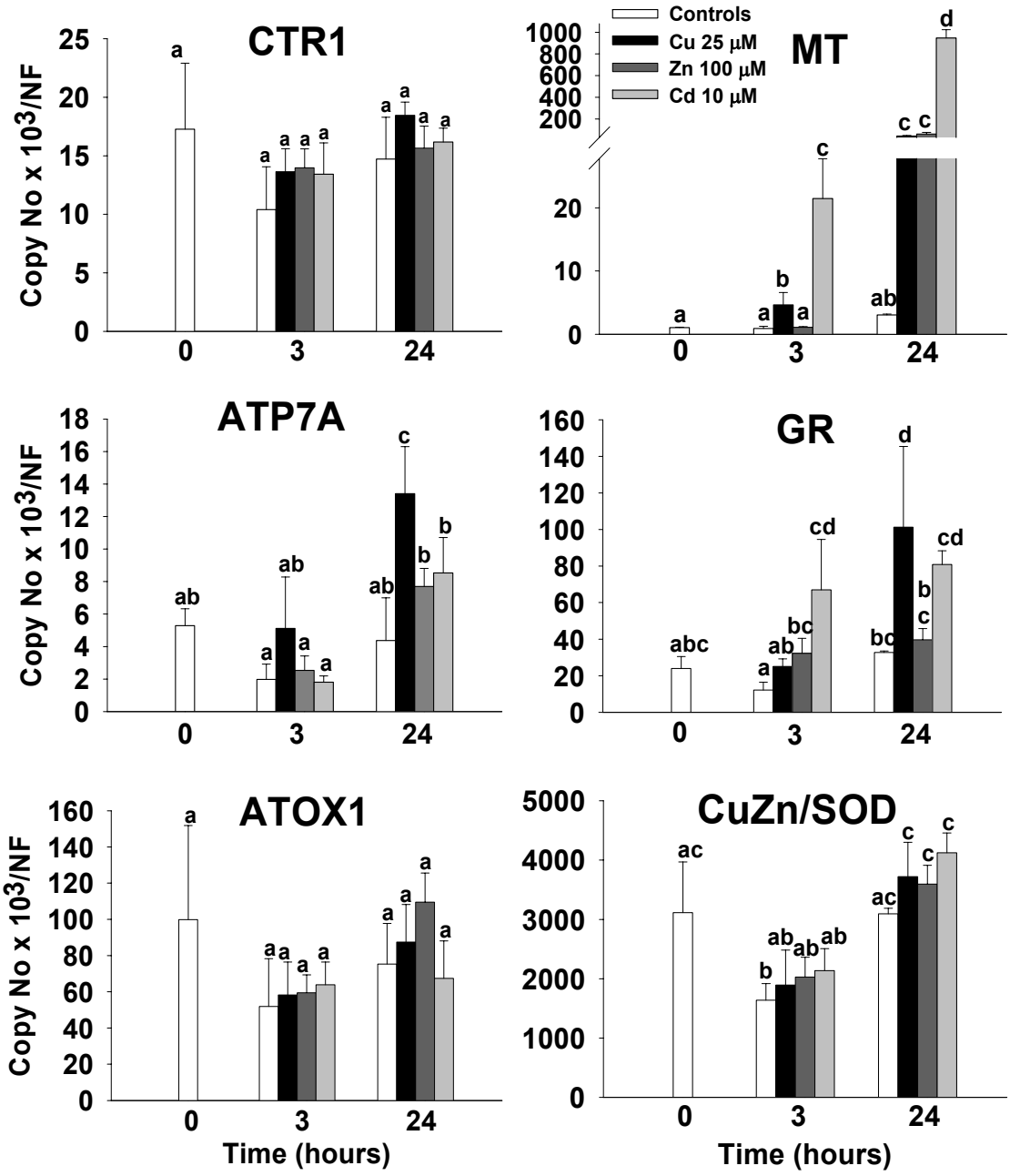


Figure 5-4 SAF1 transcriptional response to 25 µM Cu, 100 µM Zn and 10 µM Cd. Gene expression measured by QPCR of Cu transporter genes Ctr1, ATP7A and Atox1 and MT and oxidative stress genes GR and CuZn/SOD. Ctr1, ATP7A, ATP7B, MT, GR and CuZn-SOD copy numbers were normalized by geNorm using a normalization factor (NF) based on the geometric mean of β-actin, EF1α reference genes. Values are means ± S.D. n = 4. Bars bearing different lettering are significantly different ($P<0.05$; ANOVA, Tukeys test).

5.3.3 Microarray analysis and QPCR validation

Following statistical analysis, performed in Genespring (ANOVA t-test, $p<0.05$) (5.2.7.2) the expression of 235 clones (117 sequenced, unique genes, sequence contigs) were scored as changed by Cu exposure. Of these unique clones 116 were provided with UniProt IDs, of which 62 included GO annotations. Thus, using the GO ontology browser tool in Genespring, it was possible to determine which pathways were likely to be changed based on genes with overlapping ontologies (Table 5-2). In addition, to enable the use of UniProt IDs and therefore a more comprehensive analysis, DAVID functional annotation analysis was performed and the results are reported in Figure 5-5. DAVID enables Uniprot IDs from diverse organisms to be associated with GO terms, often resulting in a greater proportion of genes annotated. GO ontology analysis, showed that all terms enriched, under biological process category, were physiological processes (GO:7582), daughter terms were: 1) secretion and protein secretion, 2) protein localization and establishment of protein localization, 3) intracellular protein transport and protein targeting. Enrichments in all of these physiological processes were attributable to changes in 3 genes which are indicated. In addition, those genes which were not automatically grouped into GO categories were further considered manually to assign a possible gene ontology term. This supervised analysis of the genes whose expression was significantly changed by Cu, and following integration with the results of DAVID functional annotation analysis, showed that other genes involved in secretion and protein trafficking and cytoskeleton reorganization were present (Figure 5-5; Table 5-3). This manual analysis has also highlighted the presence of a group of stress related genes. Terms under cell component category were under Cell (GO:5623) or Organelle (GO:43226). Cell daughter terms indicate that mainly membrane proteins were involved in response to Cu,

specifically in the vesicular fraction and microsomes. Similarly organelle daughter terms were membrane bound proteins (GO terms are indicated in Table 5-2).

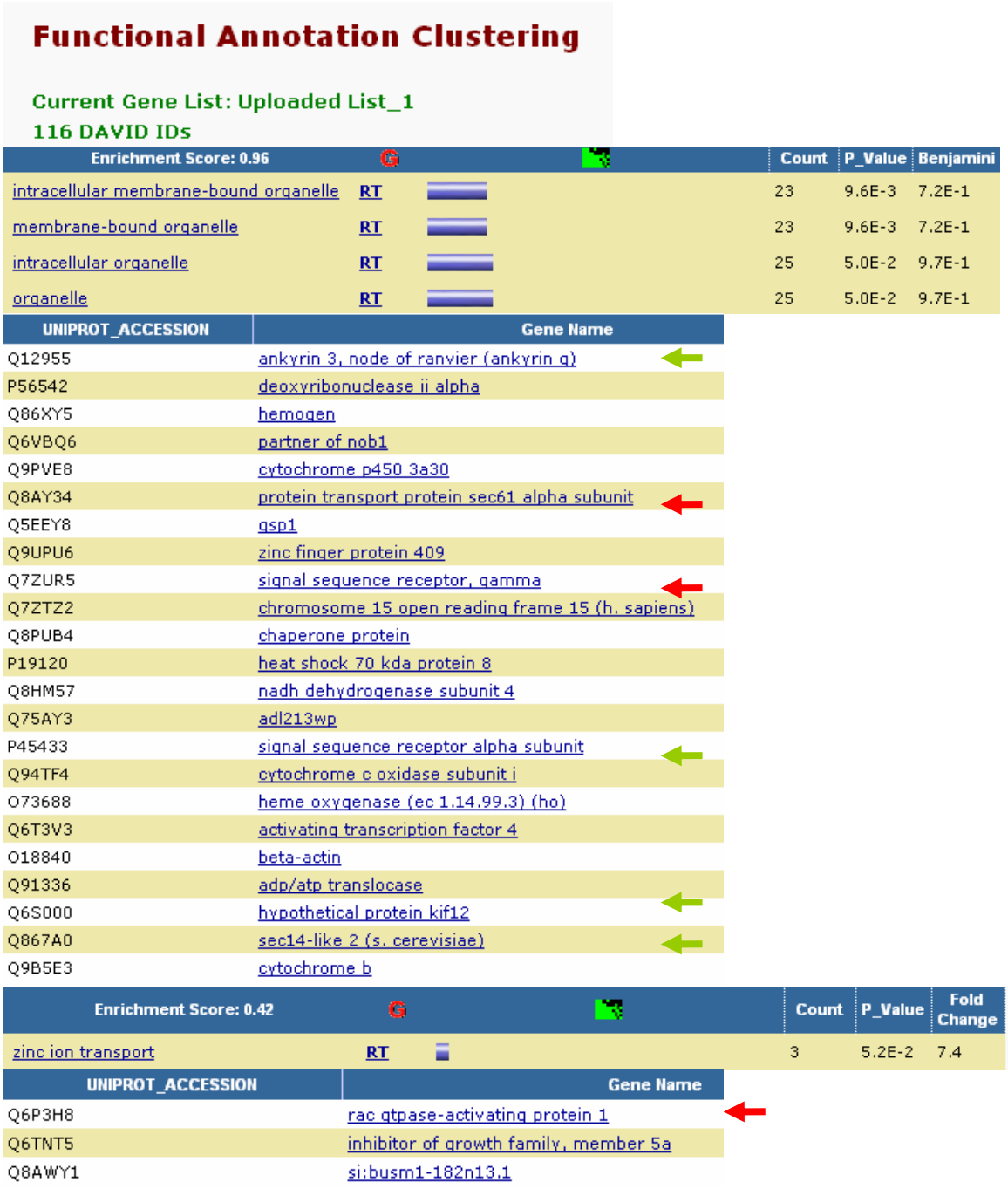
Selected genes which were changed according to microarray analysis were also measured by QPCR (Table 5-3). With the exception of cytochrome *c* oxidase and peroxiredoxin 6, whose expression was opposite to the one measured by microarray, all other genes measured by QPCR showed similar expression to the microarray.

Table 5-2 Gene ontology analysis (Genespring).

GO Biological Process Category	***Genes with GO term in input list***	***Genes with GO term in output list***	***Uniprot ID, BLASTX annotation***	***Enrichment p-Value***
GO:46903: secretion	5	3	(Q6P3H8) Rac-GTPase activating protein 1 (Q8AY34) Sec 61 α sub (Q7ZUR5) TRAP γ	0.0013
GO:9306: protein secretion	2	2	Q6P3H8 Q8AY34	0.0028
GO:8104: protein localization	14	3	Q6P3H8 Q8AY34 Q7ZUR5	0.0346
GO:45184: establishment of protein localization	14	3	Q6P3H8 Q8AY34 Q7ZUR5	0.0346
GO:6886: intracellular protein transport	7	2	Q8AY34 Q7ZUR5	0.0504
GO:6605: protein targeting	5	2	Q8AY34 Q7ZUR5	0.0257
GO Cellular component Category				
GO:5624: membrane fraction	14	3	(O73688) Heme oxygenase (Q9PVE8) Cytochrome P450 3A30 Q7ZUR5	0.0285
GO:42598: vesicular fraction	11	3	O73688 Q9PVE8 Q7ZUR5	0.0142
GO:5792: microsome	11	3	O73688 Q9PVE8 Q7ZUR5	0.0142
GO:5783:endoplasmic reticulum	26	4	O73688 Q9PVE8 Q7ZUR5 Q8AY34	0.034

GO:43226: organelle	109	10	(P56542) Deoxyribonuclease II alpha (acid DNase) (Q8JHX9) Glutamate dehydrogenase 3 O73688 Q9PVE8 (O18840) β-actin Q8AY34 Q7ZUR5 (Q5RKQ3) Zgc:101598 (Q7ZTZ2) Probable ribosome biogenesis protein RLP24 (Q8HM57) NADH-ubiquinone oxidoreductase chain 4	0.0132
GO:43227:membrane-bound organelle	84	8	P56542 Q8JHX9 Q9PVE8 O73688 Q8AY34 Q7ZUR5 Q5RKQ3 Q8HM57	0.0317
GO:43231:intracellular membrane-bound organelle	84	8	P56542 Q8JHX9 Q9PVE8 O73688 Q8AY34 Q7ZUR5 Q5RKQ3 Q8HM57	0.0317

The gene annotation information attributed by BLASTX analysis of submitted sequences is reported only once and then identical genes are highlighted in the same colour.



Functional Annotation Clustering

Current Gene List: Uploaded List_1
116 DAVID IDs

Enrichment Score: 0.96		Count	P_Value	Benjamini
intracellular membrane-bound organelle	RT	23	9.6E-3	7.2E-1
membrane-bound organelle	RT	23	9.6E-3	7.2E-1
intracellular organelle	RT	25	5.0E-2	9.7E-1
organelle	RT	25	5.0E-2	9.7E-1

UNIPROT_ACCESSION	Gene Name
Q12955	ankyrin 3, node of ranvier (ankyrin g)
P56542	deoxyribonuclease ii alpha
Q86XY5	hemogen
Q6VBQ6	partner of nob1
Q9PVE8	cytochrome p450 3a30
Q8AY34	protein transport protein sec61 alpha subunit
Q5EEY8	gsp1
Q9UPU6	zinc finger protein 409
Q7ZUR5	signal sequence receptor, gamma
Q7ZTZ2	chromosome 15 open reading frame 15 (h. sapiens)
Q8PUB4	chaperone protein
P19120	heat shock 70 kda protein 8
Q8HM57	nadh dehydrogenase subunit 4
Q75AY3	adl213wp
P45433	signal sequence receptor alpha subunit
Q94TF4	cytochrome c oxidase subunit i
O73688	heme oxygenase (ec 1.14.99.3) (ho)
Q6T3V3	activating transcription factor 4
O18840	beta-actin
Q91336	adp/atp translocase
Q6S000	hypothetical protein kif12
Q867A0	sec14-like 2 (s. cerevisiae)
Q9B5E3	cytochrome b

Enrichment Score: 0.42		Count	P_Value	Fold Change
zinc ion transport	RT	3	5.2E-2	7.4

UNIPROT_ACCESSION	Gene Name
Q6P3H8	rac gtpase-activating protein 1
Q6TNT5	inhibitor of growth family, member 5a
Q8AWY1	si:busm1-182n13.1

Figure 5-5 DAVID functional annotation analysis. Significantly enriched terms are indicated followed by gene names. Genes represented in Genespring GO ontology analysis (red arrow) and those found after manual analysis (green arrow) which are involved in secretion and protein trafficking are indicated.

Table 5-3 Supervised analysis of the output list integrated with QPCR results

Process	*Uniprot ID + Annotations (BlastX)*	*Microarray*		*QPCR*		*% of total clones present on Array*
		p-value	**Fold Change**	**p-value**	**Fold Change**	
Stress	(Q8UVY1) Metallothionein (MT)	0.0003	1.441	0.031	4.8	25% (1/4)
	(Q6USB8) Glutathione S-transferase	0.0071	0.629	-	-	100% (1/1)
	(Q6PBK9) Peroxiredoxin 6 (Novel protein)	0.0133	0.629	0.049	**6.4**	50% (1/2)
	(O73688) Heme oxygenase	0.0049	1.46	0.042	2.7	25% (1/4)
	(Q9PVE8) Cytochrome P450 3A30	0.0056	1.332	-	-	100% (1/1)
	(P19120) Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	0.0124	1.257	-	-	100% (1/1)
	(Q75UL8) Warm-temperature-acclimation-65kDa-protein-like	0.0396	1.269	-	-	9% (3/32)
	(Q8PUB4) Chaperone protein	0.0414	1.396	-	-	100% (1/1)
Protein trafficking and Secretion	(P45433) Translocon-associated protein alpha subunit precursor (TRAP-alpha) (Signal sequence receptor alpha subunit) (SSR-alpha)	0.00006	1.331	0.032	2.1	100% (1/1)
	(Q7ZUR5) Signal sequence receptor, gamma (Translocon-associated protein gamma)	0.00212	1.452	0.057	2.1	100% (1/1)
	(Q8AY34) Protein transport protein Sec61 alpha subunit	0.0036	0.537	-	-	50% (1/2)
	(Q867A0) Sec14 like- Liver tocopherol-associated protein	0.0167	1.207			100% (1/1)
Cytoskeleton organization	(Q6P3H8) RacGTPase activating protein1	0.0161	1.251	0.032	2.2	67% (2/3)
	(Q12955) Ankyrin-3 (ANK-3) (AnkyrinG)	0.01	1.313	0.420	1.6	50% (1/2)
	(O18977) Tenascin-X	0.0023	1.468	-	-	100% (1/1)
	(Q58XP5) Fibronectin 1b	0.0024	1.168	-	-	33% (1/3)
	(Q6S000) Kinesin family member 12 (Hypothetical protein kif12)	0.04	1.404	-	-	33% (1/3)
	(O18840) β-actin cytoplasmic 1	0.0271	0.761	0.670	1.1	50% (1/2)
Apoptosis	(P56542) Deoxyribonuclease II alpha precursor (DNase II alpha) (Acid DNase) (Lysosomal DNase II)	0.0278	1.315	-	-	20% (3/15)
Others	(Q8JI26) C1q-like adipose specific protein	0.0122	0.584	0.066	0.15	24% (14/59)
	(Q7T0L1) Gastrula specific embryonic protein 1	0.0196	1.477	0.042	5.1	14% (9/65)
	(Q94TF4) Cytochrome c oxidase subI	0.0414	0.804	0.031	**5.4**	11% (7/66)
	(Q6Y243) Elongation Factor 1 alpha	-	-	0.742	0.9	0% (2/2)
	ATP7A 3h	-	-	0.082	2.4	-
	ATP7A 24h	-	-	0.039	2.6	-

Shaded genes were not provided with GO annotations. QPCR fold change values and p-values (n = 4; $p<0.05$) were calculated by REST software. Disagreements in fold changes or p-values between QPCR and microarray analysis are indicated in red. Excluding ATP7A that has been analysed at 3 and 24 h all other QPCR results are relative to 3h exposure.

5.4 Discussion

The results reported here show that SAF1 cells are a suitable *in vitro* system for Cu homeostasis studies in fish. For instance, with the exclusion of ATP7B, the transcript levels of Cu transporters in control SAF1 cells were similar to the transcript in tissues isolated from fish (Figure 5-3). In addition, an element of the Cu homeostatic machinery, ATP7A showed an increase in its mRNA levels after Cu exposure similar to that observed in some tissues after waterborne exposure *in vivo*. This suggests that active Cu homeostasis machinery is present in this established cell line.

In sea bream exposed to excess waterborne Cu, the transcriptional response of tissue Cu transporters was distinct to that of sea bream exposed to excess dietary Cu, suggesting different homeostatic response mechanisms. Moreover, waterborne Cu resulted in the induction of hepatic MT and GR, genes which respond to metal toxicity and oxidative stress. SAF1 cells exposed to Cu sulphate dissolved in the growing media showed a very similar transcriptional response (induction of ATP7A, MT and GR) to hepatic tissue of fish exposed to waterborne Cu. The exception was ATP7B. As mentioned before, ATP7B was expressed at negligible levels in SAF1 cells. Conversely, in sea bream liver ATP7B was expressed and regulated by excess Cu (Figure 4.1, Figure 5-4). Indeed ATP7B in mammals and sea bream is mainly expressed in the liver, intestine, brain and kidney (Kuo et al., 1997). As SAF1 is a fibroblast-like cell line and since fibroblasts are derived from connective tissue, the low level of ATP7B expression in SAF1 cells is in line with the characteristics of this cellular type. This result suggests that Cu sulphate dissolved into the growing media, which contained 10% foetal bovine serum, is presented to the SAF1 cell, bound not specifically to ligands such as free histidines and serum albumin and a similar scenario may happen when Cu is presented to the hepatic cell in fish

exposed to waterborne Cu. In addition, induction of ATP7A in SAF1 exposed to Cu but not after exposure to Cd or Zn, implies that this gene is responding specifically to Cu. In contrast, MT was induced by all metals and GR was induced by Cu and Cd. Overall these results - specific induction of ATP7A by Cu and aspecific induction of MT - suggest that there may be a specific intracellular Cu sensor which mediates ATP7A transcription.

The mechanism of transcriptional regulation of Cu homeostasis genes in vertebrates is still not clear. The metal transcription factor-1 (MTF1) is the main metal sensing transcription factor in eukaryotic cells (Selvaraj et al., 2005; Andrews 2000). On the other hand, Zn homeostasis has been shown to be regulated by MTF1 which binding to *cis*-acting elements (MREs) induces MT and Zn transporter 1 (ZnT-1), a specific Zn transporter which mediates its excretion in response to Zn toxicity in mammals (Palmiter 2004). A similar situation has recently been confirmed in fish (Balesaria and Hogstrand 2006). MT is also induced by other heavy metals including Cu and cadmium, however their mechanism of induction is still not clear (Andrews 2000; Mayer et al., 2003) although it is hypothesised that it is by displacement of Zn from MTF-1. Moreover, Mayer et al. (2003) have shown that MT is induced by silver through a mechanism other than Zn displacement from Zn binding proteins, indicating that silver may be a direct inducer of MT. Interestingly, in the promoter areas of Ctr1, ATP7A and ATP7B putative MREs have been found (Mackenzie et al., 2004; Harris et al., 2003; Oh et al., 1999; Oh et al., 2002) suggesting that intracellular Cu homeostasis like Zn homeostasis could be mediated by MTF1. Specific Cu sensors are known in yeast (van Bakel et al., 2005) and recent studies have shown that in mammals the regulation of genes involved in Cu homeostasis, such as MT and the prion gene (PRNP) require the interaction of MTF1 with another transcription factor, SP1 (Li et al., 2008; Bellingham et al., 2008). These transcription factors bind MREs, located in the promoter area, and modulate gene expression. More importantly,

SP1 has also been demonstrated to specifically regulate the mRNA levels of hCTR1 mRNA in response to intracellular Cu, being induced by depletion and repressed by repletion of Cu, and also in response to intracellular hCtr1 mRNA, suggesting a negative feed-back mechanism of regulation (Song et al., 2008). The mechanism by which SP1 senses Cu is dependent on zinc finger motifs, and the down-regulation of hCTR1 expression is suggested to be the cause of "poisoning" of SP1 by excess Cu, which disrupts Zn-finger structure. Conversely, Cu depletion would restore normal SP1 structure, allowing binding of Zn and Ctr1 expression would be enhanced enabling Cu uptake. In our experiment, Ctr1 was not affected by Cu, Cd or Zn. However, only one Cu concentration (25 μM) was tested while in the experiment of Song and colleagues, cells were exposed to concentrations up to 4 times higher. Exposure of cells to a wider range of Cu concentration and to Cu chelators would be necessary to clearly compare these studies and show if, in fish, a similar regulation of Ctr1 exists. Interestingly, in fish intestine exposed to excess dietary Cu, Ctr1 mRNA levels were reduced compared to controls (Figure 4-1A).

Transcriptomic analysis of SAF1 cells exposed to excess Cu (25 μM for 4 hours) indicated that the pathways most significantly affected were secretion, protein trafficking and cytoskeleton reorganization (Table 5-2; Table 5-3). Previous studies suggested cytoskeleton reorganization as a mechanism of response to Cu overload in both mammals and fish (Armendariz et al., 2004; Walker et al., 2008). ATP7A regulates intracellular Cu concentration by translocating from a compartment localized within the *trans*-Golgi network to a compartment near the plasma membrane in response to Cu (Nyasae et al., 2007). Small GTPases are small G proteins (20-25kD) that bind guanosine triphosphate (GTP). Small GTPase activity is regulated by guanine nucleotide exchange factors, GTPase activating proteins and guanine nucleotide inhibitors which accelerate or inhibit

GTP hydrolysis (Sirokmany et al., 2006). The RAS superfamily of small GTPases is divided in subfamilies, Ras, Rho, Rab Rap, Arf, Ran, Rheb and Rad which are involved in a complex and diverse range of cellular processes including cellular signalling, trafficking and fusion of endocytic vesicles at different cellular compartments (Wennerberg et al., 2005). Importantly, recent studies have shown the importance of two small GTPases, Rab7-5 and Arf1 for ATP7A trafficking (Pascale et al., 2003; Holloway et al., 2007). An activating protein of a sub-member of the Rho subfamily, the RacGTPase activating protein 1 (Rac1GAP Uniprot ID Q6P3H8), was induced by Cu in SAF1 cells. Interestingly another member of the Rho subfamily, RhoA UniProt ID P61589 was induced by Cu in an *in-vitro* model of rainbow trout gill (Walker et al., 2008). Rho GTPases are involved in different cytoskeleton reorganization mechanisms including endocytosis and exocytosis (Ridley 2001). Moreover, in a recent study using an Ab-microarray, protein expression in human fibroblasts over expressing ATP7A or with ATP7A knock-out was compared. ATP7A over-expression resulted in Cu deficiency and knockout resulted in Cu accumulation. Remarkably, the expression of two small GTPase activating proteins Rap1GAP (uniprot ID P47736) and RasGAP (Uniprot ID P20936) were among the most significantly changed (Du et al., 2008) (both induced by Cu excess similarly to Rac1GAP in SAF1). Rap and Ras small GTPases are involved in the regulation of different signalling pathways which include vesicular transport and cytoskeleton reorganization (Wennerberg et al., 2005). The shared outcome of our study with other genomic (Walker et al., 2008) and proteomic (Du et al., 2008) investigations puts a particular focus on this family of proteins' importance in the regulation of Cu homeostasis. However, even though these small GTPases have been shown to be involved in vesicular trafficking, their relation with Cu homeostasis has not been demonstrated and further study is required to better understand their involvement. Nevertheless, QPCR assays showed increases in ATP7A,

MT, oxidative stress markers, RacGAP1 and in pathways of protein trafficking and cytoskeleton reorganization which might indicate changes in vesicular Cu transport and secretion as major responses to Cu excess.

In summary, this transcriptomic approach enabled the exploration of other aspects of the intracellular response to Cu excess with less bias than using an approach based on candidate genes or pathways. Using an available cross-species cDNA microarray is advantageous for examining multi-gene expression patterns in non-model organisms, saving the need for construction of species-specific arrays. The striped sea bream microarray has been shown to be an informative tool also on cross species such as sea bream. However this array has also some drawbacks that should be mentioned such as the limited number of cDNAs spotted which only enabled the response of a small percentage of the transcriptome, and that for some genes, the QPCR validation was not possible because the primers designed on striped sea bream sequences did not perform PCR with sufficient efficiency (data not shown). Nevertheless, the high percentage of gene annotations (1101 UniProt annotation and 563 GO annotations on 1119 unique clones) enabled a satisfactory functional annotation analysis (by DAVID and Genespring).

Chapter 6. General discussion

6.1 Meeting the objectives

1. In this study, for the first time, Cu transporter genes for cellular uptake (Ctr1), intracellular distribution (Atox1), delivery to cuproenzyme and excretion (ATP7A and ATP7B) and Cu storage and heavy metal detoxification (MT) were cloned from a fish species, sea bream. Moreover biomarkers of oxidative stress under Cu exposure, GR and CuZn-SOD cDNAs were also cloned.
2. Based on the cDNA sequences obtained, probes were designed and quantitative real time PCR assays developed for each gene. Tissue expression profiles were measured for each gene.
3. The analysis of Cu homeostasis and oxidative stress gene expression and Cu and Zn tissue levels in sea bream exposed to dietary or waterborne Cu showed for the first time that fish have different regulatory machanisms dependent upon route of exposure. Transcriptional regulation may be important.
4. Excluding ATP7B all other Cu homeostasis genes were expressed in SAF1 cells and ATP7A was induced by Cu showing that they are a suitable and informative system for Cu homeostasis studies. Moreover the microarray analysis showed that secretion, cytoskeleton reorganization and protein trafficking were the biological processes affected by excess Cu in SAF1 cells.

6.2 Structure of copper homeostasis genes

In the present work, homologous cDNAs to characterized mammalian Cu transporters (Ctr1, Atox1, ATP7A and ATP7B) have been cloned in a commercially important fish species, gilthead sea bream. Searches for homologous genes in the genomes of other fish (*Danio rerio, Tetraodon negroviridis, Takifugu rubripes, Oryzias latipes, Gasterosteus aculeatus*) and lower vertebrate species (*Xenopus tropicalis* and *Gallus gallus*) (using www.ensebl.org), have shown high levels of protein conservation especially in specific structural domains (Chapter 2). The precise mechanism of how Cu is "handled" and transferred between proteins and across the membranes is not completely understood, however the high level of conservation of specific domains throughout evolution highlights their importance. Furthermore, mutation analyses of these proteins from mammals have shown that some amino acids are essential for function. These critical amino acids were conserved in all vertebrate sequences analyzed including sea bream (see 2.1.4; 2.3).

The occurrence of two rounds of whole genome duplication (WGD) is thought to have influenced vertebrate evolution and have driven their evolutionary success (Dehal and Boore 2005). Moreover, in fish, a third WGD occurred, giving rise to some duplicated genes which exist only in fish (Meyer and van de Peer 2005). Following WGD, duplicated genes can be retained or lost in a lineage dependent manner, and a small percentage of the duplicated genes can undergo sub-functionalization or neo-functionalization (Brunet et al., 2006) to generate new phenotypes on which natural selection can act. Cu-ATPases provide an interesting example of these processes. Yeast (*S. cerevisiae*), arthropods (*D. melanogaster*) and chordates (urochordata - *C. intestinalis*) have only one isoform of Cu-ATPase whilst higher vertebrates, including fish possess two isoforms of Cu-ATPases,

ATP7A and ATP7B. Therefore, ATP7A and ATP7B are likely to have arisen from a single ancestral Cu-ATPase following an early WGD, and subsequently sub- or neo-funtionalized (see 1.5.4). Since none of the fish species with sequenced genomes have more than two ATP7 genes, it would appear that, early in their evolution, fish lost a further two ATP7 genes arising from the fish specific WGD.

Notably, the duplication of ATP7 occurred in the vertebrate lineage coincident with the evolution of the hepatic system. 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). In contrast vertebrates excrete excess Cu through the hepato-biliary system through the action of ATP7B, and indeed this is known to be the main function of ATP7B in mammals. Thus the duplication, and subsequent neofunctionalization of ATP7 genes may be an example of an evolutionary advance in Cu homeostasis brought about initially by WGD.

Such evolutionary elaboration of Cu-homeostasis might also be evident from consideration of the number of metal binding domains (MBDs) in various ATP7 enzymes. The number of MBDs increases from yeast, where there are two MBDs, to *Drosophila*, with four, the sea squirt, which has 5, and then mammals which have six MBDs in both ATP7A and ATP7B. Similarly to mammals in sea bream ATP7A has six MBD as in mammals whilst sea bream ATP7B has only four MBDs. The proposed function of MBDs is to sense the intracellular concentration of Cu and regulate the activity of the protein

accordingly (Guo et al., 2005). In mammals, the role of the MBDs has been investigated in several studies with a structural (Banci et al., 2004; Achila et al., 2006; DiDonato et al., 2000) and functional (Mercer et al., 2003; Cater et al., 2004) approach in both ATP7A and ATP7B, and their role has been demonstrated to be to coordinate Cu, enhancing its transport across the Cu-ATPase channel. This mechanism seems to occur by delivery of Cu from MBD 5 or MBD 6 to the CPC domain, located in TMD6 (Figure 2-3). Moreover, the CPC motif has been shown to be essential for ATP7B activity by mutational analysis (Forbes and Cox 1998). Therefore, not all the MBDs have the same role, only the last two, MBD5 and/or MBD6, have been demonstrated to be required for protein function as deletion of MBD1-5 have no effect on protein trafficking (Cater et al., 2004), and MBD1-4 do not complement the lack of MBD5-6. Nevertheless, the first four MBDs seem to have a regulatory, autoinhibitory, role as deletion of these domains facilitates hydrolysis of ATP suggesting an involvement in the regulation of the enzyme turnover (Huster and Lutsenko 2003). Moreover, in a recent mutational study Cater et al. (2007) have demonstrated that Cu-ATPase activity is not entirely dependent on the N-terminal MBDs and/or the CPC motif, although these sites clearly enhance the efficiency of the trafficking process. The authors hypothesise that some other Cu binding site or sites may contribute to the efficiency of ATP7B trafficking. Thus, the numbers of MBDs at the N-terminus of the protein have increased from yeast to mammals probably under the pressure of the requirement for a finer intracellular tuning of Cu homeostasis. Interestingly, fish ATP7B show between 3 and 5 MBDs, in contrast to fish ATP7A which all have 6 MBDs. Considering that MBD5 and MBD6, required for normal activity, are always conserved in fish ATP7B, the requirement for a finer regulation of Cu ATPase activity, given by MBD1-4, seems to be evolutionarily stronger for ATP7A. This hypothesis agrees with the evidence that ATP7A has a higher turn-over rate, transporting more Cu per minute

(Barnes et al., 2005) therefore a finer regulation, achieved by a higher number of MBDs, could have been more evolutionarily successful.

6.3 Transcriptional response of copper homoeostasis genes to excess copper: *in vivo/in vitro* comparison

The ability to regulate intracellular and body Cu homeostasis in normal, excess or deficient conditions depends on a complex network of Cu transporters. In response to intracellular Cu status, Cu transporters adjust intracellular and intra-tissue Cu concentrations.

Considering the results shown in chapter 4, it is clear that Cu has different effects on gene expression depending on its route of exposure. Although Cu absorption across the gill, following waterborne Cu exposure, repressed branchial ATP7A, Cu accumulated in the liver and resulted in a hepatic gene expression pattern which was distinct from that following Cu absorption from the diet across the intestine. Intestinal, dietary Cu uptake, although resulting in similar, even if slower, increasing tissue Cu levels (intestine and liver 30d), did not induce markers of toxicity such as GR, CuZn-SOD or MT expression in liver or other tissues. This suggests that Cu from dietary sources is associated with different hepatic components or compartments than Cu from gill uptake, in turn inferring 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. 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. Within the hepatocyte, a complex system of Cu chaperone proteins delivers Cu to various intracellular compartments and cupro-enzymes. In excess conditions, Cu is excreted into the bile whilst, at the same time, intestinal uptake is reduced (Linder et al. 1998). The results of the

studies presented herein suggest that dietary Cu entering the hepatic portal vein from the intestine may be complexed with a specific carrier, and is either taken up as a complex or "hands" on the Cu to specific uptake transporter at the liver cell membrane. In contrast, Cu entering the arterial blood via the gill bypasses intestinal absorption and reaches all tissues, including the liver directly, possibly as a different complex (Figure 6-1)(Groman 1982). This Cu may exist in a form which bypasses a metabolic step required for its normal absorption and excretion by the liver, and thus causes induction of hepatic GR and MT, markers of redox and metal stress. Thus export of Cu from the intestinal epithelial cell as specific complex is likely to be the step that is bypassed during waterborne Cu exposure. To test this SAF1 cells were exposed to sub-toxic levels of Cu dissolved in the growth medium and the resultant gene expression profile, induction of ATP7A, MT and GR mRNA levels followed that observed in fish hepatocytes after exposure of the fish to waterborne Cu (Figure 4-1; Figure 5-4). This would indicate that when Cu is added directly to L15 media supplemented with 10% FBS it is complexed in the same manner as Cu in plasma after branchial uptake. To date, three candidates for circulatory Cu transport have been suggested. Under normal conditions, approximately 95% of blood Cu is bound to ceruloplasmin, a cuproprotein essential for iron homeostasis. However, deficiency of the protein does not appear to alter Cu homeostasis indicating that this is not an essential function of the protein (Meyer et al., 2001). Similarly, deficiency of serum albumin, to which Cu^{2+} binds with high affinity, has no Cu-transport or distribution effects (Vargas et al., 1994). Furthermore serum albumin binds Cu in a state which is not available for cellular uptake, although cells will take up Cu^{2+} complexed with L-histidine which in blood may couple albumin-bound Cu with cellular uptake systems (Deschamps et al., 2005). In the present study with SAF-1 cells the major Cu-complexes present in the growth medium are likely to be Cu-albumin and His-Cu-albumin. The third carrier

candidate is transcuprein (also known as α2-macroglubulin and α1-inhibitorIII) and the bulk of Cu derived from the intestine is bound to this protein. Thus it is probable that this is the normal carrier to the liver. Moreover, serum albumin readily exchanges Cu^{2+} with transcuprein (Lui et al., 2007). Although it is currently the best candidate for an entero-hepatic Cu carrier, conclusive evidence for its central role in Cu transport is still lacking.

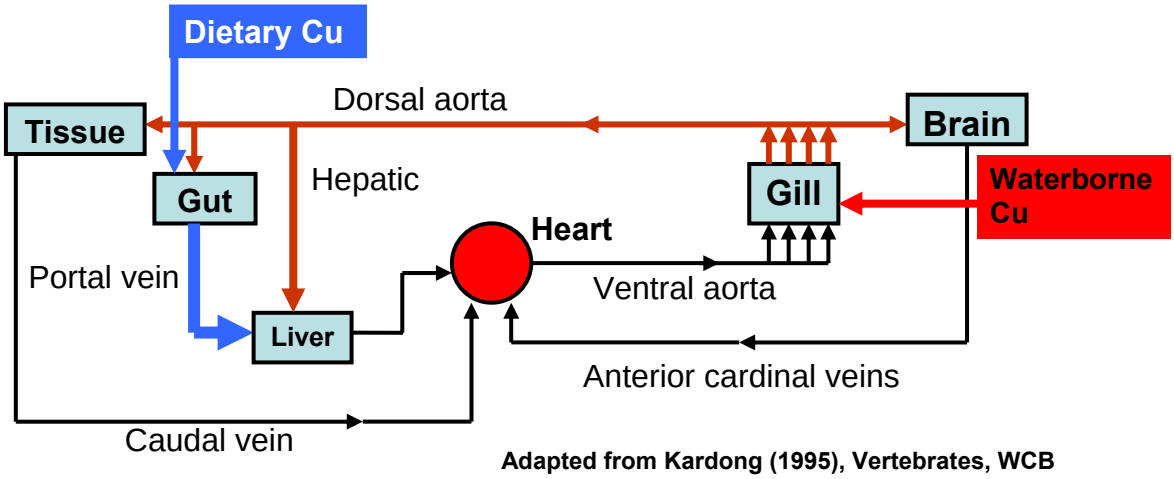


Figure 6-1 Fish circulation.

A clear determination of the localization of Cu transporters is of crucial importance to understand their homeostatic role and also to interpret experimental results. While the basolateral Cu export is attributed to the functional localization of ATP7A in delivering Cu to the blood portal vein (Lutsenko et al., 2008), and in trout also to a Cu-Cl^- symport (Handy et al., 2000), the proteins or mechanisms responsible for apical uptake at the intestinal brush border are not clear (Zimnicka et al., 2007). Copper uptake in the intestine occurs with different kinetics across the apical and basolateral membrane. The rate limiting step of Cu uptake is basolateral transport, presumably mediated by ATP7A (Handy et al., 2000; Zerounian et al., 2003). Apical Cu transport is passive and there is evidence for both saturatable and non saturatable transport suggesting that apical intestinal transport is mediated by two different mechanisms, one carrier mediated which has been

shown to be specific for Cu bound to histidine (Glover and Wood 2008) and the other not carrier mediated such as endocytosis or mediated by a low affinity process (Arredondo et al., 2000; Zerounian et al., 2003; Zimnicka et al., 2007). The main candidates for intestinal, energy independent, apical Cu uptake are the high affinity Cu transporter 1 (Ctr1) and the divalent metal transporter 1 (DMT1) (see 1.5.2). Both are expressed in the enterocyte, however the main point of controversy is the localization of these proteins. DMT1 has been localized at the apical membrane (Tandy et al., 2000) and has been demonstrated to be essential for intestinal non haem- iron transport after birth, as well as transporting Cu with lower affinity (Gunshin et al., 2005; Arredondo et al., 2003). The non saturatable uptake kinetic of absorption (Arredondo et al., 2000; Zimnicka et al., 2007) and the fact that Cd^{2+}, another substrate of DMT1 (Gunshin et al., 1997), does not inhibit Cu uptake argues against the role of this transporter for apical Cu uptake. Although, to exclude DMT1's role in apical Cu transport its function should be tested under the full range of pH as it could affect its function (Zimnicka et al., 2007). Importantly, partial knock-down of DMT1, with an antisense DMT1 oligonucleotide, results in 48% decrease in apical uptake of Cu (Arredondo et al., 2003). The localization of Ctr1 is not as clear, it has been observed at both the apical (Nose et al., 2006a; Kuo et al., 2006) and basolateral membranes (Zimnicka et al., 2007) in mammalian intestinal cells with a portion also present in the cytoplasm. If DMT1 and Ctr1 are both localized at the apical membrane, where dietary uptake occurs, the latter would be more likely to uptake Cu due to its higher affinity for Cu (Puig and Thiele 2002). Ctr1 is essential for Cu absorption from the diet in neonates, as specific intestinal Ctr1 knock-out in mice results in death approximately 10 days after birth (Nose et al., 2006a), and show overall tissue Cu deficiency with Menkes disease symptoms including decreased activities of Cu-dependent enzymes and hepatic iron accumulation due to lack of holo-ceruloplasmin. However, the most interesting and

unexpected result was that intestine specific Ctr1 knockout mice accumulated Cu (8-10 fold higher compared to wild-type) in the intestine. This Cu was accumulated in a non bio-available form as shown by low intestinal hephaestin and high CCS (chaperone for CuZn-SOD), both known biomarkers for low Cu availability. This evidence supports the hypothesis that an apical Cu transporter other then Ctr1 exists and that Ctr1 may function at the basolateral membrane delivering blood-borne Cu specifically for incorporation into intracellular intestinal proteins. This hypothesis has been recently investigated in studies by Zimnicka et al. (2007) which found that Ctr1 was mainly localized at the basolateral membrane, and by measuring Cu uptake at both poles of Caco-2 cells (enterocyte cell line), they showed that Cu uptake was more efficient at the basolateral membrane (high affinity/saturating) than at the apical membrane where Cu uptake was 20 fold lower and non saturatable. In addition, basolateral Cu uptake was inhibited by silver which is known to mimic Cu (Solioz and Odermatt 1995). A basolateral functional localization of Ctr1 could also explain the increase in Cu accumulation rates following serosal application of the anion transport inhibitor 4,4-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS) in perfused catfish intestine (Handy et al., 2000) which may result in acidification leading to increased Ctr1 activity (Lee et al., 2002a). A diagram summarizing these results is represented in Figure 6-2. Although it is clear that Ctr1 is the main systemic Cu uptake transporter delivering Cu from the blood to the cell (Zimnicka et al., 2007; Nose et al., 2006a), its role in intestinal uptake requires further study.

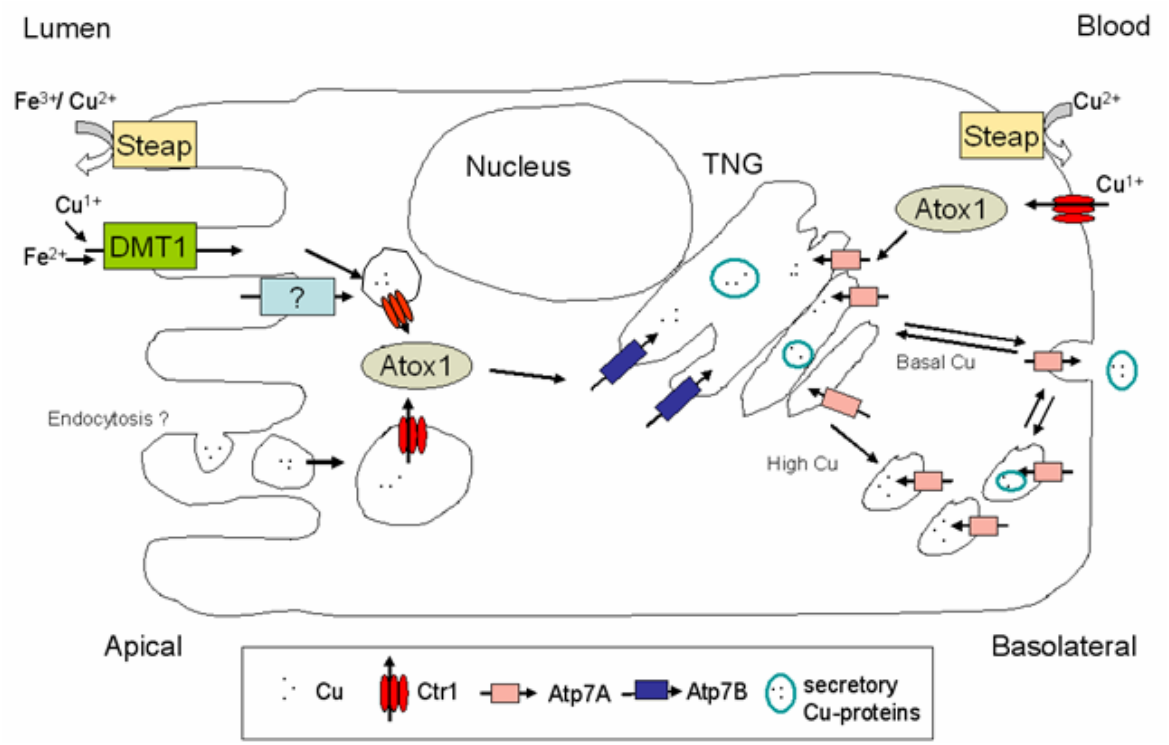


Figure 6-2 Proposed model of copper transporters trafficking and their intracellular localization in enterocytes. Copper enters via Ctr1 at the basolateral membrane or via DMT1, endocytosis or other unidentified transporters at the apical membrane. Previous entry via Ctr1 and DMT1 Cu is reduced by the metalloreductase Steap. After entry Cu is bound by the chaperone Atox1 which delivers it 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, ATP7A cycles between the TGN and the basolateral membrane. When intracellular Cu levels increase, ATP7A is sorted into vesicles that move toward the basolateral membrane and Cu-loaded vesicles may fuse with the membrane to release Cu incorporated into secretory proteins by exocytosis. (Adapted from Zimnicka et al., 2007 and Nyasae et al., 2007).

These considerations may now clarify the unexpected response to waterborne Cu by the intestine. Assuming that Cu is toxic when present in its free or not specifically bound form, the gut, like the liver, might be considered an organ of detoxification and accumulation. The increase in intestinal Ctr1 and ATP7A mRNA may be induced by arterial Cu present in the blood stream in a toxic form. This response could be part of a homeostatic defensive mechanism increasing the rate of intestinal Cu uptake and detoxification through its incorporation into specific Cu protein carriers, by ATP7A and

ATP7B in the secretory pathway. This mechanism would allow the delivery of Cu into the portal vein in a form that could be metabolized and excreted by the liver. An "anti homeostatic" increase in Cu uptake by excess Cu has been previously reported (Arredondo et al., 2000; Zerounian et al., 2003) and was interpreted as an adaptive mechanism, increasing storage and efflux capacity and also uptake. Interestingly, bacteria regulate both uptake and efflux proteins in response to Cu overload (Solioz and Stoyanov 2003). In fish a similar adaptation may guarantee a more dynamic regulation of Cu homeostasis and prevent acute changes in plasma Cu concentrations (Arredondo et al., 2000). As marine fish drink, and waterborne Cu has been shown to increase the drinking rate, it is also possible that Cu is absorbed across the intestinal apical membrane (Grosell et al., 2004a). However, dietary Cu resulted in an opposite effect on Ctr1 and ATP7A mRNA expression levels and Cu rapidly accumulated in the liver and gill but not the intestine following waterborne Cu exposure. Thus, the effect on intestinal Ctr1 and ATP7A expression, following waterborne Cu exposure, is likely to be in response to increases in Cu in the arterial blood coming from the gill. The kidney is another organ involved on synthesis of ceruloplasmin (Linder et al., 1998), where interestingly Ctr1 was induced by waterborne Cu. This metabolic function, may not be as efficient as in the gill, especially considering its low level in ATP7B expression (Figure 3-7) which is responsible for the incorporation of Cu into ceruloplasmin, but may also be involved in the incorporation of Cu into other essential cuproproteins (see 3.4). The gill tissue architecture with one single layer of cells may be more permeable to Cu than the gut and branchial ATP7A reduction may not be sufficient to avoid the release of Cu into the blood in its free or not specifically bound form.

Alternatively, the increase in intestinal Ctr1 and ATP7A by waterborne Cu, could be a defence mechanism removing Cu from the blood stream involving

compartmentalization of Cu in vesicles (Nose et al., 2006a) or binding to MT (Handy 1996). Excess Cu could then be excreted into the faeces via sloughing of enterocytes. The results from sea bream, however, do not support this second hypothesis as no accumulation of Cu, or induction of MT by waterborne Cu was detected in the intestine.

In summary, the tissue mRNA expression of Cu homeostasis genes is consistent with the physiologic evidence from various fish species for the involvement of functional high affinity (Ctr1-like) and ATP dependent (ATP7A-B-like) transporters in Cu homeostasis under conditions of both normal and excess dietary Cu exposure. Furthermore, mRNA levels of Cu transporters, Ctr1 and ATP7A are differently regulated depending upon the route of exposure. In addition, Cu derived from uptake via the gill was associated with the induction of markers of metal toxicity, whilst uptake from intestine using the synthetic diets used in this study was not. In view of the potential for toxic metal contamination below aquaculture sites, it is also notable that the concentration of Zn in a normal commercial diet may also result in increased MT in intestine and liver, indicating that dietary Zn levels might be excessive, from both a fish health and environmental health perspective.

The transcriptomic analysis of SAF1 exposed to sub-toxic Cu, althought performed on a relatively small number of cDNAs (1119 unique contigs), has shown that the biological processes more affected by Cu were secretion, cytoskeleton reorganization and protein trafficking. Moreover, the comparison of this study with available genomic and proteomic studies have highlighted the presence of small GTPases among the most significantly changed genes (Du et al., 2008; Walker et al., 2008). Therefore, although perhaps important in the response to excess Cu, small GTPases are involved in many adaptative cellular responses and the specific relation between the induced Rac GTPase

activating protein 1 and Cu excess require further study. However the involvement of other members small GTPases with trafficking of ATP7A from the Golgi apparatus towards the plasma membrane (PM) have been previously shown (Pascale et al., 2003; Holloway et al., 2007). In mammals, Cu-ATPases regulate intracellular Cu homeostasis trafficking from the Golgi apparatus to the PM, therefore elements which control protein trafficking are crucial for its function. These results suggest that fish, similarly to mammals, may regulate their intracellular concentration of Cu by a similar trafficking mechanism. Overall this study shows that SAF1 cells are an informative and suitable *in vitro* system for Cu homeostasis and heavy metal toxicity studies. Moreover, these results suggest that ATP7A may be regulated at the transcriptional level directly by Cu because it was the only metal to affect ATP7A mRNA levels while the induction of MT may be regulated by a different mechanism (e.g. Zn displacement) as all metals tested (Cu, Zn and Cd) induced MT mRNA levels. Although preliminary, the microarray results presented here show a transcriptional fingerprint which showed some overlap with the transcriptional response of fish gills exposed to Cu (Walker et al., 2008) which may characterize Cu toxicity in fish. However, comparative transcriptomic *in vivo* studies are now required.

6.4 Future studies

The results reported in this thesis show that genes involved in the homeostasis of Cu are very highly conserved in all vertebrates including fish. Some of these genes have been cloned in sea bream, their deduced protein sequence has been studied and their mRNA level measured in tissues, however, the cloning of more components of the Cu homeostasis machinery would allow a more comprehensive understanding of this process. For example, it would be interesting to measure the expression of DMT1 in the intestine to

determine if it is regulated by Cu and thus determine if it may be involved in apical Cu uptake.

Variations in mRNA levels of Cu transporter genes depending upon route of exposure and tissue type have been shown, suggesting transcriptional regulation. To determine if this variation is also evident at the protein level, it would now be required to evaluate Cu transporter protein levels by western blot analysis. Similar mRNA/protein regulation by Cu overload have been described previously in mammals (Bauerly et al., 2005). The lack of specific fish antibodies for Cu transporters may present an issue however the high level of conservation between fish and mammalian protein sequences and the availability of mammalian antibodies specific for different protein locations could enable cross-specificity, and this should be tested. Moreover, in order to investigate the mechanism of transcriptional regulation of Cu transporters, the putative promoters of each gene should be isolated and tested *in-vitro*. Each sea bream promoter sequence should then be compared with promoter sequences available from other published fish (and mammals) genomes and searched using automated software designed to highlight evolutionary conserved sequences (Frazer et al., 2004). This "in silico" analysis could highlight sequence similarities identifying putative *cis*-acting elements and suggesting the involvement of specific transcription factors. In addition, a synthetic construct containing the promoter attached to a reporter gene (e.g. luciferase) then transfected into a cell line could be used to test the activity of the promoters in presence of different stimuli including Cu excess or Cu chelators.

The toxic response in sea bream liver exposed to waterborne Cu and in SAF1 exposed to Cu dissolved into the growing media compared to the response in sea bream exposed to dietary Cu raise questions. To what protein is Cu complexed when it enters the

blood stream coming from the intestine or from the gill? If Cu is complexed to a different protein when coming from the intestine or the gill, are these proteins interacting differently with the hepatocyte? What is the mechanism of interaction? If in the intestine Cu is incorporated to a specific serum carrier, what is the protein? Studies with Cu isotopes and evaluation of metal complexation of serum proteins in the portal blood and in the total blood of sea bream could show which specific carrier is that delivers bioavailable Cu to the liver.

In conclusion, the age related abnormal accumulation of Cu in the liver of white perch (*Morone Americana*) (Bunton et al., 1987) could be a form of Cu toxicosis in fish. However, the molecular mechanism of this possible Cu toxicosis has not been determined. Analysis of the genes involved in the hepatic excretion of excess Cu such as Atox1, ATP7B and COMMD1 could highlight a mutation responsible for this toxicosis. Moreover, this fish species could be used as a model to study the effects of excess hepatic Cu.

An overall objective of future studies should be to integrate the emerging information on Cu homeostatic gene and protein function in fish with the large amount of information available for ion, and particularly Cu, physiology in fish, both in freshwater and saltwater.

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Ref Type: Abstract

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APPENDIX 1 – Degenerate Alphabet

Degenerate alphabet used for primers design

A = Adenine

C = Cytosine

G = Guanine

T = Thymine

R = AG

Y = CT

M = AC

K = GT

W = AT

S = CG

B = CGT

D = AGT

H = ACT

V = ACG

N = ACGT

APPENDIX 2 – Microarray Analysis complete Output

Output list of genes significantelly changed in SAF1 cells exposed to 25µM Cu for 4 hours

	Uniprot ID + Annotation (BLASTX)	No significant in analysis	Total on Array	Percentage	Av.Fold Change
1	(Q51FR9) Hypothetical protein	3	3	100%	1.665
2	(Q7ZTZ2) Probable ribosome biogenesis protein RLP24	2	2	100%	1.267
3	(Q8VC12) Probable urocanate hydratase (EC 4.2.1.49) (Urocanase) (Imidazolonepropionate hydrolase)	2	2	100%	1.552
4	(O18977) Tenascin-X	1	1	100%	1.468
5	(O64463) Gene, complete cds, similar to reverse transcriptase genes of various retrotransposons	1	1	100%	1.252
6	(P19120) Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)	1	1	100%	1.257
7	(P45433) Translocon-associated protein alpha subunit precursor (TRAP-alpha) (Signal sequence receptor alpha subunit) (SSR-alpha)	1	1	100%	1.452
8	(P46570) Serpentine receptor class gamma 1 (Srg-1 protein)	1	1	100%	1.223
9	(Q26806) Surface glycoprotein	1	1	100%	1.801
10	(Q4VBR7) Hypothetical protein wu:fc15d01	1	1	100%	1.267
11	(Q4WDG3) Hypothetical protein	1	1	100%	1.219
12	(Q4YAY8) Hypothetical protein (Fragment)	1	1	100%	1.226
13	(Q51VV9) Hypothetical protein	1	1	100%	1.314
14	(Q5CMV9) Synthetic antigen	1	1	100%	1.225
15	(Q5J7W6) Growth-inhibiting gene 2 protein	1	1	100%	1.306
16	(Q5RKQ3) Zgc:101598	1	1	100%	1.259
17	(Q6NJ97) Putative transport system secreted protein	1	1	100%	1.365
18	(Q6T3V3) Activating transcription factor 4	1	1	100%	1.219
19	(Q6UAM7) Class I helical cytokine receptor number 29	1	1	100%	1.16
20	(Q6USB8) Glutathione S-transferase (EC 2.5.1.18)	1	1	100%	1.206
21	(Q75AY3) ADL213Wp	1	1	100%	1.529
22	(Q7Q1G1) ENSANGP00000015727	1	1	100%	1.158
23	(Q7RAY2) Hypothetical protein (Fragment)	1	1	100%	1.752
24	(Q7ZUR5) Signal sequence receptor, gamma (Translocon-associated protein gamma)	1	1	100%	1.331
25	(Q84UB2) GMP synthetase	1	1	100%	1.244
26	(Q86361) G protein (Fragment)	1	1	100%	1.36

27	(Q867A0) Liver tocopherol-associated protein	1	1	100%	1.207
28	(Q8A5G0) Putative ribose phosphate pyrophosphokinase	1	1	100%	1.288
29	(Q8CEH1) Erytroid membrane-associated protein	1	1	100%	1.17
30	(Q8HM57) NADH-ubiquinone oxidoreductase chain 4	1	1	100%	1.357
31	(Q8IIT5) Hypothetical protein	1	1	100%	1.215
32	(Q8PUB4) Chaperone protein	1	1	100%	1.396
33	(Q92GE9) Hypothetical protein	1	1	100%	1.241
34	(Q9PVE8) Cytochrome P450 3A30 (CYPIIIA30)	1	1	100%	1.332
35	(Q9SNH1) Hypothetical protein	1	1	100%	1.174
36	(Q9XZ12) CG7600-PA	1	1	100%	1.282
37	(Q6P3H8) Rac GTPase activating protein 1	2	3	67%	1.251
38	(O18840) Actin, cytoplasmic 1 (Beta-actin)	1	2	50%	0.761
39	(Q12955) Ankyrin-3 (ANK-3) (Ankyrin G)	1	2	50%	1.313
40	(Q40879) Myristyl-ACP desaturase	1	2	50%	1.188
41	(Q5A188) Hypothetical protein	1	2	50%	1.344
42	(Q5EEY8) GSP1	1	2	50%	1.289
43	(Q5JKD2) Hypothetical protein	1	2	50%	1.22
44	(Q6AGH5) Hypothetical protein	1	2	50%	1.241
45	(Q6PBK9) Peroxiredoxin 6 (Novel protein) (Zgc:73360)	1	2	50%	0.629
46	(Q6TNT5) Inhibitor of growth family, member 5	1	2	50%	1.156
47	(Q6VBQ6) Partner of Nob1	1	2	50%	1.245
48	(Q7R1E8) GLP_306_4392_4646	1	2	50%	1.299
49	(Q86XY5) Hemogen	1	2	50%	1.233
50	(Q8AY34) Protein transport protein Sec61 alpha subunit	1	2	50%	0.537
51	(Q8GHF9) Cytochrome C oxidase assembly protein	1	2	50%	1.228
52	(Q8JHX9) Glutamate dehydrogenase3	1	2	50%	1.22
53	(Q8QFS3) H01CJC protein	1	2	50%	1.229
54	(Q8UVU5) Insulin-like growth factor binding protein-2 precursor	1	2	50%	1.683
55	(Q91336) ADP/ATP translocase	1	2	50%	1.379
56	(Q9GPA9) Ribosomal protein S18	1	2	50%	1.293
57	(Q9ZQC2) Putative receptor-like protein kinase	1	2	50%	1.268
58	(Q9XS13) MHC class I antigen (Fragment)	3	9	33%	1.213
59	(Q9UPU6) Zinc finger protein 409	2	6	33%	1.783
60	(Q54E55) Hypothetical protein	1	3	33%	1.272
61	(Q58XP5) Fibronectin 1b	1	3	33%	1.168
62	(Q5PQ19) LOC496010 protein	1	3	33%	1.298
63	(Q6DFT5) MGC79476 protein	1	3	33%	1.216
64	(Q6P2V4) Hgd protein	1	3	33%	1.196
65	(Q6S000) Kinesin family member 12 (Hypothetical protein kif12)	1	3	33%	1.404
66	(Q6Y213) Ribosomal protein L4 (Fragment)	1	3	33%	1.317

67	(Q7NB50) Unique hypothetical	1	3	33%	1.29
68	(Q7SYK7) Zgc:66329	1	3	33%	1.503
69	(Q7T2C2) Acyl-Coenzyme A dehydrogenase, very long chain	1	3	33%	1.662
70	(Q9B5E3) Cytochrome b (Fragment)	1	3	33%	1.389
71	(Q6DCC9) MGC83638 protein	3	12	25%	1.265
72	(Q9DFJ2) Adenylate cyclase type v-like protein	2	8	25%	1.360
73	(O73688) Heme oxygenase (EC 1.14.99.3) (HO)	1	4	25%	1.46
74	(Q66S03) Nattectin precursor	1	4	25%	1.256
75	(Q6GNF7) MGC82838 protein	1	4	25%	1.169
76	(Q8AWY1) SI:dZ182N13.2 (Last exon of novel protein)	1	4	25%	1.406
77	(Q8MY11) Amyloid protein A	1	4	25%	1.102
78	(Q8UVY1) Metallothionein (MT)	1	4	20%	1.414
79	(Q8JI26) C1q-like adipose specific protein	14	59	24%	0.584
80	(P56542) Deoxyribonuclease II alpha precursor (DNase II alpha) (Acid DNase) (Lysosomal DNase II)	3	15	20%	1.315
81	(Q54EF7) Hypothetical protein	1	5	20%	1.405
82	(Q6FRC2) Similar to sp|P40009 S. cerevisiae YER005w YND1	1	5	20%	1.318
83	(Q8H049) Hypothetical protein	1	5	20%	1.16
84	(Q9DFJ1) Chemotaxin (Fragment)	2	12	17%	1.462
85	(O42364) Apolipoprotein Eb precursor (Apo-Eb)	1	6	17%	0.876
86	(Q7QNI6) ENSANGP00000013812	1	6	17%	0.784
87	(Q9JG53) ORF1	1	6	17%	1.21
88	(Q6Y227) MHC class II antigen	1	7	14%	1.302
89	(Q6ZUR3) Hypothetical protein	1	7	14%	1.253
90	(Q9I9M7) Vitelline envelope protein beta	1	7	14%	1.274
91	(Q9YGE7) Complement factor Bf-1	1	7	14%	1.556
92	(Q7T0L1) Gastrula specific embryonic protein 1	9	65	14%	1.477
93	(Q68M53) Hepcidin 5	3	23	13%	1.366
94	(Q7T127) Novel protein similar to vertebrate gliacolin (C1Q) (Fragment)	2	16	13%	0.573
95	(Q8AYR7) Vitellogenin	1	9	11%	1.305
96	(Q94TF4) Cytochrome c oxidase subunit I	7	66	11%	0.804
97	(Q503P4) Hypothetical protein zgc:110377	2	20	10%	1.265
98	(Q5BXC0) Hypothetical protein	2	20	10%	1.264
99	(Q98TS6) Complement component C3	1	10	10%	1.317
100	(Q75UL8) Warm-temperature-acclimation-related-65kDa-protein-like-protein	3	32	9%	1.269
101	(Q9W7P9) Elastase 4 (Fragment)	2	25	8%	1.297
102	(Q589T1) Vitellogenin	3	38	8%	1.305
103	(Q9PTY3) Complement component C7	2	27	7%	1.233
104	(P53447) Fructose-bisphosphate aldolase B (Liver-type aldolase)	1	15	7%	1.252
105	(Q98TH0) Trypsinogen	1	16	6%	1.19

106	(O42175) Apolipoprotein A-I precursor (Apo-AI) (ApoA-I)	19	364	5%	1.312
107	(Q95I98) Major histocompatibility class I receptor	1	20	5%	1.355
108	(Q8AVL6) MGC53587 protein	8	161	5%	1.385
109	(P06596) Phospholipase A2 precursor (Phosphatidylcholine 2-acylhydrolase)	3	64	5%	1.332
110	(Q6Q5X4) Hepcidin-like	11	240	5%	1.309
111	(Q9PTY1) Complement component C3	1	22	5%	1.317
112	(Q645Q0) Fucolectin (Fragment)	2	46	4%	1.283
113	(Q804W7) Prothrombin (EC 3.4.21.5)	2	51	4%	1.492
114	(Q8JJ05) Liver-basic fatty acid binding protein (Fragment)	2	59	3%	1.360
115	(P79893) Chorion protein	2	65	3%	1.627
116	(Q6NYE1) Hypothetical protein fgb	6	259	2%	1.337
117	(Q9PFK6) Transport protein	1	58	2%	1.335
118	not sequenced	20	304	7%	
	Grand Total	**235**	**2418**		

APPENDIX 3 – Dissemination

PUBLICATIONS

- **Minghetti, M.**, Leaver, M. J., Carpenè, E., and George, S. G. 2008, Copper transporter 1, metallothionein and glutathione reductase genes are differentially expressed in tissues of sea bream (*Sparus aurata*) after exposure to dietary or waterborne copper. Comparative Biochemistry and Physiology Part C, vol. 147, no. 4, pp. 450-459.

In preparation:

- **Minghetti, M**., Leaver, M. J., and George, S. G. Cloning and expression of copper transporting P-type ATPases in fish.
- **Minghetti M**., Leaver, M. J., Taggart, J.B., and George, S.G., Identification of a suitable in vitro system for copper homeostasis and heavy metal toxicity in a fish species, Sea bream (*Sparus aurata*).

CONFERENCE CONTRIBUTIONS

Institute of Aquaculture PhD Research Conference, (2008), Stirling, UK.

- Minghetti, M., Leaver, M.J., George, S.G., Distinct expression of copper transport genes in sea bream (*Sparus aurata*) exposed to copper in water and diet - (**oral presentation**) - **Awarded price for best presentation**.

6th International Copper Meeting: Copper and Related Metals in Biology, (2008), Alghero, Italy.

- Minghetti M., Leaver, M. J., Taggart, J.B., Auslander, M., Tom, M. and George, S.G., Identification of a suitable in vitro system for copper homeostasis and heavy metal toxicity in a fish species, Sea bream (*Sparus aurata*) - (**oral presentation**).
- Minghetti, M., Leaver, M. J., Carpenè, E., and George, S. G. Cloning and expression of copper transporting P-type ATPases in fish - (**poster**).

50th National Congress of the Italian Society of Biochemistry, (2005), Riccione, Italy.

- Minghetti, M., Carpenè, E., Leaver, M.J., and George, S.G. (2005) Expression of genes involved in copper homeostasis in sea bream (*Sparus aurata*) exposed to waterborne and dietary copper - (**oral presentation**)

PRIMO 13 "Pollutant responses in marine organisms", (2005), Alessandria, Italy.

- Minghetti, M., Diab, A., Sabine, V., and George, S.G. Glutathione reductase probes as a marker for oxidative stress - (**poster**)
- Minghetti, M., Carpenè, E., Leaver, M.J., and George, S.G. Expression of genes involved in copper homeostasis in sea bream (*Sparus aurata*) exposed to waterborne and dietary copper - (**poster**).

23rd ESCPB Conference "Coping with environmental factors at sea: a molecular approach", (2004), Cesenatico. Italy.

- Minghetti, M., Carpenè, E., and S.G. George. Expression of genes for copper metabolism in Sea bream and Flounder - (**oral presentation**) **Awarded price for best presentation.**