

# Zebrafish (*Danio rerio*) as a model for studying the genetic basis of copper toxicity, deficiency, and metabolism<sup>1–4</sup>

Pedro P Hernández and Miguel L Allende

## ABSTRACT

Unicellular eukaryotes and cultured cells from several animal species were invaluable in discovering the mechanisms that govern incorporation, handling, and excretion of copper at the cellular level. However, understanding the systemic regulation of copper availability and distribution among the different tissues of an intact multicellular organism has proven to be more challenging. This analysis is made even more difficult if the genetic variability among organisms is taken into account. The zebrafish has long been considered a powerful animal model because of its tractable genetics and embryology, but it has more recently become a player in environmental studies, pharmaceutical screening, and physiologic analysis. In particular, the use of the larvae, small enough to fit into a microtiter well, but developed enough to have full organ functionality, represents a convenient alternative for studies that aim to establish effects of environmental agents on the intact, living organism. Studies by our group and others have characterized absorption and tissue distribution of copper and have described the acute effects of the metal on larvae in terms of survival, organ stress, and functionality of sensory organs. A large body of work has shown that there is strong conservation in mechanisms and genes between fish and mammals, opening the possibility for genetic or small molecule screens or for generating fish models of human diseases related to copper metabolism. The variability within humans in terms of tolerance to copper excess or deficiency requires a genetic approach to be taken to understand the behavior of populations, because markers and vulnerabilities need to be identified. The zebrafish could represent a unique tool to move in this direction. *Am J Clin Nutr* 2008;88(suppl):835S–9S.

## INTRODUCTION

The opposing effects of copper in the organism, essential and toxic, pose an unparalleled challenge to cells such that internalization, homeostasis, and excretion have to be finely balanced. Perturbations of this balance, through diet or disease, can lead to a number of disorders. To date, a handful of experimental systems have been used to describe the basic mechanisms of copper handling in the eukaryotic cell, most notably yeast cells and mammalian cells in culture. However, we still lack suitable whole-animal models that can be used to define mechanisms, risk factors, and genetic predisposition to copper-related metabolic disease in humans. Solving some of the uncertainty may be reached by using the fruit fly *Drosophila melanogaster* or the nematode *Caenorhabditis elegans*, 2 powerful invertebrate systems amenable to genetic screens that have provided valuable

information related to copper homeostasis (1, 2). However, there are likely to be vertebrate-specific mechanisms that will escape analysis in these systems. The zebrafish (*Danio rerio*) is a small freshwater teleost favored by developmental biologists for the optical transparency of its embryos and rapid development. A sequenced genome and large-scale mutagenesis screens have contributed to establish the zebrafish as a bona fide genetic system and have provided numerous examples of the structural and functional conservation of genes across all vertebrates. In consequence, this organism is also becoming valued for studies in basic physiology because, despite its small size, analysis at the level of the whole organ, tissue, or the intact organism is possible (3). The real power of the fish though may be that during the larval stages, 2–5 d after fertilization, zebrafish are small and can thus be easily manipulated in large numbers, while possessing fully functional organs, not unlike those present in an adult animal. The nervous system, heart, vasculature, muscles, digestive tract, liver, pancreas, and immune system, among others, are differentiated and support the animals' physiology at these stages. Importantly, the larvae do not require food until after the fifth day of life; therefore they can be analyzed with complete disregard to diet (other than the nutrients provided by the yolk sac). Moreover, habitation in an aqueous environment allows the addition of compounds directly to the water or growth medium, facilitating dosage of test substances and timing of delivery. These features have now made the zebrafish of value to the pharmaceutical industry, which sees the opportunity to carry out large-scale drug discovery screens at a fraction of the cost and time compared with other vertebrate models (4).

Here, we review current progress on our understanding of copper metabolism in the zebrafish and on the identification of the relevant genes. We explore some of the strategies being used to generate new knowledge that can be of use to clinicians and nutritionists in terms of markers and potential targets.

<sup>1</sup> From the Center for Genomics of the Cell, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

<sup>2</sup> Presented at the symposium "Molecular Biomarkers of Copper Homeostasis," held in Viña del Mar, Chile, September 26–29, 2007.

<sup>3</sup> Supported by the Millennium Science Initiative (ICM P06-039-F) and the International Copper Association (MLA).

<sup>4</sup> Reprints not available. Address correspondence to ML Allende, Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile. E-mail: allende@uchile.cl.

## COPPER HANDLING IN THE ZEBRAFISH EMBRYO AND LARVA

Like most freshwater species, adult zebrafish satisfy their dietary need for copper mainly from food sources. In the ovary, maturation of the oocytes is accompanied by accumulation of copper in the yolk, reaching amounts of 3.5 ng by the late vitellogenic stages (5). After fertilization, copper concentrations drop, suggesting that there is excretion and possibly metal exchange with the environment. Elevated copper concentrations in the water can induce accumulation in larvae, indicating that the basic transport mechanisms are in place by these stages (Hernández et al, unpublished observations, 2008).

Zebrafish embryos and larvae can tolerate dissolved copper in the water in the low micromolar range, but lethality increases rapidly with increasing concentrations. Copper can affect hatching at early stages, especially if treatments begin before chorion hardening, which may protect the embryos somewhat (6). Exposure of larvae to a concentration of 250  $\mu\text{mol/L}$  kills all of the fish within a few hours (Hernández et al, unpublished observations, 2008). Although low micromolar concentrations of waterborne copper do not kill zebrafish larvae, deleterious effects were observed in sensory organs, such as the lateral line system (7, 8), and it is likely to affect the olfactory system as it does in other freshwater fish (9). Cells in these sensory systems are directly exposed to the environment; therefore, they escape the protection offered by the integument. The mechanisms of copper ingestion into sensory cells are unknown, although there is ubiquitous expression of the high-affinity copper transporter 1 (Ctr-1) beginning early in embryogenesis (10).

When the metal is in excess, cellular stress becomes rapidly evident in the gills and later in the liver, intestine, and nervous system, indicative of a mechanism that restricts distribution of the metal among the different tissues. This was studied with transgenic zebrafish that express green fluorescent protein under the control of a heat-shock promoter, a useful indicator of the stress response (Hernández et al, unpublished observations, 2008; 11). In embryos, a strong response of metallothionein genes is detected, evidence that embryonic cells are capable of inducing expression of copper-handling proteins (12). In adults, a consequence of acute copper toxicity is oxidative stress in the liver and gill and elevated expression of the copper chaperone COX-17 and catalase, although these effects strongly depend on water chemistry (13).

As in other organisms, copper deficiency in zebrafish is also detrimental to embryonic growth and survival. Assays in zebrafish with drugs that chelate copper show that the notochord is extremely sensitive to this deficiency, a phenotype shown to be due to the inactivation of the cuproenzyme lysyl oxidase (14). The essentiality of this micronutrient was also shown genetically, as a mutation in the *atp7a* transporter, the Menkes disease gene, produces a similar phenotype (15). In addition, in Ctr-1 knockdown experiments (in which translation of Ctr-1 was abrogated by antisense morpholinos), the larvae die at around day 4 after fertilization (10). Therefore, as in mammals, copper absorption and transport into cells are essential for normal development in the zebrafish.

In embryos and larvae, the copper stores present in the yolk are depleted within a few days, and they most likely begin to acquire the metal through food and water sources. Under conditions of low dietary copper, fish absorb copper directly from the water by

the gills. Exposure of zebrafish to ion-depleted water (soft water) elicits changes in expression of several key transporters and enzymes related to copper metabolism. Epithelial calcium channel,  $\text{Na}^+\text{K}^+$  adenosine triphosphatase,  $\text{Na}^+/\text{H}^+$  exchanger, carbonic anhydrase and Ctr-1 show increases in mRNA amounts after a few days of acclimation in soft water (16). Thus, the zebrafish shows an adaptive response at the genomic level to situations of copper deficit, as is the case for excess.

## CONSERVATION OF GENES INVOLVED IN COPPER TRANSPORT AND METABOLISM

Many genes previously known from other organisms to be critical for copper homeostasis and transport were identified in the zebrafish, either by *in silico* examination of the genome or expressed sequence tag databases or by deliberate cloning of homologs. The 20 zebrafish genes described thus far in the literature that are associated with copper regulation are listed in **Table 1**. To date, only a handful of these genes has been characterized by mutation, but the zebrafish offers the possibility of analyzing embryonic genes by inhibiting translation with antisense morpholinos, as was the case for the Ctr-1 high-affinity copper transporter (10) or lysyl oxidase (13). Transporters, chaperones, and other regulatory proteins (such as the metal-response transcription factor-1 and metallothionein genes) were studied in embryos and larvae only in terms of their expression (12, 22, 23). Likewise, most proteins that require copper as a cofactor for their activity are found in the fish, and some of these are characterized (see Table 1).

Some copper homeostasis genes have yet to be described in the zebrafish, absences that could be due to incomplete annotation of the genome or to lack of proof for true orthology to their human cognates. The *CutC* (copper(I) thiophene-2-carboxylate) gene is a good example, because there is an uncharacterized zebrafish sequence (Ensembl gene ENSDARG00000039853) encoding a protein with 76% amino acid identity to the human CutC protein. For some families of proteins with multiple members in humans (eg, metallothioneins and lysyl oxidases) not all fish counterparts have been found, possibly showing differences in metabolic complexity among fish and mammals. Nonetheless, database searches show that, in *Danio*, there are numerous homologous sequences for the human copper metabolism genes. We must await their further characterization to establish whether these genes show functional conservation as well.

## SEARCHING FOR NEW RELEVANT GENES AND MARKERS

The potential of the zebrafish as a discovery tool in copper research has yet to be realized because the identification of new participants and markers is only beginning to be explored. Pioneering work was recently carried out by Mendelsohn et al (15), who have performed large-scale screens with small molecules to generate a copper-deficiency model in the zebrafish. In a screen carried out in larvae, which relies on function of the tyrosinase enzyme for pigment synthesis, several candidate molecules that can effectively limit copper availability to the organism were identified (15). The deficiency phenotype included defects in notochord formation, neural cell survival, cartilage formation, and hematopoiesis, indicating that certain tissues are differentially sensitive to the lack of this micronutrient. They also identified a mutation in *atp7a* that shows an identical phenotype to

**TABLE 1**

Copper metabolism genes identified in the zebrafish

Gene <sup>1</sup>	Human homolog	Molecular function	Expression in zebrafish	Reference
<i>atp7a</i> (mutant: <i>calamity</i> )	ATP7A (Menkes)	Copper-exporting ATPase activity	Neural tube, notochord, whole organism	15
<i>atp7b</i>	ATP7B (Wilson's)	Copper-exporting ATPase activity	Liver	15
<i>slc31a1</i> ( <i>ctr1</i> )	CTR1	High-affinity copper ion transmembrane transporter activity	Whole embryo; larval brain liver gut; adult gill, liver, gut, ovary	10
<i>cox17</i> (Cytochrome <i>c</i> oxidase assembly)	COX17	Copper chaperone activity	Lens, myotome, pectoral fin musculature, liver, gill	13, 17
<i>sod1</i>	SOD1	Copper, zinc superoxide dismutase activity	Brain, gill, liver, musculature system, whole organism	13, 18
<i>mt-co2</i> (Cytochrome <i>c</i> oxidase subunit 2)	MT-CO2	Cytochrome <i>c</i> oxidase	—	19
<i>mt-co1</i> (Cytochrome <i>c</i> oxidase subunit 1)	MT-CO1	Cytochrome <i>c</i> oxidase	Brain, gill, liver, musculature system	18
<i>slc11a2</i> (DMT1; mutant: <i>chardonnay</i> )	SLC11A2	Divalent metal transporter	Blood, lens, liver, optic tectum, ventral mesoderm, YSL	20
<i>mt</i> (Metallothionein)	13 described genes of the metallothionein superfamily (INTERPRO: IPR003019)	Copper ion binding	Brain, gill, liver, musculature system, cerebellum, common cardinal vein, neuromast, olfactory pit, pronephric duct, pronephros, retina, whole organism	18, 21
<i>mt2</i>	13 described genes of the metallothionein superfamily (INTERPRO: IPR003019)	Copper ion binding	Blastoderm, blastomere, brain, cerebellum, common cardinal vein, gill, hyosymplectic cartilage, kidney, lens, liver, musculature system, olfactory pit, ovarian follicle stage I–IV, pancreas, proliferative region, pronephric duct, pronephros, retina, whole organism	17, 18, 22
<i>mtf-1</i>	MTF-1	Induction of metallothionein in response to metals	Cerebellum, eye, forebrain, hindbrain, hatching gland, lens, midbrain, mesencephalon, retina, neural tube, optic primordium, telencephalon	23
<i>tyrp1b</i> (Tyrosinase-related protein 1b)	TYRP1	Oxidoreductase activity	Hatching gland, pigment cell, retina, retinal pigmented epithelium, melanoblast, melanocyte, presumptive pigmented epithelium, presumptive retina	17, 24
<i>sparc</i> (Secreted acidic Cysteine rich glycoprotein)	SPARC	Calcium channel activity	Apical ectodermal ridge pectoral fin, cleithrum, epidermis, floor plate, notochord, otic placode, otic vesicle, periderm, pharyngeal arch 3–7 skeleton, somite, regenerating fin	17, 25
<i>f5</i> (Coagulation factor V)	F5	Copper ion binding, oxidoreductase activity	—	26
<i>lox</i> (Lysyl oxidase)	LOX	Protein-lysine 6-oxidase activity	—	14
<i>dbh</i> (Dopamine beta hydroxylase)	DBH	Dopamine $\beta$ -monooxygenase activity	Neurons, interrenal gland, interrenal primordium, steroid hormone-secreting cell, medulla oblongata, peripheral nervous system, chromaffin cell, locus coeruleus	27, 28
<i>dct</i> (Dopachrome tautomerase)	DCT	Oxidoreductase activity	Melanoblast, melanocyte, pigment cell, presumptive pigmented epithelium, retinal pigmented epithelium	29, 30
<i>tyr</i> (9 mutant lines)	TYR	Tyrosinase	Melanoblast, melanocyte	30, 31
<i>cp</i> (Ceruloplasmin)	CP	Ceruloplasmin (ferroxidase)	Gut, hepatic duct, hepatocyte, liver, whole organism, YSL	17, 32–34
<i>appb</i> (Amyloid beta (A4) precursor protein b)	APP	amyloid beta (A4) precursor protein (peptidase nexin-II, Alzheimer disease)	Axial vasculature, brain, central nervous system, cranial ganglia, lateral line ganglion, retina, retinal ganglion cell layer	17

<sup>1</sup> To define this list of genes we used the Gene Ontology term “copper ion binding” (GO:0005507; Ensembl release 46, August 2007), which produced 64 human genes. These were then used individually to search for zebrafish orthologs for which there is published confirmation of identity. Some of these genes have not been formally published, but their expression is described in a public database (reference 17). ATPase, adenosine triphosphatase; YSL, yolk syncytial layer.

that produced by exposure to the identified drugs (15). Obviously, this study paves the way for further small molecule screens because these models for human diseases can be used as testing assays for a desired biological activity, eg, recovery of cuproenzyme function in the mutant background.

Mutations can be efficiently recovered in fish with chemicals that induce single nucleotide substitutions, the traditional way for mutagenizing the genome on a large scale. In addition, a significant collection of insertional mutants now have the advantage of simpler access to the mutated gene (avoiding the laborious mapping of the mutations implicit with chemical mutagenesis). Many genes of interest to the copper biology community are present in these collections.

Various types of genetic screens aimed at finding copper metabolism genes can be envisioned, such as resistance to deficiency or to toxicity. The possibility of looking through large numbers of mutagenized animals under controlled conditions (eg, a specific concentration of copper in the water in which larvae are raised) could identify new genes and potential therapeutic targets as well. To our knowledge, this type of forward genetic approach has been used before only in *Drosophila* among multicellular animals (1). The robust lethality values we have for zebrafish larvae should provide a suitable assay for even minor deviations from the average population. Likewise, quantitative trait locus analysis could be performed to assist in the identification of genes that confer resistance or susceptibility after classic genetic selection; the large numbers of organisms and rapid generation time of the zebrafish should be instrumental in this type of approach.

The availability of genomic expression tools such as microarrays or other expression-profiling methods (serial analysis of gene expression, etc) were crucial developments for the emerging fields of toxicogenomics or nutrigenomics. The zebrafish has already been providing relevant information in these fields, and copper-related research stands a chance for significant advancement. Besides the obvious acute-effect screens for changing patterns of gene expression, it should be straightforward to carry out screens in fish that combine copper status with other influencing variables such as water hardness, pH, or the presence of interacting ions or metals such as zinc or iron. In addition, it should be possible to carry out long-term studies in adults, in which the effects of a slight excess or deficiency of copper over a significant portion of the animals' life span can be analyzed. Reliable methods for assaying copper status in humans suffer from a lack of adequate markers, an aspect that could clearly benefit from new candidates identified with nonbiased approaches (35).

Expression profiling can provide new markers and players, but it has the disadvantage of potentially generating many false-positive genes. Candidates will have to be tested for relevance at least in terms of expression, which, again, is amenable to large format processing in the fish through in situ hybridization in larvae. Large-scale functional analysis will be more challenging though, because a reverse genetic approach to study known genes by gene targeting ("knockouts") is still unavailable in fish. A strategy of targeting induced local lesions in genomes (TILLING) has been developed (36) and could become a useful alternative if the desired mutants are not part of the currently available collections. At least as far as embryonic and early larval stages go, essentiality and function of candidate genes may be tested by knock-down with antisense morpholinos.

In summary, the zebrafish shows strengths in specific areas that can be useful for gene and marker discovery and for unraveling some of the more complex aspects of the genetics of copper metabolism in vertebrates.

We thank Natalia Mackenzie, Mónica Brito, Viviana Gallardo, Cristian Undurraga, and Ariel Reyes who have participated in different aspects of copper-related research in our laboratory.

The author's responsibilities were as follows—PPH: researched the literature and performed the database searches for the genes listed in Table 1; MLA: did the writing; and both authors revised the submitted manuscript. None of the authors had a personal or financial conflict of interest.

## REFERENCES

1. Norgate M, Southton A, Zou S, et al. Copper homeostasis gene discovery in *Drosophila melanogaster*. *Biometals* 2007;20:683–97.
2. Chu K-W, Chan SKW, Chow KL. Improvement of heavy metal stress and toxicity assays by coupling a transgenic reporter in a mutant nematode strain. *Aquat Toxicol* 2005;74:320–32.
3. Briggs J. The zebrafish: a new model organism for integrative physiology. *Am J Physiol Regul Integr Comp Physiol* 2002;282:R3–9.
4. Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 2005;4:35–44.
5. Riggio M, Filosa S, Parisi E, Scudero R. Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish). *Comp Biochem Physiol Part C* 2003;135:191–6.
6. Johnson A, Carew E, Sloman KA. The effects of copper on the morphological and functional development of zebrafish embryos. *Aquat Toxicol* 2007; Jul 17 (Epub ahead of print; DOI:10.1016/j.aquatox.2007.07.003).
7. Linbo TL, Stehr CM, Incardona JP, Scholz NL. Dissolved copper triggers cell death in the peripheral mechanosensory system of larval fish. *Environ Toxicol Chem* 2006;25:597–603.
8. Hernandez PP, Moreno V, Olivari FA, Allende ML. Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (*Danio rerio*). *Hear Res* 2006;213:1–10.
9. Sandahl JF, Baldwin DH, Jenkins JJ, Scholz NL. A sensory system at the interface between urban stormwater runoff and salmon survival. *Environ Sci Technol* 2007;41:2998–3004.
10. Mackenzie NC, Brito M, Reyes AE, Allende ML. Cloning, expression pattern and essentiality of the high-affinity copper transporter 1 (ctr1) gene in zebrafish. *Gene* 2004;328:113–20.
11. Seok SH, Park JH, Baek MW, et al. Specific activation of the human HSP70 promoter by copper sulfate in mosaic transgenic zebrafish. *J Biotechnol* 2006;126:406–13.
12. Chan KM, Ku LL, Chan PCY, Cheuk WK. Metallothionein gene expression in zebrafish embryo-larvae and ZFL cell-line exposed to heavy metal ions. *Mar Environ Res* 2006;62(suppl):S83–7.
13. Craig PM, Wood CM, McClelland GB. Oxidative stress response and gene expression with acute copper exposure in zebrafish (*Danio rerio*). *Am J Physiol Regul Integr Comp Physiol* 2007; Sep 12 (Epub ahead of print; DOI:10.1152/ajpregu.00383.2007).
14. Gansner JM, Mendelsohn BA, Hultman KA, Johnson SL, Gitlin JD. Essential role of lysyl oxidases in notochord development. *Dev Biol* 2007;307:202–13.
15. Mendelsohn BA, Yin C, Johnson SL, Wilm TP, Solnica-Krezel L, Gitlin JD. Atp7a determines a hierarchy of copper metabolism essential for notochord development. *Cell Metab* 2006;4:155–62.
16. Craig PM, Wood CM, McClelland GB. Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiol Genomics* 2007;30:53–60.
17. Thisse B, Thisse C. Fast release clones: a high throughput expression analysis. ZFIN direct data submission. 2004. (Material deposited at <http://zfin.org>; ZFIN ID: ZDB-PUB-040907-1).
18. Gonzalez P, Baudrimont M, Boudou A, Bourdineaud JP. Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). *Biometals* 2006;19:225–35.
19. Broughton RE, Milam JE, Roe BA. The complete sequence of the zebrafish (*Danio rerio*) mitochondrial genome and evolutionary patterns in vertebrate mitochondrial DNA. *Genome Res* 2001;11:1958–67.
20. Donovan A, Brownlie A, Dorschner M, et al. The zebrafish mutant gene



- chardonnay (cdy) encodes divalent metal transporter 1 (DMT1). *Blood* 2002;100:4655–9.
21. Chen WY, John JA, Lin CH, Chang CY. Expression pattern of metallothionein, MTF-1 nuclear translocation, and its dna-binding activity in zebrafish (*Danio rerio*) induced by zinc and cadmium. *Environ Toxicol Chem* 2007;26:110–7.
  22. Chen WY, John JA, Lin CH, et al. Expression of metallothionein gene during embryonic and early larval development in zebrafish. *Aquat Toxicol* 2004;69:215–27.
  23. Chen W-Y, John ACJ, Lin C-H, Chang C-Y. Molecular cloning and developmental expression of zinc finger transcription factor MTF-1 gene in zebrafish, *Danio rerio*. *Biochem Biophys Res Comm* 2002;291:798–805.
  24. Zou J, Beerermann F, Wang J, Kawakami K, Wei X. The Fugu tyrp1 promoter directs specific GFP expression in zebrafish: tools to study the RPE and the neural crest-derived melanophores. *Pigment Cell Res* 2006;19:615–27.
  25. Whitehead GG, Makino S, Lien CL, Keating MT. fgf20 is essential for initiating zebrafish fin regeneration. *Science* 2005;310:1957–60.
  26. Hanumanthaiah R, Day K, Jagadeeswaran P. Comprehensive analysis of blood coagulation pathways in teleostei: evolution of coagulation factor genes and identification of zebrafish factor VIIi. *Blood Cells Mol Dis* 2002;29:57–68.
  27. Ryu S, Mahler J, Acampora D, et al. Orthopedia homeodomain protein is essential for diencephalic dopaminergic neuron development. *Curr Biol* 2007;17:873–80.
  28. Lucas ME, Muller F, Rudiger R, Henion PD, Rohrer H. The bHLH transcription factor hand2 is essential for noradrenergic differentiation of sympathetic neurons. *Development* 2006;133:4015–24.
  29. Montero-Balaguer M, Lang MR, Sachdev SW, et al. The mother superior mutation ablates foxd3 activity in neural crest progenitor cells and depletes neural crest derivatives in zebrafish. *Dev Dyn* 2006;235:3199–212.
  30. Yang CT, Johnson SL. Small molecule-induced ablation and subsequent regeneration of larval zebrafish melanocytes. *Development* 2006;133:3563–73.
  31. Mellgren EM, Johnson SL. A requirement for kit in embryonic zebrafish melanocyte differentiation is revealed by melanoblast delay. *Dev Genes Evol* 2004;214:493–502.
  32. Shin D, Shin CH, Tucker J, et al. Bmp and Fgf signaling are essential for liver specification in zebrafish. *Development* 2007;134:2041–50.
  33. Cheng W, Guo L, Zhang Z, et al. HNF factors form a network to regulate liver-enriched genes in zebrafish. *Dev Biol* 2006;294:482–96.
  34. Ober EA, Verkade H, Field HA, Stainier DY. Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* 2006;442:688–91.
  35. Danzeisen R, Araya M, Harrison B, et al. How reliable are current biomarkers of copper status? *Br J Nutr* 2007; Aug 1 (Epub ahead of print; DOI:10.1017/S0007114507798951).
  36. Sood R, English MA, Jones M, et al. Methods for reverse genetic screening in zebrafish by resequencing and TILLING. *Methods* 2006;39:220–7.