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Mechanisms of Copper Deficiency in the Zebrafish Embryo

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MECHANISMS OF COPPER DEFICIENCY IN THE ZEBRAFISH EMBRYO

by

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ABSTRACT OF THE DISSERTATION

Mechanisms of Copper Deficiency in the Zebrafish Embryo

By

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Doctor of Philosophy in Biology and Biomedical Sciences
(Molecular Cell Biology)

Washington University in St. Louis, 2010

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Proper maternal nutrition is critical for early embryonic development. Despite overwhelming epidemiologic data indicating the benefits nutrient supplementation for the developing organism we do not fully understand the genetics of predisposition to abnormal developmental phenotypes when faced with suboptimal nutrient levels.

Copper is an essential nutrient required for critical biochemical processes. Severe defects in copper homeostasis lead to significant disease typified by the X-linked recessive disorder Menkes disease. Patients with Menkes disease have cutis laxa, bone deformities, hypopigmentation, arterial malformation, and neurodegeneration due to copper deficiency caused by loss-of-function mutations in *ATP7A*, a copper transport protein. Despite the critical requirement for adequate copper nutrition and the characterization of key copper transport proteins there remain significant gaps in our understanding of copper metabolism.

In order to better understand both the cell metabolic and developmental requirements for copper our laboratory has defined a zebrafish model of severe copper deficiency. The copper chelator neocuproine causes a Menkes-like phenotype in wild-

type zebrafish embryos and the mutant *calamity* which phenocopies neocuproine treated embryos contains a null allele of *atp7a*.

In this thesis we build upon this previous model in two ways in order to address the problem of treatment of Menkes disease and to define novel pathways important for copper metabolism. The first body of work uses small-molecule modifiers of mRNA processing (morpholinos) to correct splicing defects which cause Menkes disease phenotypes in zebrafish embryos. Since about 20% of human disease causing mutations are due to splicing defects this approach may be directly applicable to a wide array of human diseases. The second body of work uses a forward chemical-genetic screen for zebrafish mutants sensitive to mild copper deficiency. Screening mutagenized embryos exposed to subthreshold doses of the chelator neocuproine for copper deficiency phenotypes resulted in two mutants. The first, containing a hypomorphic allele of *atp7a* demonstrates the effect of maternal nutrition and genetics on embryonic development. The second contains a loss-of-function mutation in the vacuolar proton pump leading to a severe embyronic lethal phenotype which is sensitive to copper chelation. This implicates pH gradients in the metabolism of copper in zebrafish.

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Chapter 1

Introduction: Copper Deficiency

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Copper Deficiency

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Purpose of review

Reports of the neurologic findings in adults with acquired copper deficiency as well as the development of novel models for Menkes disease have permitted a greater understanding of the role of copper in the central nervous system. A role of mitochondrial copper homeostasis in cellular energy metabolism suggests roles for this metal in cellular differentiation and biochemical adaptation.

Recent findings

Acquired copper deficiency in adults is reported with increasing frequency, often without any identifiable cause. Chemical genetic studies identified a zebrafish model of Menkes disease that can be used for high-throughput therapeutics and revealed a hierarchy of copper distribution during development. Studies in mice reveal that the copper transport protein Ctr1 is essential for intestinal copper absorption and suggest a unique role for copper in axonal extension, excitotoxic cell death and synaptic plasticity in the central nervous system. Lastly, recent biochemical studies indicate a central role for the mitochondrial matrix in cellular copper metabolism.

Summary

The recent developments in our understanding of copper deficiency and copper homeostasis outlined in this review provide an exciting platform for future investigations intended to elucidate the role of copper in central nervous system development and disease.

Introduction

Copper is an essential nutrient in humans, readily available in the diet and rapidly absorbed through the stomach and duodenum into the portal circulation for subsequent uptake by the liver. When incorporated into specific cuproenzymes, copper serves as a critical cofactor, catalyzing the facile electron transfer reactions required for cellular respiration, iron oxidation, pigment formation, neurotransmitter biosynthesis, antioxidant defense, peptide amidation, and connective tissue formation (Balamurugan and Schaffner, 2006). The liver serves as the predominant organ of copper storage and the only physiological mechanism of copper elimination is through excretion into the bile. Hepatic copper stores are easily depleted if copper intake is impaired by dietary copper deficiency or malabsorption and thus copper deficiency can develop under such circumstances (Schilsky and Fink, 2006). Although loss of cuproenzyme function accounts for the signs and symptoms of copper deficiency, there are several clinical findings in copper deficient patients that are not readily attributable to loss of activity of known enzymes, suggesting either novel functions for known cuproenzymes, the presence of as yet unidentified enzymes or unique physiological roles for copper.

Physiology of copper deficiency

Copper can enter cells via Ctr1, a high affinity polytopic membrane transport protein found in most tissues. Previous studies in a mouse model of Ctr1 deficiency revealed a critical role for this protein in embryonic development but early lethality precluded definitive analysis of the role of Ctr1 in copper physiology. The Thiele group has recently addressed this issue through the generation of intestinal epithelial cell-specific Ctr1 knockout mice. These mice are profoundly copper deficient and exhibit

striking neonatal defects in copper accumulation in most tissues. By a few weeks of age these mice demonstrate severe cardiac hypertrophy, marked growth impairment and increased mortality (Nose et al., 2006a). These defects can be overcome by administration of parenteral copper treatment, demonstrating the essential role of Ctr1 in intestinal copper uptake critical for early postnatal development in the mouse. These findings are supported by recent studies in suckling mice from copper-adequate or deficient dams that reveal that Ctr1 expression is increased in the intestine following perinatal copper deficiency (Kuo et al., 2006). Although the mechanisms of increased Ctr1 expression have not yet been determined, these findings are consistent with a critical role for this transporter in intestinal copper acquisition. These studies of Ctr1 are critical to our understanding of human copper deficiency and strongly suggest that this transporter is required for apical membrane enterocyte copper uptake in the human intestine. The 6-angstrom projection structure of human Ctr1 based upon electron crystallography of two dimensional protein crystals in a native phospholipid barrier reveals the formation of a putative pore for metal ions at the interface of three identical subunits and reveals a novel architecture not previously described for other transport proteins (Aller and Unger, 2006). This protein structure is based upon a Ctr1 trimer and is structurally most closely related to channel proteins, suggesting a novel mechanism for copper entry across membranes (Nose et al., 2006b). Understanding the precise mechanisms by which Ctr1 transports copper will be critical for our understanding of acquired copper deficiency in adults where functions of environmental dietary factors such as zinc may directly interact with Ctr1 and thus disrupt intestinal copper uptake. Such knowledge may also be critical in the design of therapeutics such as cis-platin

compounds that utilize this transporter for cellular uptake (Nose et al., 2006b). Inherited polymorphismin the gene encoding human Ctr1 might be predicted to account for variations in copper uptake amongst individuals and here again detailed structural information may be of great value in interpreting the molecular outcome of these genetic variants.

Acquired copper deficiency in adults

Acquired copper deficiency in adults may occur following increased zinc intake, long-term parenteral nutrition or malabsorption from a variety of causes. The most commonly reported manifestations of copper deficiency are hematological and may include neutropenia, thrombocytopenia and anemia. Most recently increasing numbers of patients have been identified with acquired copper deficiency of unknown etiology. Harless and colleagues (Harless et al., 2006) describe two healthy young women with copper deficiency of unclear cause, which resolved after copper supplementation. The authors suggest that dietary factors, including soft drink consumption, may have been involved and that the problem may be more widespread than is currently realized, a concept supported by similar case reports from multiple institutions in the past few years. Accompanying such reports has been the recognition of a syndrome of adult-onset copper deficiency with neurological manifestations (Kumar et al., 2004). Affected individuals present with sensory ataxia, hyperreflexia and a spastic gait similar to the progressive ataxic myelopathy in goats and sheep raised on copper-deficient diets. If detected early, copper supplementation always resolves the hematological manifestations and may prevent further neurological deterioration. Neurologic recovery may, however, be slow

and preferentially involves the sensory symptoms. Recent studies in three copperdeficient adults, two of whom were referred with a presumptive diagnosis of amyotrophic lateral sclerosis, revealed progressive asymmetric weakness and electrodiagnostic findings of proximal and distal denervation suggestive of lower motor neuron disease. Copper replacement resulted in improvement in this weakness suggesting that the neurologic spectrum of copper deficiency should include lower motor neuron disease (Weihl and Lopate, 2006). The neurologic signs and symptoms of copper deficiency may be present without the hematological manifestations and relapse has been reported on adequate copper supplementation (Prodan et al., 2006), suggesting caution in both the diagnosis and long-term follow-up of patients with acquired copper deficiency. While this syndrome has been reported following recognized causes of copper deficiency including zinc ingestion (Willis et al., 2005) and malabsorption (Everett et al., 2006; Tan et al., 2006), it would appear that the majority of cases occur without any identifiable cause. Increased serum concentrations of zinc have been reported in several patients even in the absence of exogenous zinc ingestion, but this is not a universal observation and the significance of this finding is unknown. Magnetic resonance imaging reveals increased T2 signal in the dorsal midline cervical and thoracic cord that is reversible with normalization of serum copper (Kumar et al., 2006). Taken together, these clinical and radiologic findings are very similar to the subacute combined degeneration reported in patients with vitamin B12 deficiency. Consistent with this finding, a pilot study reports undiagnosed copper deficiency in a number of elderly patients with vitamin B12 deficiency, implying a role for copper in the pathogenesis of this disease (Prodan et al., 2006).

Developmental copper deficiency and Menkes disease

Clinical and experimental data indicate that copper is essential for normal development as copper deficiency during pregnancy results in neurologic impairment and defects in organogenesis in multiple tissues. Menkes disease, a fatal neurodegenerative disorder resulting in seizures, hypotonia, and failure to thrive, is due to inherited loss-of-function mutations in the gene encoding the copper-transporting ATPase atp7a. Although affected patients exhibit signs and symptoms of copper deficiency, the mechanisms resulting in neurologic disease remain unknown. To elucidate the mechanisms for these defects a chemical genetic screen was undertaken in zebrafish, revealing a role for copper in notochord formation and demonstrating a hierarchy of copper metabolism within the developing embryo (Mendelsohn et al., 2006). A genetic screen for embryos phenocopied by copper deficiency, identified *calamity*, a mutant defective in the zebrafish ortholog of atp7a. The availability of a zebrafish model of Menkes disease permits a precise characterization of the genesis of the developmental abnormalities associated with this disorder and permits high-throughput chemical suppression screens for compounds of immediate clinical relevance that restore cuproenzyme function in the setting of atp7a deficiency. Of direct relevance to our understanding of copper deficiency in humans, the gene dosage of atp7a in these studies was found to determine the sensitivity to copper deprivation, revealing that the developmental hierarchy of copper metabolism is informed by specific genetic factors (Merchant and Sagasti, 2006). These data suggest the intriguing possibility that suboptimal copper availability due either to dietary factors or genetic variation may contribute to structural birth defects and suggest the need for a

more critical analysis of copper deficiency in the setting of specific, isolated phenotypes in the newborn infant.

Studies of a loss-of-function allele of DmATP7, the *Drosophila* orthologue of the Menkes and Wilson genes, reveal that copper is essential in embryogenesis in this organism, as well as early larval development and adult pigmentation. The data also suggest the possibility of novel functions for this transporter in the developing larva that may be relevant to our understanding of human copper deficiency (Norgate et al., 2006). Characterization of the neurodevelopmental expression and localization of atp7a in the murine brain reveals that this transporter is most abundant in the early postnatal period and increases in specific regions localized to CA2 hippocampal pyramidal and cerebellar Purkinje neurons (Niciu et al., 2006). In this study, atpt7a was detected in the axons of postnatal, but not adult, optic nerve suggesting unique stage-specific roles for this protein. The precisely regulated neurodevelopmental expression of atp7a observed in these studies provides an important histological correlate for the limited therapeutic window in patients with Menkes disease. Analysis of atp7a expression by this same group within the developing olfactory system suggests a role for this transporter in axon extension, a finding that could account for a component of the neurodegeneration in Menkes disease (El Meskini et al., 2005). Recent analysis of global changes in gene expression in the brain tissue from patients with Menkes disease reveals downregulation of genes involved in myelination, energy metabolism, and translation and may offer further insight into the pathogenesis of copper deficiency within the developing brain (Liu et al., 2005).

Cell biological analysis within hippocampal neurons reveals a critical role for atp7a in the availability of an N-methyl-D-aspartate (NMDA) receptor-dependent, releasable pool of copper, demonstrating a unique mechanism linking copper homeostasis and neuronal activation within the central nervous system (Schlief et al., 2005). These findings suggest a role for copper in activity-dependent modulation of synaptic activity and in support of this hypothesis, copper chelation exacerbates NMDAmediated excitotoxic cell death in primary hippocampal neurons, whereas the addition of copper is protective (Schlief et al., 2006). This protective effect of copper depends on endogenous nitric oxide production in hippocampal neurons, demonstrating in-vivo links among neuroprotection, copper metabolism, and nitrosylation. Atp7a is required for these copper-dependent effects because hippocampal neurons isolated from a murine model of Menkes disease reveal a marked sensitivity to endogenous glutamate-mediated NMDA receptor-dependent excitotoxicity in vitro, and mild hypoxic/ischemic insult to these mice in vivo results in significantly increased neuronal injury. Taken together, these data reveal a unique connection between copper homeostasis and NMDA receptor activity that is of broad relevance to the processes of synaptic plasticity and excitotoxic cell death. The observation that copper deficiency exacerbates excitotoxic cell death reveals a unique paradigm for neuronal injury in patients with Menkes disease and suggests a novel approach to the treatment of intractable seizures in such patients via blockade of excitatory neuronal pathways (Schlief and Gitlin, 2006).

Patients with Menkes disease reveal many of the classic signs and symptoms of copper deficiency including marked connective tissue abnormalities secondary to impaired activity of copper-dependent lysyl oxidases. Recent reports reveal that

metaphyseal dysplasia and fractures in such patients may be initially confused with child abuse (Bacopoulou et al., 2006). Unusual clinical findings have also recently been reported that include severe brachial artery aneurysms (Godwin et al., 2006) and bilateral panlobular emphysema and pulmonary arterial hypoplasia (Grange et al., 2005) also due to copperdependent impairment in connective tissue biosynthesis and cross-linking. While Menkes disease is the only well defined genetic cause of copper deficiency during human development, the recent report of a 4-month-old infant presenting with congenital cataract, severe muscular hypotonia, developmental delay, sensorineural hearing loss and cytochrome c oxidase deficiency with repeatedly low copper and ceruloplasmin levels suggests the possibility of additional such disorders. Consistent with this idea, the family history in this case revealed parental consanguinity and although genetic analysis of all known copper genes was normal, copper histidine supplementation resulted in a remarkable improvement of clinical symptoms, suggesting as yet unidentified proteins controlling copper homeostasis (Horvath et al., 2005).

Intractable seizures are a universal feature in almost all patients with Menkes disease and a recent analysis in affected children with prolonged survival defined three stages of epilepsy development that include early focal status, followed by infantile spasms and subsequent myoclonic and multifocal epilepsy after 2 years of life (Bahi-Buisson et al., 2006). These clinical observations may have relevance for the development of treatment optimization for the severe copper deficiency observed in Menkes disease. Iinuma and colleagues (Munakata et al., 2005) used proton magnetic resonance spectroscopy to examine the metabolic changes in the brain of a boy with Menkes disease treated with parenteral copper histidine supplementation from 5 months

of age. Therapy initiated after the critical period still improved the neuronal metabolism, suggesting that some copper was delivered to neurons despite the fact that brain atrophy, impaired myelination, and severe neurological symptoms were largely unaffected. In this case and another recent report (Olivares et al., 2006) the authors hypothesize that some correlation exists between genotype and clinical outcome after copper histidine therapy, although this remains a very poorly understood clinical issue. The Kaler group at the US National Institutes of Health has had considerable experience with this treatment approach and in a thoughtful review of their experience suggest that in light of the limited clinical benefits and potential risks, the decision whether or not to offer copper-replacement treatment to symptomatic Menkes patients represents an ethical challenge best made in each case in consultation with the patient's parents (Sheela et al., 2005). While not advancing our knowledge of treatment mechanisms, this approach is welcome and critical to the appropriate care of the family in the circumstances of such a devastating diagnosis.

Mitochondrial copper and Sco2

Under physiological circumstances, the availability of free intracellular copper is restricted, and therefore following uptake by Ctr1 copper is bound to a family of proteins termed metallochaperones that function to deliver this metal to specific pathways, protecting against intracellular copper chelation (Culotta et al., 2006). Copper trafficking to the mitochondria is necessary for the proper metallation of cytochrome c oxidase and requires the metallochaperones Cox17, Sco1 and Sco2 (Cobine et al., 2006b). As the copper-binding subunits of cytochrome c oxidase are encoded by the mitochondrial

genome, copper insertion into this protein must occur within this organelle. Consistent with a role for these chaperones in cytochrome c oxidase function, inherited deficiency of Sco2 results in a fatal encephalocardiomyopathy from impaired respiratory chain function (Bohm et al., 2006). Interestingly, a recent report examining patient and parental genotypes identified a child with hemizygosity for the common Sco2 missense mutation E140K, a finding with important implications for genetic counseling (Leary et al., 2006). The relevance of these Sco2 mutations to copper deficiency has recently become more apparent with the observation that a mitochondrial matrix copper pool serves as the source of copper for cytochrome c oxidase metallation (Cobine et al., 2006a). Most interestingly, this process is dependent upon a unique mitochondrial matrix copper ligand that may serve as a sensor of copper homeostasis within cells.

Although the identity of this mitochondrial matrix copper ligand has not yet been determined, this finding is of potential broad significance in our understanding of the mechanisms of cellular copper homeostasis and the pathogenesis of neuronal injury in copper deficiency. This concept is underscored by recent studies that identify Sco2 as a critical downstream mediator between the utilization of respiratory and glycolytic pathways in cells (Matoba et al., 2006). In this work, p53, a gene frequently mutated in cancer, was shown to regulate Sco2 expression. Furthermore, disruption of the Sco2 gene in cancer cells with wild-type p53 shifted metabolism toward glycolysis analogous to that observed in p53-deficient cells. This metabolic switch to glycolysis, commonly known as the Warburg effect, is proposed to provide a significant growth advantage to cancer cells, permitting proliferation and possibly promoting invasive characteristics (Assaily and Benchimol, 2006; Kruse and Gu, 2006). The finding that Sco2 is a direct metabolic target

of p53 reveals an essential and previously unappreciated role for copper in the regulation of cellular energy metabolism. Although still in the early stages, such observations suggest novel mechanisms for neuronal injury in copper deficiency directly related to the mitochondrial regulation of aerobic respiration, energy production and metabolism.

Conclusion

Copper deficiency remains an important, albeit uncommon clinical problem. Clinical and experimental studies in affected patients continue to reveal new insights into the fundamental roles of copper in cellular biochemistry. Exciting new findings regarding the role of copper in synaptic plasticity and excitotoxicity offer new therapeutic approaches and suggest unique functions of copper in neurobiology. Lastly, observations on the role of copper and Sco2 in mitochondrial biology may herald new insights into the role of copper in mitochondrial biology, energy homeostasis and neurodegenerative disease.

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Chapter 2

Introduction: Copper and Iron Disorders of the Brain

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Copper and Iron Disorders of the Brain

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Abstract

Copper and iron are transition elements essential for life. These metals are required to maintain the brain's biochemistry such that deficiency or excess of either copper or iron results in central nervous system disease. This review focuses on the inherited disorders in humans that directly affect copper or iron homeostasis in the brain. Elucidation of the molecular genetic basis of these rare disorders has provided insight into the mechanisms of copper and iron acquisition, trafficking, storage, and excretion in the brain. This knowledge permits a greater understanding of copper and iron roles in neurobiology and neurologic disease and may allow for the development of therapeutic approaches where aberrant metal homeostasis is implicated in disease pathogenesis.

Introduction

Copper and iron function as cofactors in specific proteins, catalyzing electron transfer reactions required for cellular metabolism and binding inorganic ions (e.g., oxygen) that serve as substrates for this biochemistry (Andrews and Schmidt, 2007; Balamurugan and Schaffner, 2006). Deficiency in these metals results in metabolic abnormalities due to loss of function of these iron- and copperdependent proteins. Excess of these metals can result in the unregulated oxidation of proteins, lipids, and other cellular components causing subsequent tissue injury. Thus pathways of copper and iron metabolism have evolved to ensure adequate amounts of each metal for cellular survival while protecting the organism from the consequences of metal excess. In the past decade, elucidation of the genetic basis of many of the inherited copper and iron metabolism disorders has begun to provide insight into these pathways, permitting an understanding of the mechanisms involved in cellular homeostasis of these metals.

The inherited copper and iron metabolism disorders that cause deficiency or excess of these metals in the brain result in central nervous system disease (Ponka, 2004; Waggoner et al., 1999). Although these disorders reveal that copper and iron homeostasis is essential for normal brain function, little is currently known about the mechanisms of copper and iron acquisition, trafficking, storage, and excretion within the central nervous system. How and where are iron and copper stored in the brain and under which circumstances do these metals accumulate? How do cells in the brain acquire these metals when needed? Why is iron deficiency in utero associated with significant long-term cognitive impairment? How are the uptake and excretion of these metals regulated in the brain, and what is the relationship to these processes in systemic circulation? How does excess copper accumulation within the brain result in psychiatric disease? Does impaired homeostasis of copper or iron contribute to the pathogenesis of common neurodegenerative disorders such as Parkinson or Alzheimer disease? One only has to view the clinical consequences of accumulation or deficiency of these metals in the human brain to appreciate the critical nature of such questions. Mechanistic understanding of the human genetic disorders that impair copper or iron homeostasis in the brain provides insight into the role of these metals in neurobiology and disease.

COPPER

Overview

Copper is required for cellular respiration, iron oxidation, pigment formation, neurotransmitter biosynthesis, antioxidant defense, peptide amidation, and connective tissue formation (Pena et al., 1999). This metal is essential for central nervous system

development, and disruption of copper homeostasis during fetal life leads to perinatal mortality, severe growth retardation, and neurodegeneration (Keen et al., 1998). Experiments in mice reveal that the developmental timing of perinatal copper deficiency influences the severity of neurological outcome, suggesting a critical period for brain copper acquisition (Prohaska and Brokate, 2002). Acquired copper deficiency in adults results in myelopathy with lower limb spasticity and sensory ataxia due to ascending sensory tract dysfunction and neurodegeneration of the dorsal column (Kumar et al., 2004; Prodan et al., 2006).

Menkes disease and Wilson disease are the known inherited disorders of copper metabolism in humans (**Table 1**). The essential role of copper in the developing central nervous system is evidenced by Menkes disease, during which impaired copper transport into and within the developing brain results in demyelination and neurodegeneration (Kaler, 1998; Kodama et al., 1999). Brain copper accumulation inWilson disease results in dystonia, dysarthria, and other Parkinsonian symptoms, as well as psychiatric symptoms of depression, cognitive deterioration, personality change, psychosis, and schizophrenia (Ferenci, 2004; Oder et al., 1991). Although the signs and symptoms of Menkes and Wilson diseases are distinct, each disorder results from inherited loss-of-function mutations in genes encoding homologous copper-transporting P-type adenosine triphosphatases (Atpases) Atp7a and Atp7b (**Figure 1**) (Culotta and Gitlin, 2001a).

Copper Metabolism

Copper is readily available in the diet and, following absorption through the stomach and duodenum, is rapidly removed from the portal circulation by hepatocytes in the liver. Biliary excretion is the only physiological mechanism of copper elimination,

and at steady state the amount of copper excreted into the bile is equivalent to that absorbed from the intestine (Gitlin, 2003). The rate of copper excretion into the bile increases promptly in response to an increase in dietary copper, and excess of this metal does not occur in the absence of an underlying metabolic defect (Gollan and Deller, 1973). This aspect of copper homeostasis is critical to the interpretation of any study that implicates increases in dietary copper content with neurologic disease (Sparks and Schreurs, 2003).

Ctr1 is a plasma membrane protein essential for early embryonic development and intestinal copper uptake (Kuo et al., 2001; Lee et al., 2001; Nose et al., 2006a) (**Figure 2**). Ctr1 is present on endothelial cells of the bloodbrain barrier, and expression at this site increases following perinatal copper deficiency (Kuo et al., 2006). These observations suggest that copper is transported from the plasma into the brain via Ctr1. Intracellular copper metabolism is dependent on the copper transport Atpases, Atp7a and Atp7b. One of these Atpases resides in the late Golgi of every cell, delivering copper to the secretory pathway for incorporation into cuproenzymes and excretion (**Figure 1**). In the brain, Atp7a is expressed in endothelial cells of the bloodbrain barrier and facilitates copper movement across the basolateral membrane into the extravascular space of the brain. Atp7a is also ex- pressed within specific populations of neurons in several brain regions including the cerebellum and hippocampus (**Figure 3**). Atp7b is expressed in hepatocytes and is required for copper excretion from these cells into the bile. Copper-dependent trafficking of these Atpases serves as the primary mechanism determining intracellular copper homeostasis (Lutsenko and Petris, 2003; Petris et al., 1996) (**Figure 2**).

Intracellular trafficking of copper also requires proteins termed metallochaperones that direct this metal to specific cellular pathways (O'Halloran and Culotta, 2000; Rae et al., 1999). These chaperones include Atox1, which delivers copper to the coppertransporting Atpases in the late Golgi (Hamza et al., 2001; Hamza et al., 2003), CCS, which is required for copper incorporation into cytoplasmic Cu/Zn superoxide dismutase (Culotta et al., 2006; Wong et al., 2000), and Cox17, Sco1, and Sco2, which deliver copper to mitochondrial cytochrome c oxidase (Hamza and Gitlin, 2002) (**Figure 2**). Sco1 and Sco2 also function in a pathway of mitochondrial copper homeostasis (Leary et al., 2007). Metallothionein is a cysteine-rich cytoplasmic protein that chelates copper and is essential to protect against the toxicity caused by excess copper (Kelly and Palmiter, 1996).

Copper is distributed throughout most regions of the brain and is most abundant in the basal ganglia. Studies have detected this metal in the cell bodies of cortical pyramidal and cerebellar granular neurons, in the neuropil of the cerebral cortex, in the hippocampus and cerebellum, and in synaptic membranes of afferent nerves (Kozma et al., 1981; Sato et al., 1994; Trombley and Shepherd, 1996). In some neurons, copper is released at the synapse (Brown et al., 1997; Hartter and Barnea, 1988) reaching micromolar concentrations (Kardos et al., 1989) that can abrogate long-term potentiation in the hippocampus (Doreulee et al., 1997). Copper is an antagonist at *N*-methyl- D-aspartate (NMDA) receptors in cultured hippocampal neurons (Vlachova et al., 1996; Weiser and Wienrich, 1996), modulating activation of calcium-dependent cascades that contribute to synaptic plasticity (Lu et al., 2001). Synaptic NMDA receptor activation results in rapid and reversible trafficking of Atp7a in cultured hippocampal neurons in

association with copper release, suggesting the presence of a mechanism linking copper homeostasis and neuronal activation within the brain (Schlief et al., 2005) (**Figure 3**).

Menkes Disease

Menkes disease is an X-linked disorder characterized by growth failure, brittle hair, hypopigmentation, arterial tortuosity, and neuronal degeneration due to loss-of-function mutations in the gene encoding Atp7a (Chelly et al., 1993; Mercer et al., 1993; Vulpe et al., 1993). The pleiotropic features of this disease are the result of impaired activity of specific cuproenzymes resulting from impaired Atp7a function (**Figure 2**). The neurologic features are present in early infancy, revealing a critical role for Atp7a and copper in neuronal development (Mercer, 1998). Magnetic resonance imaging of the brain reveals deficient myelination with cerebellar and cerebral atrophy (Geller et al., 1997; Leventer et al., 1997), and neuropathologic examination demonstrates focal degeneration of the gray matter and neuronal loss most prominent in the hippocampus and cerebellum (Barnard et al., 1978; Okeda et al., 1991) (**Figure 4**).

Although several of the known cuproenzymes play critical roles in brain biochemistry, the mechanisms of neurodegeneration in Menkes disease are unknown and not explained by impaired activity of any of these enzymes (**Table 2**). Studies in a murine model of Menkes disease suggest a role for Atp7a and copper in axon extension and synaptogenesis during development (El Meskini et al., 2005). Atp7a mediates the availability of an NMDA receptor–dependent, releasable pool of copper in hippocampal neurons (Schlief et al., 2005), and the absence of Atp7a activity in Menkes disease markedly accentuates NMDA receptor–mediated excitotoxicity in these neurons (Schlief et al., 2006). These data suggest a model whereby loss of Atp7a contributes to both

seizures and neuronal degeneration in affected patients and raise the possibility of therapeutic approaches based on NMDA receptor blockade (Hardingham and Bading, 2003; Schlief et al., 2006).

Systemic copper treatment is not effective in patients with Menkes disease because copper transport into the brain is dependent on Atp7a. Rare patients with some residual Atp7a activity have less central nervous system pathology despite significant systemic disease (Moller et al., 2000) and when treated with copper evidence neurodevelopmental improvement (Christodoulou et al., 1998; Kaler et al., 1995). This clinical observation reveals a hierarchy of copper distribution preferential to the brain under circumstances of limited copper availability, a concept supported by recent observations in a zebrafish model of Menkes disease (Mendelsohn et al., 2006). The mechanisms of this hierarchy are unclear but this process illustrates copper's essential role in brain development.

Wilson Disease

Wilson disease is an autosomal recessive disorder resulting in hepatic cirrhosis and progressive basal ganglia degeneration due to loss-of-function mutations in the gene encoding the copper-transporter Atp7b (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1993). The resulting impairment in biliary copper excretion leads to hepatocyte copper accumulation, copper-mediated liver damage, activation of cell-death pathways, leakage of copper into the plasma, and eventual copper overload in all tissues (**Figure 4**) (Gitlin, 2003; Tao and Gitlin, 2003). Although Atp7b is expressed in some regions of the brain, in Wilson disease copper overload in extrahepatic tissues is due to excess

accumulation from the plasma following liver injury because this is entirely reversed following liver transplantation (Emre et al., 2001; Schumacher et al., 2001).

Ceruloplasmin is an essential ferroxidase that contains greater than 95% of the copper present in plasma. This protein is synthesized in hepatocytes and secreted into the plasma following the incorporation of six copper atoms in the late secretory pathway. In Wilson disease, loss of function of Atp7b results in synthesis of apoceruloplasmin that is rapidly degraded in the plasma (**Figure 5**). As a result, the serum ceruloplasmin concentration is a useful diagnostic indicator ofWilson disease (Hellman and Gitlin, 2002).

Almost half of all patients with Wilson disease present with signs and symptoms of neuropsychiatric illness (Gollan and Gollan, 1998). Although such neurological features may initially be subtle, without chelation patients will progress to severe Parkinsonian symptoms, consistent with the neuropathologic findings of basal ganglia copper accumulation and neurodegeneration (Oder et al., 1991). Neurodegeneration in Wilson disease results directly from copper accumulation, but the precise mechanisms of cellular injury are unknown. Psychiatric symptoms are also common and range from behavioral problems to psychosis (Dening, 1991). These abnormalities, although without neuropathologic correlates, are clearly the result of brain copper accumulation because prompt improvement is observed upon treatment with oral chelating agents to restore copper homeostasis. The reversible nature of many of these psychiatric symptoms is consistent with the concept that copper can modulate synaptic function (Schlief and Gitlin, 2006) and suggests novel avenues for investigation in common psychiatric disorders.

IRON

Overview

Iron is required as a cofactor in central nervous system metabolic processes including oxidative phosphorylation, neurotransmitter production, nitric oxide metabolism, and oxygen transport (Ponka, 1999). The observation that brain iron content is increased in patients with Parkinson disease and other neurodegenerative disorders has created significant interest in the possibility that disturbances of brain iron homeostasis may contribute to the pathogenesis of these diseases (Thomas and Jankovic, 2004). Although numerous clinical and experimental studies have attempted to address this issue, a causative role for impaired iron homeostasis has yet to be established in these more common neurodegenerative diseases. Nevertheless, interest in this neuropathology has focused increased attention on elucidating the mechanisms of brain iron homeostasis and on defining iron's role in neurologic disease.

The inherited diseases of iron metabolism are more common and more numerous than those of copper, and the genetic basis of many of these disorders has been characterized (Andrews, 2002). Although many of the proteins shown to be essential for systemic iron homeostasis are expressed within the brain (Wu et al., 2004; Zecca et al., 2004), genetic disorders resulting in loss of function of these proteins rarely result in either brain iron overload or deficiency or neurologic disease. For example, although ferroportin is abundantly expressed in the brain microvasculature and is the only known cellular iron exporter, patients with mutations that impair this protein's function have no evidence of brain iron accumulation (Pietrangelo, 2006a). These observations suggest the

presence of unique mechanisms regulating iron metabolism within the central nervous system.

Iron Metabolism

Genetic analysis of the inherited iron metabolism disorders has identified many of the proteins necessary for systemic iron homeostasis (Hentze et al., 2004; Pietrangelo, 2006b) (**Figure 6**). Ferrous iron (Fe2+) is taken up into cells by the divalent membrane transporter DMT1, whereas ferric iron (Fe3+) enters cells via endocytosis of the transferrin receptor following binding to transferrin (Andrews and Schmidt, 2007). The most compelling feature of systemic iron homeostasis is that iron requirements far exceed the gastrointestinal absorption capacity, and thus almost all the iron utilized each day is continuously recycled from internal stores (Koury and Ponka, 2004) (**Figure 7**). Central to this process is the hepatocyte-derived peptide hepcidin, which regulates iron availability in response to hypoxia, inflammation, erythropoietic needs, and iron stores (Nemeth and Ganz, 2006). Hepcidin binds to and induces the endocytosis and turnover of the plasma membrane iron exporter ferroportin, which is the primary determinant of gastrointestinal iron absorption and iron release from reticuloendothelial stores (Nemeth et al., 2004). Ceruloplasmin plays a critical role in this process by establishing a rate of plasma iron oxidation sufficient for the continued release of this metal from the storage sites (Harris et al., 1998) (**Figure 7**).

Iron is taken up into the brain from the plasma via transferrin receptor–mediated endocytosis in the brain capillaries and returned to circulation via absorption of cerebrospinal fluid (Moos et al., 2006). Brain iron accounts for less than 2% of the total

body iron content and varies greatly in amount and concentration depending on the specific region of the brain. The concentration of iron in the brain is greatest in the basal ganglia where it is equivalent to that found in the liver, suggesting a role in brain iron storage and distribution (Haacke et al., 2005). Iron is widely distributed in all cell types within the central nervous system but is abundant especially in astrocytes, supporting the idea that these cells and other types of glia function in iron storage and regulation.

Iron requirements in the brain are much greater than the observed rate of iron uptake into this tissue (Bradbury, 1997). This finding suggests most brain iron used each day is derived from recycling behind the bloodbrain barrier analogous to what occurs in the periphery (**Figure 7**). This mechanism would protect the brain from the effects of systemic iron overload or deficiency and is supported by observations that disturbances of systemic iron homeostasis exhibit minimal effects on central nervous system iron content or metabolism (Moos and Morgan, 2004). The rate of brain iron uptake is greatest during fetal life and postnatal iron repletion is ineffective to correct the cognitive defects arising from iron deficiency in utero; this concept indicates that following birth recycling, rather than uptake from the circulation, is the major iron source for brain function (Lozoff et al., 2006). Existence of this brain iron cycle has critical implications for interpreting any finding of brain iron accumulation in disease. The inherited disorders aceruloplasminemia and neuroferritinopathy support the concept of a brain iron cycle and demonstrate that dysregulation of brain iron homeostasis can be a primary cause of neurodegeneration (Ponka, 2004) (**Table 3**).

Aceruloplasminemia

Aceruloplasminemia is an autosomal recessive disorder of iron homeostasis caused by loss-of-function mutations in the ceruloplasmin gene (Harris et al., 1995; Yoshida et al., 1995). Patients have absent serum ceruloplasmin, decreased serum iron, elevated serum ferritin, anemia, and insulin-dependent diabetes mellitus (Gitlin, 1998; Nittis and Gitlin, 2002). Despite these systemic features, most patients present with progressive dementia, dysarthria, and dystonia secondary to basal ganglia iron accumulation (Logan et al., 1994; Miyajima et al., 1987; Morita et al., 1995). The neurologic disease in aceruloplasminemia is always associated with increased brain iron as detected by magnetic resonance imaging or autopsy (Kono and Miyajima, 2006). Histological findings from affected brain regions include neuronal cell loss, abnormal astrocyte architecture, and excess iron deposition in glia and neurons (Kaneko et al., 2002; Morita et al., 1995; Oide et al., 2006) (**Figure 4**).

The absence of ceruloplasmin results in the slow accumulation of iron in the reticuloendothelial cells where this metal is normally stored and then mobilized for recycling (Harris et al., 1999; Patel et al., 2002) (**Figure 7**). This mechanism of systemic iron accumulation also occurs in patients with dominant-negative mutations in the gene encoding the iron exporter ferroportin (De Domenico et al., 2006). Loss of ceruloplasmin also results in increased nontransferrinbound iron that rapidly accumulates in the liver, pancreas, and other tissues following removal from the plasma by DMT1 (Andrews, 2002; Hentze et al., 2004) (**Figure 7**). This mechanism of tissue iron accumulation also occurs in atransferrinemia, where the absence of serum transferrin increases the relative amount of nontransferrin-bound plasma iron (Craven et al., 1987); in HFE hemochromatosis, where excess iron absorption elevates nontransferrin-bound plasma

iron (Andrews, 2002); in the thalassemias and other forms of secondary iron overload, where transfusiondependency elevates nontransferrin-bound plasma iron (Pietrangelo, 2006b); and in hemochromatosis, owing to loss-of-function mutations in ferroportin that impair hepcidin regulation inappropriately increasing nontransferrin-bound iron (De Domenico et al., 2006).

Although these disorders of iron metabolism share common mechanisms of tissue iron overload, accumulation of iron in the brain is unique to aceruloplasminemia. The absence of brain iron accumulation in other diseases with increased nontransferrinbound plasma iron supports the idea that brain iron accumulation in aceruloplasminemia results directly from impaired iron homeostasis within the central nervous system. Consistent with this concept, ceruloplasmin is synthesized in astrocytes (Klomp et al., 1996; Klomp and Gitlin, 1996) as a glycophosphatidylinositol-linked isoform (Jeong and David, 2003; Patel et al., 2000), suggesting that this membrane-bound ferroxidase facilitates the rate of iron release from storage cells within the central nervous system. Ceruloplasmin is one of very few proteins established as playing a critical role in brain iron homeostasis.

Neuroferritinopathy

Ferritin is a ubiquitously expressed cytoplasmic iron storage protein consisting of heavy and light chains encoded on separate genes (**Figure 6**). Iron-dependent translational regulation of this protein by the cytosolic iron regulatory proteins Irp1 and Irp2 ensures that ferritin is a readily available source of intracellular iron in all cells including neurons and glia (Rouault, 2006). Neuroferritinopathy, an autosomal dominant extrapyramidal disease resulting from mutations in the gene encoding the light chain of ferritin, has revealed a primary role for ferritin in brain iron homeostasis (Curtis et al.,

2001; Maciel et al., 2005; Mancuso et al., 2005; Vidal et al., 2004). Pathological examination of affected patients reveals cavitary degeneration in the basal ganglia nuclei, neuronal loss, and iron and ferritin in both extracellular and cytoplasmic inclusion bodies of microglia. The autosomal dominant inheritance and the multimeric structure of ferritin suggest that these mutations impair ferritin assembly resulting in loss of iron storage capacity within brain cells and subsequent iron-mediated cell injury (Levi et al., 2005). Although there are some phenotype-genotype correlations specific to each light chain mutation, all patients with neuroferritinopathy evidence dystonia in association with basal ganglia iron accumulation (Chinnery et al., 2007).

Patients with neuroferritinopathy express the ferritin light chain mutation in all cells yet evidence no abnormalities in systemic iron homeostasis with the exception of decreased serum ferritin (Chinnery et al., 2007). This finding suggests either a unique role for the ferritin light chain in some cell types within the brain or a greater sensitivity of these cells to this form of iron-dependent injury. Although the mechanism of neurodegeneration in neuroferritinopathy is unknown, the similar pathology to aceruloplasminemia suggests a role for ferritin in the brain iron cycle and implies a common iron-dependent mechanism of neurodegeneration in these two diseases (**Figure 7**). Recent studies in aceruloplasminemia suggest that neuronal loss in this disease arises from iron deficiency secondary to impaired iron movement from astrocytes within the brain iron cycle (Jeong and David, 2006). Support for a similar mechanism in neuroferritinopathy comes from a murine model of Irp2 deficiency that impairs ferritin regulation, results in iron accumulation within oligodendrocytes, and leads to

neurodegeneration most likely from secondary neuronal iron deficiency (LaVaute et al., 2001).

Friedreich's Ataxia

Friedreich's ataxia is an autosomal recessive disorder characterized by sensory neuron, cerebellar, and cardiomyocyte degeneration (Durr et al., 1996) due to loss of function of frataxin, a mitochondrial protein involved in inorganic iron-sulfur (Fe/S) cluster biogenesis (Babcock et al., 1997). Although not specific to the brain, the effect of impaired mitochondrial iron homeostasis on neuronal survival in this disease warrants mention here. Mitochondrial iron is required for heme biosynthesis and for the generation of Fe/S clusters that are prosthetic groups in many essential enzymes (Ajioka et al., 2006; Lill and Muhlenhoff, 2006). Iron is imported into mitochondria by mitoferrin, a transporter expressed in the inner mitochondrial membrane (Shaw et al., 2006), and exits mitochondria largely in the form of Fe/S clusters (Rouault and Tong, 2005). Although excess mitochondrial iron is detected in cardiomyocytes and sensory neurons from affected patients (Puccio et al., 2001), impairment of heme and Fe/S cluster biosynthesis is the likely proximate cause of neurodegeneration in Friedreich's ataxia (Lill and Muhlenhoff, 2006; Voncken et al., 2004).

Neurodegeneration with Brain Iron Accumulation

Neurodegeneration with brain iron accumulation describes a heterogeneous group of patients with progressive neurodegeneration and iron deposition in the basal ganglia (Gregory and Hayflick, 2005; Hayflick et al., 2003). Most of these patients present in childhood with dystonia, dysarthria, and pigmentary retinopathy and have autosomal recessive, loss-of-function mutations in the gene-encoding mitochondrial pantothenate

kinase 2, an essential mitochondrial enzyme involved in coenzyme A biosynthesis (Hayflick et al., 2003; Johnson et al., 2004; Zhou et al., 2001). The mechanisms of iron accumulation and the role of iron, if any, in the pathogenesis are unknown. However, these disorders deserve mention because recent studies reveal mutations in the gene encoding a calcium-independent group VI phospholipase A2 in some children with brain iron accumulation and neurodegeneration, suggesting a novel link between phospholipid and iron metabolism in the brain (Morgan et al., 2006).

CONCLUSIONS

The inherited disorders of copper metabolism reveal copper's essential role in brain development and neuropsychiatric disease. The mechanisms of psychiatric disease observed in patients with Wilson disease are of particular interest. Chelation is an effective therapy in such individuals, indicating copper's direct role in pathogenesis and suggesting avenues for study that may be widely applicable to brain function and mental health. Although elucidation of the genetic basis of Menkes andWilson diseases has revealed much about the cell biology of copper metabolism, much more needs to be learned about the normal mechanisms of copper homeostasis in the brain. In particular, the role of the basal ganglia in copper and iron storage and distribution needs further study as do the mechanisms of this brain region's vulnerability to excess copper and iron. Recent studies have focused on copper's role in the pathogenesis of prion-mediated encephalopathy and Alzheimer disease, including the proposed treatment of affected patients with metal chelating drugs (Bush, 2000; Doraiswamy and Finefrock, 2004; Gaeta and Hider, 2005). However, caution is warranted because more knowledge of copper homeostasis within the brain and copper's role in specific neurologic functions is

required before any such therapeutic approaches can be thoughtfully undertaken or interpreted.

The discovery of aceruloplasminemia and neuroferritinopathy demonstrates that dysregulation of brain iron homeostasis can be a primary cause of neurodegeneration. These inherited disorders provide a platform for further mechanistic investigations that should reveal new insights into brain iron homeostasis. Nevertheless, absent further understanding of the molecular mechanisms of iron homeostasis in the central nervous system, investigators currently cannot interpret the significance of iron accumulation in the pathogenesis of other neurologic diseases (Lee et al., 2006; Zecca et al., 2004). This concept is illustrated by recent studies in aceruloplasminemia that suggest that neurodegeneration arises from iron deficiency despite the marked brain iron overload. A cautionary tale is also provided by the numerous iron chelator and antioxidant trials initiated in patients with Friedreich's ataxia on the basis of the findings of mitochondrial iron accumulation, none of which have any demonstrated clinical benefit in doubleblind placebo-controlled trials. These data remind us of the need for critical, mechanistic approaches to defining disease pathogenesis when normal physiology remains poorly understood. In this regard, attention is given to recent studies identifying a signaling cascade in neurons where stimulation of NMDA receptors mediates iron uptake via the divalent iron transporter DMT-1. This work provides a physiologic link between neuronal function and iron homeostasis and suggests mechanisms linking iron and NMDA neurotoxicity that would direct future investigation (Cheah et al., 2006).

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Table 1

	Wilson Disease	Menkes Disease
Genetics	Autosomal recessive	X-linked
	Loss-of-function mutations	Loss-of-functions mutations
	ATP7B gene	*ATP7A* gene
Presentation	Late childhood: liver	Early infancy
	Second-third decated, neuropsychiatric	
Defect	Biliary copper excretion	Copper transport across the placenta, brain, and gastroinestinal tract
Pathogenesis	Copper accumulation	Copper deficiency
Clinical	Cirrhosis, dystonia, dysarthria, Parkinsonian tremor, psychiatric	Hypothermia, hypopigmentation, abnormal hair, tortuous arteries, intractable seizures, failure to thrive
Pathology	Basal ganglia copper accumulation	Cerebral and cerebellar degeneration
	Neuronal cell loss	Purkinje cell axonal swelling
		Abnormal arborization

Table 1: The hereditary disorders of copper metabolism

Table 2

Enzyme	Function	Consequences of Loss
Tyrosinase	Melanin formation	Albinism
Lysyl oxidase	Collagen and elastin cross-link formation	Perinatal death: arterial aneurysms, diaphragmatic rupture (Hornstra et al. 2003)
Peptidylglycine α-amidating monooxygenase	Acitavtion of peptides with α-terminal glycine	Embryonic lethality: cardiac failure (normal CNS) (Czyzyk et al. 2005)
Cu/Zn superoxide dismutase	Antioxidant defense (superoxide dismutation)	Impaired pulmonay defenses to paraquat; infertility (Ho et al. 1998)
Ceruloplasmin	Ferroxidase	Parenchymal iron overload, anemia, diabetes, neurodegeneration
Hephaestin	Ferroxidase	Impaired iron absorption, anemia (Vulpe et al.1999)
Dopamine-β-hydroxylase	Norepinephrine synthesis	Impaired sympathetic regulation, hypotension, hypoglycemia
Cytochrome c oxidase	Oxidative phosphorylation	Encephalopathy, muscle weakness, cardiac failure - neonate

Table 2: The cuproenzymes

Table 3

	Aceruoloplasminemia	Neuroferritinopathy
Genetics	Autosomal recessive	Autosomal dominant
	Loss-of-function mutations	Dominant negative
	Ceruloplasmin gene	Ferritin light chain gene
Presentation	Third decade – diabetes	Third through sixth decade
	Fifth decade – neurologic	
Defect	Brain iron recycling	Brain iron storage
Pathogenesis	Brain iron accumulation Systemic iron accumulation	Brain iron accumulation
Clinical	Diabetes, anemia, dementia, dystonia, dysarthria	Dementia, dystonia, dysarthria
Pathology	Iron accumulation in astrocytes	Iron accumulation in astrocytes
	Neuronal cell loss	Neuronal loss

Table 3: The hereditary disorders of brain iron metabolism

Figure 1

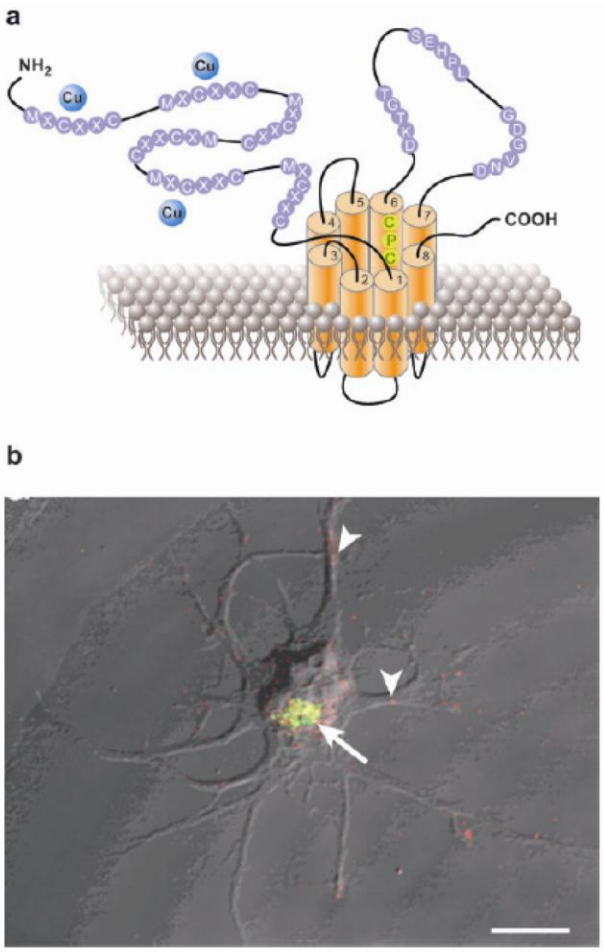

Figure 1

The copper-transporting P-type Atpases. a: Proposed structure of the Atpase homologues Atp7a and Atp7b. Motifs highlighted are the MXCXXC copper-binding motifs in the amino terminus, the DKTGT sequence that is the canonical phosphorylation site, the GDGVND Atp binding domain and the conserved SEHPL, which contains the most common mutation (H1069Q) inWilson disease. Modified from Schaefer & Gitlin (1999) b. Merged image of Atp7a (red) and late Golgi resident syntaxin 6 (green) in a Nomarski differential interference contrast image of a developing hippocampal neuron. Arrowheads reveal substantial overlap of Atp7a in Golgi, whereas the arrow indicates Atp7a in processes. Scale bar 25 μM. Modified from Schlief et al. (2006).

Figure 2

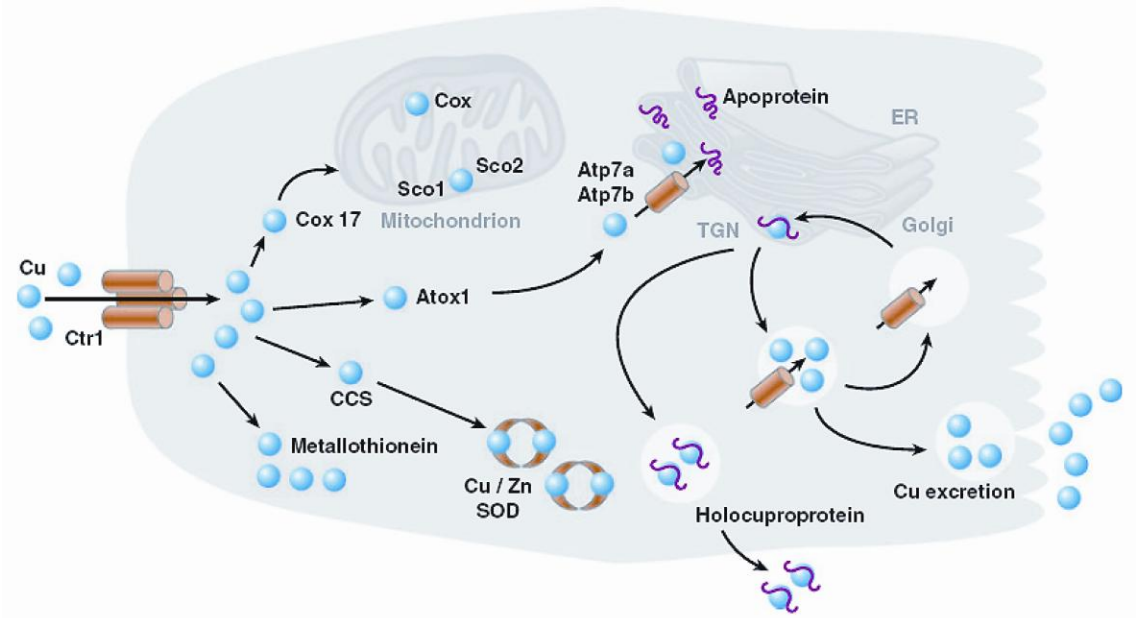

Figure 2

Cellular copper homeostasis. Model of copper trafficking in polarized cell reveals copper entry via Ctr1 followed by distribution to the copper chaperones. The copper chaperone for superoxide dismutase (CCS) delivers copper to Cu/Zn superoxide dismutase (SOD1), Atox1 delivers copper to one of the Atpases (Atp7a/Atp7b) in the late Golgi and Cox17, and Sco1 and Sco2 are involved in the pathway of copper trafficking to mitochondria and cytochrome oxidase (Cox). The Atpases transport copper into the secretory pathway for incorporation into newly synthesized cupropro-teins and for export from the cell. Metallothionein serves to chelate most available copper and is critical for cell survival in copper excess.

Figure 3

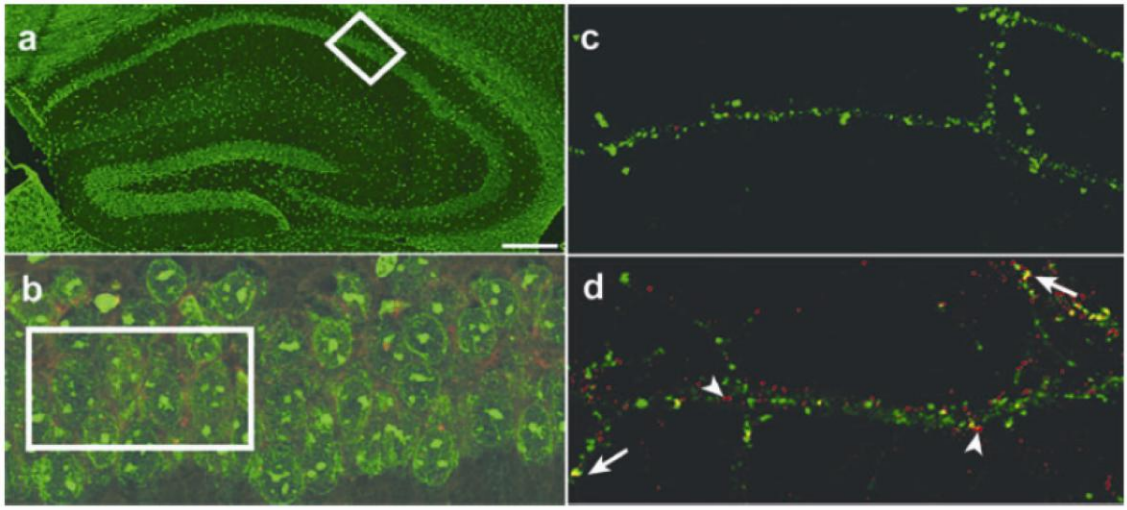

Figure 3

Atp7A expression and NMDA receptor–mediated trafficking. a: NeuroTrace staining of neurons revealing the overall architecture of the mouse hippocampus. Scale bar 150 μ m. b: Boxed area in a enlarged as merged image of Atp7a (red) and NeuroTrace reveals Atp7a expression in perinuclear neuronal compartment. c: Hippocampal neurons treated for 24 h with a 200-μM concentration of the copper chelator BCS and immuno-labeled for PSD-95 (green) and Atp7a (red). d: Hippocampal neurons treated for 24 h with a 200-μM concentration of the copper chelator BCS and 50 μM glutamate, 5 μM glycine, and immunolabeled for PSD-95 (green) and Atp7a (red). Upon activation of NMDA receptors, Atp7a traffics from cell bodies down axonal processes localizing in part with postsynaptic densities (PSD). Arrows indicate regions of Atp7a overlap; arrowheads indicate regions distinct from PSD. Modified from Schlief et al. (2005).

Figure 4

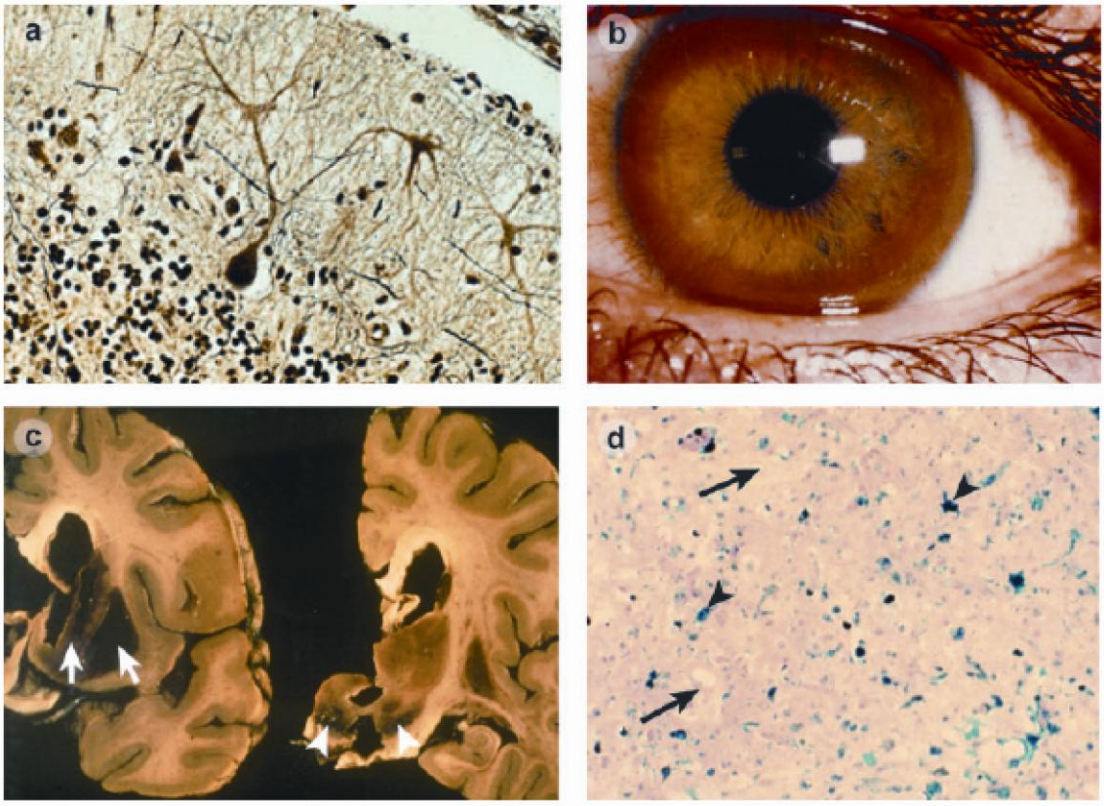

Figure 4

Pathology in copper and iron disorders of the brain. a: Section of cerebellum from Menkes patient brain reveals abnormal Purkinje cell with axonal swelling and arborization (courtesy of K. Roth). b: Copper accumulation inWilson disease visible as Kayser-Fleischer rings in Descemet's membrane at the limbus of the cornea (Gitlin 2003). c: Coronal brain section in aceruloplasminemia reveals cavitary degeneration and discoloration of basal ganglia (arrowheads). d: Prussian blue stain of putamen in aceruloplasminemia reveals iron accumulation in astrocytes and neurons (arrowheads) with neuronal loss (arrows) (Morita et al. 1995).

Figure 5

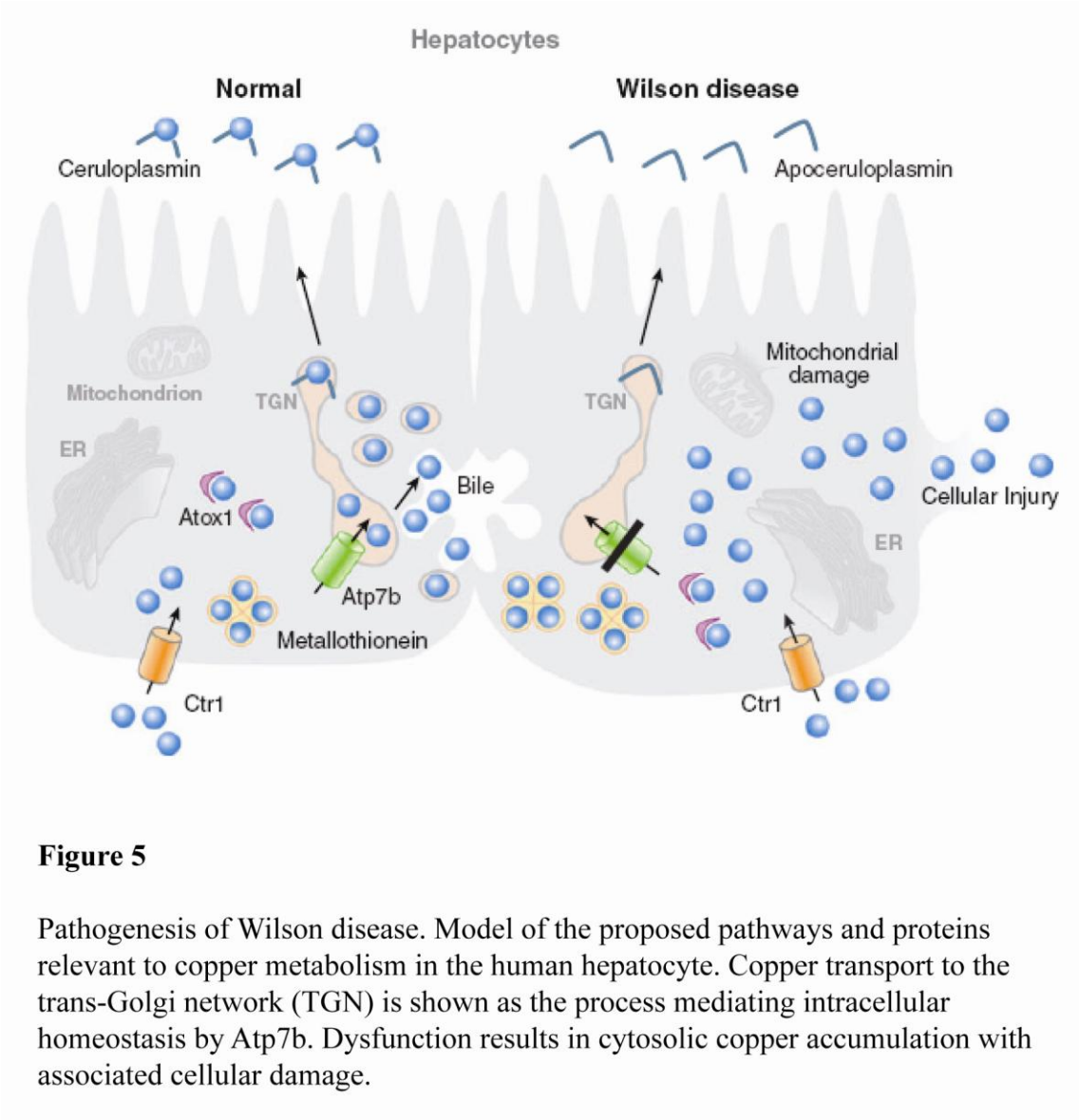

Figure 5

Pathogenesis of Wilson disease. Model of the proposed pathways and proteins relevant to copper metabolism in the human hepatocyte. Copper transport to the trans-Golgi network (TGN) is shown as the process mediating intracellular homeostasis by Atp7b. Dysfunction results in cytosolic copper accumulation with associated cellular damage.

Figure 6

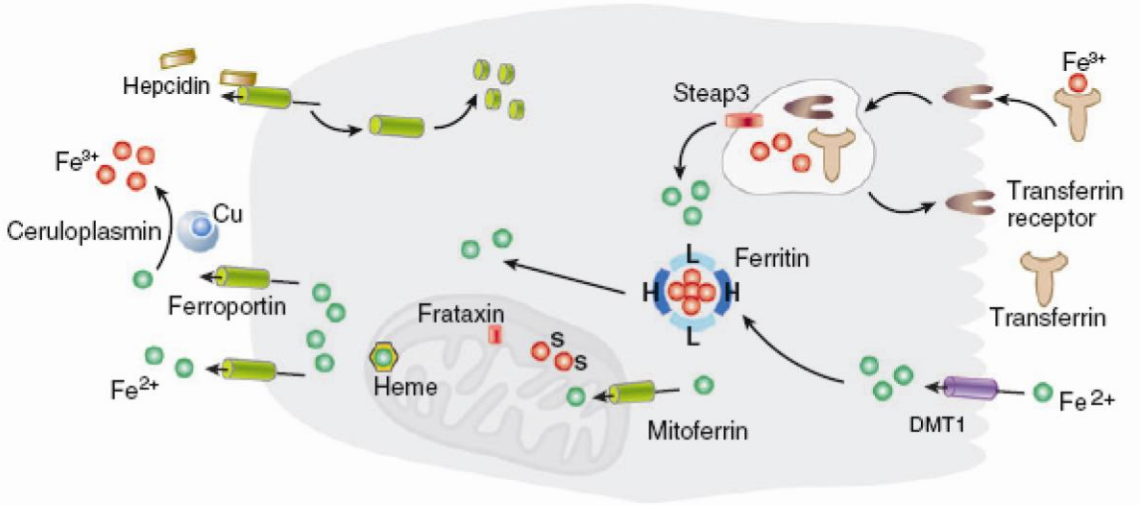

Figure 6

Pathways of cellular iron homeostasis. Iron uptake can occur via the transferrin receptor and the divalent transporter DMT1. Steap3 is a ferrireductase critical for transferrin-mediated iron release into the cell. Ferritin is the predominant storage protein consisting of heavy chains with ferroxidase activity and light chains. Ferroportin is the only known cellular iron exporter, and ceruloplasmin is a ferroxidase mediating efficient cellular iron release. Iron homeostasis is regulated by hepcidin, a circulating peptide that binds to ferroportin, mediating uptake and degradation of this exporter. Iron enters mitochondria via mitoferrin, and frataxin is a mitochondrial protein mediating Fe-S cluster formation and heme biosynthesis.

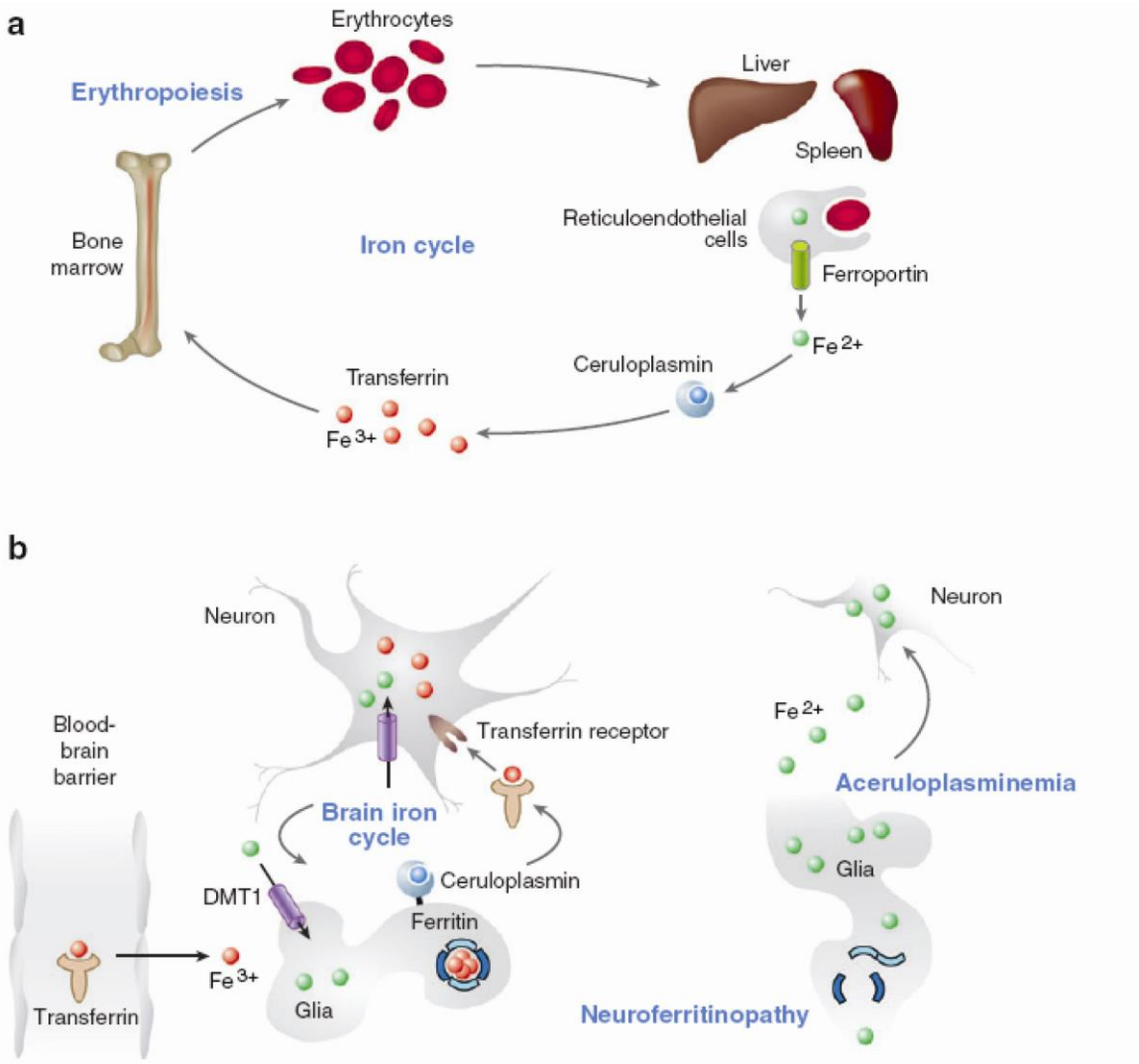

Figure 7

Iron cycles and the pathogenesis of brain iron disease. a: Systemic iron cycle allows for rapid utilization of iron and is dependent on ceruloplasmin to establish a rate of iron oxidation sufficient for mobilization from reticuloendothelium. b: Iron crosses the blood brain barrier via transferrin receptor pathway on endothelium. The brain iron cycle consists of glia and neurons, where gpi-linked ceruloplasmin functions in a similar role as in the periphery. In aceruloplasminemia, excess iron accumulation damages glia, and neurons are subsequently injured from loss of glia-derived factors, iron-deficiency, or accumulation of nontransferrin-bound iron. A similar pathogenesis is proposed for neuroferritinopathy.

Chapter 3

In Vivo Correction of a Menkes Disease Model
Using Antisense Oligonucleotides

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In Vivo Correction of a Menkes Disease Model Using Antisense Oligonucleotides

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Abstract

Although the molecular basis of many inherited metabolic diseases has been defined, the availability of effective therapies in such disorders remains problematic. Menkes disease is a fatal neurodegenerative disorder due to loss-of-function mutations in the *ATP7A* gene encoding a copper transporting P-type Atpase. To develop novel therapeutic approaches in affected patients we have identified a zebrafish model of Menkes disease termed *calamity* that results from splicing defects in the zebrafish orthologue of the *ATP7A* gene. Embryonic recessive lethal mutants have impaired copper homeostasis that results in absent melanin pigmentation, impaired notochord formation and hindbrain neurodegeneration. In this current study we have attempted to rescue these striking phenotypic alterations utilizing a series of antisense morpholino oligonucleotides directed against the splice-site junctions of two mutant *calamity* alleles. Our findings reveal a robust and complete correction of the copper-deficient defects of *calamity* in association with the generation of wild-type Menkes protein in all rescued mutants. Interestingly, quantitative analysis of *atp7a* specific transcripts suggests that competitive translational regulation may account for the synthesis of wild-type protein in these embryos. This *in vivo* correction of Menkes disease through rescue of aberrant splicing may provide new therapeutic options in this fatal disease and illustrates the potential for zebrafish models of human genetic disease in the development of treatments based on the principles of interactions of synthetic oligonucleotide analogues with mRNA.

Introduction

Menkes disease is an inherited metabolic disease due to loss of function mutations in the *ATP7A* gene encoding a P-type Atpase required for copper absorption and

homeostasis (Lutsenko et al., 2007; Lutsenko and Petris, 2003). Patients with Menkes disease have absent melanin pigmentation, impaired extracellular matrix formation and neurodegeneration resulting in intractable seizures, hypotonia, severe failure to thrive and death in early infancy (Culotta and Gitlin, 2001b; Kaler, 1998). These pleiotropic phenotypes result from loss of activity of essential cuproenzymes within distinct subcellular compartments. Treatment with exogenous copper is largely ineffective (Sheela et al., 2005) and absent development of directed pharmacotherapy, restoration of normal gene function remains the most viable treatment for affected patients. While partial correction of a murine model of Menkes disease was demonstrated utilizing a human Menkes transgene (Llanos et al., 2006), methods of safely introducing wild-type cDNAs into humans have remained elusive.

We recently utilized chemical genetics to obtain *calamity*, a zebrafish model of Menkes disease (Mendelsohn et al., 2006). Embryonic mutants reveal a striking phenotype with absent melanin pigment, impaired notochord formation and neurodegeneration. As several *calamity* alleles arise from splicing defects in the zebrafish orthologue of *ATP7A*, we reasoned that if normal splicing could be restored sufficient wild-type message might be generated to rescue these defects. Furthermore, as previous studies of copper nutrition in the zebrafish embryo revealed a hierarchy of temporal and dosage-dependent phenotypes (Mendelsohn et al., 2006) unique therapeutic windows might allow for long-term correction following transient restoration of wild-type expression. Such an approach has broad applicability as many metabolic diseases arise from mutations that interfere with normal splicing of pre-mRNA message (Wang and Cooper, 2007). In such cases the threshold of wild-type transcript necessary to restore

functional protein may require only a small shift in favor of the wild-type message in order to ameliorate the disease.

RNA targeting has recently emerged as a potential alternative to more conventional approaches in gene therapy (Wood et al., 2007). Antisense oligonucleotides which bind to pre-mRNA and sterically alter processing have been used successfully in cell culture to demonstrate the utility of this approach (Bruno et al., 2004; Du et al., 2007; Gebski et al., 2003; Suwanmanee et al., 2002). More recently, in murine models of Duchenne muscular dystrophy, systemic delivery of morpholino oligonucleotides has been shown to effect partial restoration of protein function on a cellular level in mutant mice (Alter et al., 2006; Lu et al., 2005; Moulton et al., 2007). A critical factor in such strategies for the treatment of human metabolic disorders is the availability of robust disease models that permit rapid screening for effective phenotypic rescue. In this current study we have utilized such an approach to rescue the phenotype of *calamity*, demonstrating the applicability to complex organisms of several recent cell culture studies using this technology in a number of human genetic diseases (Du et al., 2007; Moulton et al., 2007; Ugarte et al., 2007).

Results and Discussion

Recently, our lab has described the zebrafish mutant *calamity* (allele vu69) as a model of Menkes disease that recapitulates key aspects of the human phenotype (Mendelsohn et al., 2006) (Fig. 1A). The defect in *calamity*^{vu69} is caused by creation of a new 3' splice site resulting in a 7 base-pair insertion into the mRNA (Fig. 1F). The subsequent frame-shift creates an early stop codon and loss of greater than 95% of the

protein product (Fig. 1 D,E). In the course of continued screening we have identified a second allele of *calamity*, designated gw246. This allele causes an identical phenotype to the first and is non-complementary in a standard genetic cross (Fig. 1 B, C). Cloning of the mRNA in this mutant revealed a 12 base-pair in-frame deletion at the 3' end of exon 20 which removes 4 amino acids located in the critical and highly conserved ATPase domain of Atp7a (Fig. 1F). This deletion at the junction of two exons is due to the preferential use of a new 5' splice site. Interestingly, genomic sequence analysis revealed mutation of neither the wild-type 5' splice site (splice donor) nor creation of a new GT splice donor pair; rather, the mutation was five base-pairs downstream from the new mutant splice donor. Comparison of the mutant sequence to the previously established zebrafish splice donor consensus sequence indicated that a new splice consensus site had been formed (Yeo et al., 2004). Given that the mutant displays such a severe phenotype we inferred that this new consensus sequence creates a dominant splice donor to the near exclusion of the wild-type donor. High-resolution separation of a small PCR product from mutant cDNA demonstrates that this is indeed the case (Fig. 2B, lane 1). In this mutant, any wild-type splicing that does occur retains a single base change which causes a non-conservative amino acid change from Leu to Arg at position 1316 (Fig. 1F). Immunoblots with an antibody raised against a C-terminal peptide from zebrafish Atp7a revealed near-complete loss of protein product in both vu69 and gw246 (Fig. 1E). This lack of protein product in the gw246 allele suggests that there may be an early processing defect which causes rapid and early degradation of the protein.

The presence of the wild-type splice sites in both alleles of *calamity* immediately caused us to consider the use of morpholinos directed to specific sequences near the

mutant splice sites in a bid to “force” wild-type splicing events. There was no data available indicating the precise location that would be best suited for accomplishing this goal. At the same time, the nature of both alleles provided an ideal system for investigating this very question. In the case of gw246 we had two competing splice donors sufficiently far apart that we might interfere with one or the other but close enough that we might also block both.

Design of a series of morpholinos that would “walk” along the exon-intron boundary sequence was based on comparison with known consensus sequences (Fig. 2A). The basic 5' splice site consensus sequence is small and well defined making targeting straightforward for the gw246 mutant (Yeo et al., 2004). Wherever a morpholino sequence overlapped a mutation, the mutant sequence was used at that base pair. The first series of morpholinos were injected at a dose of 1.44 pmol/embryo (12ng/embryo) into intercrosses of gw246 heterozygous carriers. At 48hpf embryos were examined for phenotypic effects and mutants collected for mRNA extraction and RT-PCR. The effects on splicing are shown in Fig. 2B. Across the series of morpholinos several splice forms were seen. We confirmed that each band is a splice form of a*tp7a* via direct sequencing of the RT-PCR product. Several patterns emerged from these experiments. First, and most interesting, blocking of the mutant splice donor did indeed restore a significant amount of wild-type splice product (lanes e2-e3); however, this was not accompanied by a rescue of the phenotype. We surmised that this was due to the retention of a single base change caused by the mutation in the final, properly spliced transcript which results in the non-conservative L1316R substitution. Such a large change in polarity and charge in a highly conserved region of this ATPase might be expected to

disrupt function. Consistent with this hypothesis, when we introduced this mutation into the wild-type cDNA it resulted in an ATPase that fails to deliver copper to tyrosinase in transiently transfected *ATP7A*-deficient fibroblasts indicating that this mutation results in an inactive transporter (Sup. Fig. 1). Alternatively, the mutation may render the protein unstable leading to early degradation and subsequent loss of function.

Second, injection of the series of morpholinos revealed several other weak cryptic splice sites all contained within exon 20, immediately 5' to the mutation (Fig. 2). This is most clearly seen when the morpholino overlies both the wild-type and mutant sites (lanes e4-e5). There was no evidence of exon skipping induced by any of the morpholinos (data not shown). Bands corresponding to inclusion of the entire intron in the final product were seen, but we could not totally rule out the possibility of genomic contamination of the cDNA as the source of these bands (data not shown).

Thirdly, the morpholino that accomplished the most robust rescue of mRNA splicing (lane e3) overlay the mutant splice site on either side and remained at least 6 base-pairs 5' to the wild-type GT pair. Moving only four bases in either direction resulted in either an increased use of the mutant site or an increase in the upstream cryptic sites due to blockage of the wild-type and mutant sites (lane e4-e5).

Lastly, when the morpholino target sequence was located 3' to the mutant splice donor the mutant again became the dominant splice form (lane e7). Wild-type embryos injected with this same morpholino fully displayed the *calamity* phenotype (Sup. Fig. 2B). Combined with the RT-PCR data this indicates that when the wild-type 5' splice site is blocked by a morpholino the next most robust cryptic site is the one used in the mutant. Injection of wild-type embryos also resulted in the same pattern of splicing illustrating

why the mutation is so easily capable of co-opting splicing to the cryptic site in the mutant (Sup. Fig. 2A).

These specific observations on a trial-and-error approach allow us to propose a set of generalities regarding morpholino targeting to an exon-intron junction. First, morpholinos targeted within 30bp of an 5' splice site will have an effect on splicing, even if it is subtle. Secondly, in some cases it appears that the magnitude of the effect of the morpholino is dependent not only on its distance from the splice site, but also upon the general strength of the splice consensus (cf. e1 and e4) and surrounding cryptic sites. Thirdly, optimal blocking occurs in two forms: 1) the morpholino is entirely exonic except for 2bp overlap with the GT pair (e2 and e5), or 2) the morpholino extends at least 6 bp on either side of the exon-intron junction. Fourthly, morpholinos can reveal unappreciated enhancers and suppressors of specific splicing events located outside of the consensus sequence (e10) and can be used to change the hierarchy of cryptic splicing events. Validation of these rules will require separate experiments in a different mutant with a similar defect and distinct phenotype; however, they do establish a framework which can guide further testing.

Taken together these results demonstrate the utility of using morpholinos to correct aberrant splicing in an embryonic vertebrate in cases where wild-type splice donors are not affected. Morpholino e3 strongly rescues the mRNA. Unfortunately we did not see a corresponding rescue of the phenotype. That this mutant protein was incapable of rescue indicates one of the potential caveats of this system – that the retention of a mutation in the rescued mRNA can just as strongly affect the phenotype of the organism as the mis-spliced sequence. Promisingly however, it has been estimated

that the probability that a random amino acid change would cause protein inactivation is around 34% (Guo et al., 2004). Conversely, this would mean that just under two-thirds of random amino acid changes will result in functional protein sequence. Together with alternate approaches such as forced exon skipping the probability of rescuing any given splice mutation with morpholinos remains fairly high.

Unlike the gw246 allele, rescue of vu69 would not cause inclusion of the mutated base but would fully restore the wild-type sequence without alteration. Also unlike gw246 the mutation alters the splice acceptor. The length and placement of consensus splicing sequences around the splice acceptor are much less well defined and more degenerate making targeting predictions much more cumbersome (Yeo et al., 2004). Given the proximity of the vu69 splice acceptor to the polypyrimidine tract, we reasoned that directly overlapping the mutant splice acceptor with a morpholino would interfere with wild-type splicing. Similarly, we assumed that both mutant and wild-type splice acceptors would use the same polypyrimidine tract, making it difficult to preferentially target one splice acceptor by direct targeting of the polypyrimidine tract. However, the inherent degeneracy of the U2AF recognition sequence might permit a morpholino placed nearby to sterically interfere and alter the exact location of binding of the U2AF to the polypyrimidine tract (Sickmier et al., 2006). No structural data existed to aid in the design of such a morpholino. Therefore, we took a similar approach using a series of morpholinos that "walk" across the splice acceptor region but expanded our target area to include sequence up to 73 base-pairs 5' to the mutant acceptor (Fig. 3A). Briefly, morpholinos i1 and i2 both caused a more severe phenotype in *calamity* mutants and heterozygotes implying an interference with wild-type splice events (Data not shown).

Morpholinos i5 to i7 had no effect on phenotype. Morpholinos i3, i3.5 and i4 caused robust rescue of the phenotype of mutant *calamity* embryos (Fig. 3B-G). Melanin pigmentation was restored and the notochord did not contain any discernible defects compared with *calamity* mutant embryos. The rescue was seen in 100% of the mutant embryos with all injected mutants containing at least a few melanocytes and 95% having greater than 20; no injected mutant embryo had a discernable notochord defect when scored at 48 hpf (Fig. 3H). The rescue effect was still clearly visible at 6 days post fertilization when about 75% of the uninjected *calamity* embryos were either dead or dysmorphic (Fig. 3I) compared with nearly normal looking rescued embryos (Fig. 3J). Lowering the dose of the morpholino resulted in similar pigmentation with intermediate notochord defects consistent with previous observations on the developmental hierarchy of copper metabolism that revealed a critical threshold of lysyl oxidase activity necessary for normal notochord development (data not shown) (Gansner et al., 2007b; Mendelsohn et al., 2006). Sequencing of genomic DNA confirmed that the rescued embryos were indeed mutant (Data not shown).

As the mutant embryos had clear phenotypic rescue we wanted to confirm that this was the result of restored protein expression. Immunoblot analysis of injected and rescued mutants with a zebrafish Atp7a-specific antibody shows the restoration of full-length, wild-type protein in the rescued embryos compared with no visible protein in uninjected mutants (Fig. 4A). To our surprise however, this did not correspond with an increase in wild-type splice products as measured via qRT-PCR . Only three consistent patterns emerged from these experiments. The first is the presence of a small amount of amplifiable wild-type transcript in all of the mutants, consistent with the presence of a

wild-type band when end-point RT-PCR products are run on a polyacrylamide gel (Fig. 4C and Sup. Fig. 3B,C). The second is that there is decreased total a*tp7a* transcript in the mutants probably due to nonsense-mediated decay of mutant transcripts (Sup. Fig. 3D). The third is that there is a significant reduction of mutant transcript in the injected embryos. The most likely explanation for this reduction is the shifting of this transcript to a third splice form, which is corroborated by the RT-PCR gel analysis showing a third splice form containing an 800bp intronic insertion due to the use of a cryptic 3' splice site within the intron (Fig. 4C). Sequence analysis of the alternate splice form indicates a frame shift and early stop codon, analogous to the original mutation. It is important to note that as the antibody used for immunoblot recognizes the C-terminus of the wild-type zebrafish protein, the identification of full-length protein will only occur when wild-type transcripts are present.

Both the reduction in mutant transcript as well as the lack of any increase in wild-type transcript levels as measured by qRT-PCR were consistent across three embryo ages, 12, 24, and 48 hours post-fertilization (data not shown) indicating that there is not an early correction of splicing that decreases as the morpholino is diluted during development. The lack of a measurable substantial increase in wild-type transcript in these studies could simply reflect the limits of quantitation for the threshold necessary to result in rescue. If this is the case, then it suggests that only small changes in wild-type mRNA can result in significant phenotypic improvement on an organismal level, at least in the case of Menkes disease. Alternatively, the data suggest that the increase in protein levels could result from removal of inhibition on translation of the wild-type transcript. Such inhibition could arise from competition for translation from the mutant transcript, a

concept that would be consistent with observations on translational competition in *Drosophila (Gebauer et al., 1999)* and is supported by our finding that knock-down of the mutant transcript best correlates with rescue of the *calamity* phenotype through restoration of protein levels (Fig. 4). Although this proposed mechanism for the increase in functional protein is not directly testable under these conditions, if plausible this significantly broadens the applicability of the morpholino approach because it demonstrates that loss of one particular mutant form may be sufficient to cause rescue of protein function and phenotype, with only minimal increases in the amount of wild-type mRNA.

Taken together, the data reported here have several implications of direct relevance to the development of effective therapeutics in Menkes disease. Experimental (Gansner et al., 2007a; Mendelsohn et al., 2006) and clinical observations in affected patients (Tang et al., 2006) reveal that maintenance of central nervous system function in this disease is prioritized under circumstances of limited copper availability or decreased ATP7A function. This hierarchy of copper delivery suggests that prevention of the neurodegenerative features in Menkes disease may be possible with therapeutic interventions that result in a modest increase in ATP7A within a specific developmental window. The *in vivo* rescue observed in these studies directly supports these pathophysiologic concepts and raises the potential for *in utero* interventions in this disease. The data in this paper demonstrate the utility of zebrafish for rapid screens to identify specific antisense sequences effective in phenotypic rescue. While morpholino delivery remains problematic, given the robustness of the assay reported here and the increasing number of zebrafish models of human genetic disease (Ackermann and Paw,

2003) these findings support further study of this technology in the development of effective therapeutics for inherited metabolic disease.

Methods

Animal maintenance. Fish stock maintenance was performed according to institutionally approved procedures. Embryos for experiments were obtained through *in vitro* fertilization and incubated at 28.5°C. Wild-type fish were AB strain. The mutant *calamity* has been described previously (Mendelsohn et al., 2006). Mutagenesis of wild-type fish was performed using the chemical mutagen ethyl nitrosourea (ENU). Mutants were screened for the *calamity* phenotype comprising lack of pigmentation and notochord defects. Putative mutants were crossed to *calamity* to verify allelism. These were then out crossed to AB fish and grown to adulthood. Regions of genomic DNA containing putative mutations were sequenced in both AB and WIK strains to rule out the possibility of strain-dependent polymorphisms.

Morpholino Synthesis and Injection. Morpholinos were synthesized by GeneTools, LLC. Morpholinos were dissolved to 2mM in water and stored at -80C. Injection of morpholinos was performed on a microinjection apparatus (Harvard Instruments). A constant volume (1.44nL) of morpholinos was injected at different dilutions of the stock (1:2 to 1:10) to optimize the dose which gave the strongest phenotypic effect while minimizing off-target effects.

Molecular cloning and PCR. Total RNA was extracted from embryos using the Trizol reagent (Invitrogen). Random hexamer primed cDNA was synthesized using SuperScriptIII according to manufacturer's protocol (Invitrogen). PCR was performed

using Phusion DNA polymerase (Finnzymes). High-resolution separation of products was performed on 10% TBE-polyacrylamide gels. All sequences were verified by direct sequencing. The L1316R clone of zebrafish a*tp7a* was generated from the wild-type sequence using the QuickChangeII site-directed mutagenesis kit (Stratagene). The altered clone was sequenced and other PCR errors excluded.

qPCR. qRT-PCR was performed using primers specific to either the mutant or wild-type form of the Menkes transcript. This was accomplished by designing primers that were complementary to the exon-exon junction affected in the mutant (Sup. Fig. 3A). The following primer sequences were used: Common forward primer ATGATGAGCTCCGGACAGAC. *calamity* specific reverse GGAATGATCTTTTCCACCTGAG. Wild-type specific reverse GGAATGATCTTTTCCACTGTCG. Total a*tp7a* primers TGGAGCTTGTGGTCAGAGG and AGGGCAACTGAAGCGTAGAG. Ornithine decarboxylase primers ATCTGGATCTCCGTTTTGCT and CCGTTTTACGCAGTGAAGTG. Primer efficiencies were calculated in triplicate for each primer set using either wild-type or mutant cDNA respectively. Random hexamer primed cDNA was synthesized using SuperScript III (Invitrogen) according to manufacturer's protocol. Quantitative PCR was carried out on an iCycler (BioRad) with iQ Sybr Green SuperMix (BioRad). Calculations were performed using the Pfaffl method and each bar in the figures is the average of 3 to 5 individually prepared embryo RNA samples. Relative to wild-type, all cal embryos had a 50% decrease in total *atp7a* transcript (data not shown).

Immunoblots. Twenty to thirty 48hpf zebrafish embryos were gently homogenized in sucrose homogenization buffer (250mM sucrose, 5mM Tris pH 7.4) plus protease inhibitors (Roche) and then spun at 100,000rpm in a table-top Beckman ultracentrifuge for 30 min. The pellet was then resuspended in 50μL RIPA buffer. Alternatively, 48hpf embryos were manually deyolked and homogenized directly in RIPA buffer. Equal amounts of protein were loaded on 6% polyacrylamide gels. After transfer to nitrocellulose, immunoblotting was performed using a rabbit polyclonal antibody raised to a C-terminal peptide of zebrafish Atp7a. Anti-rabbit secondary antibody and ECL reagents were purchased from Pierce and used according to manufacturer's recommendations.

Acknowledgments

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Figure 1

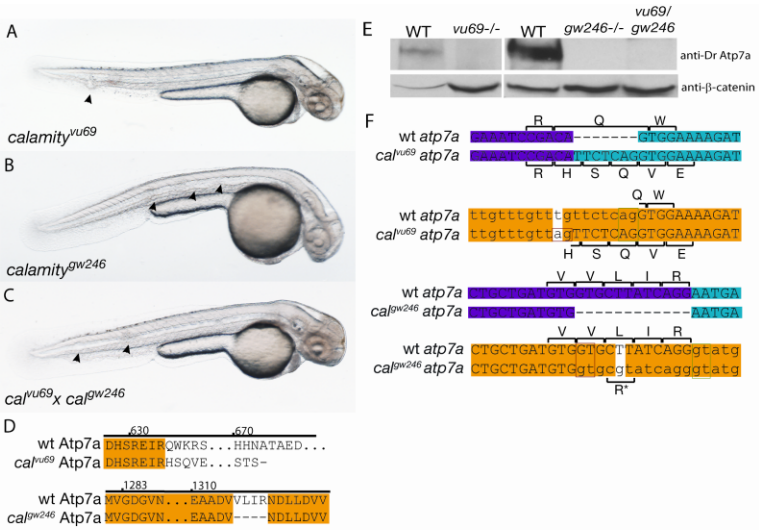


Fig. 1. Comparison of phenotype, genotype, and protein expression for two alleles of *calamity*. (A) *calamity*^{vu69}, characterized elsewhere , displays the copper deficient phenotype of loss of pigmentation, notochord abnormalities (arrowheads), and an enlarged ventricle and is due to a loss of function of atp7a. (B) *calamity*^{gw246}, a second allele of *atp7a* has the same phenotype. (C) *cal*^{gw246} fails to complement *cal*^{vu69} confirming allelism. (D) Amino acid sequence changes caused by the mutations. *cal*^{vu69} results in a frameshift and truncation of the C-terminus of the protein. *cal*^{gw246} contains an in-frame deletion in the highly conserved ATPase domain of Atp7a. (E) Immunoblot analysis using an antibody made to a C-terminal peptide from zebrafish Atp7a shows complete loss of wild-type protein in *cal*^{vu69}, *cal*^{gw246}, and in the compound heterozygote. (F) The mRNA and genomic sequences of *cal*$^{vu69-/-}$ (top) and *cal*^{gw246} (bottom) highlighting the mutation and subsequent changes in amino acid sequence due to mis-splicing of exons through aberrant use of mutant splice sites (red boxes). Exon boundaries in the mRNA are illustrated by a change in color from magenta to blue. In the genomic sequence introns are in lower case and the mutations are the bases with the white background. The green boxes delineate the wild-type splice donor/acceptor pair and red boxes the mutant pair. For *cal*$^{gw246-/-}$ a small percentage of transcripts splice at the wild-type splice junction retaining the non-synonymous mutation, a Leu to Arg amino acid subsitution(*).

Figure 2

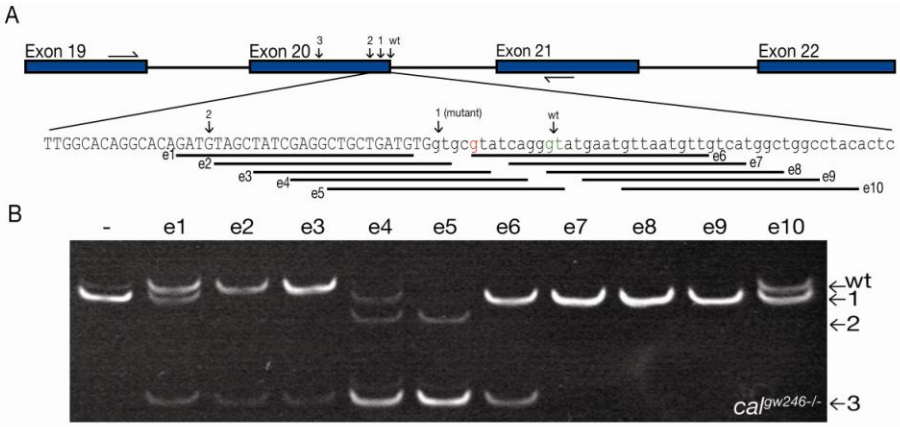


Fig. 2. A series of morpholinos causes alterations of splicing in *cal*^{gw246}.
(A) Schematic and sequence information of the morpholino design for exploring alterations in splicing caused by variable morpholino placement with respect to wild-type and mutant splice donors. Exon sequence is upper case. The mutation is red. Lines indicate the binding site of each listed morpholino (B) Injection of the series of morpholinos in (A) into *cal*^{gw246}. Splice forms were amplified by PCR of cDNA using the primers illustrated in (A). Wild-type, and 3 alternate splice forms were seen on polyacrylamide gel stained with ethidium bromide. Each was sequenced to identify exact locations of splicing which are illustrated in (A). All cryptic splice forms were located within Exon 20.

Figure 3

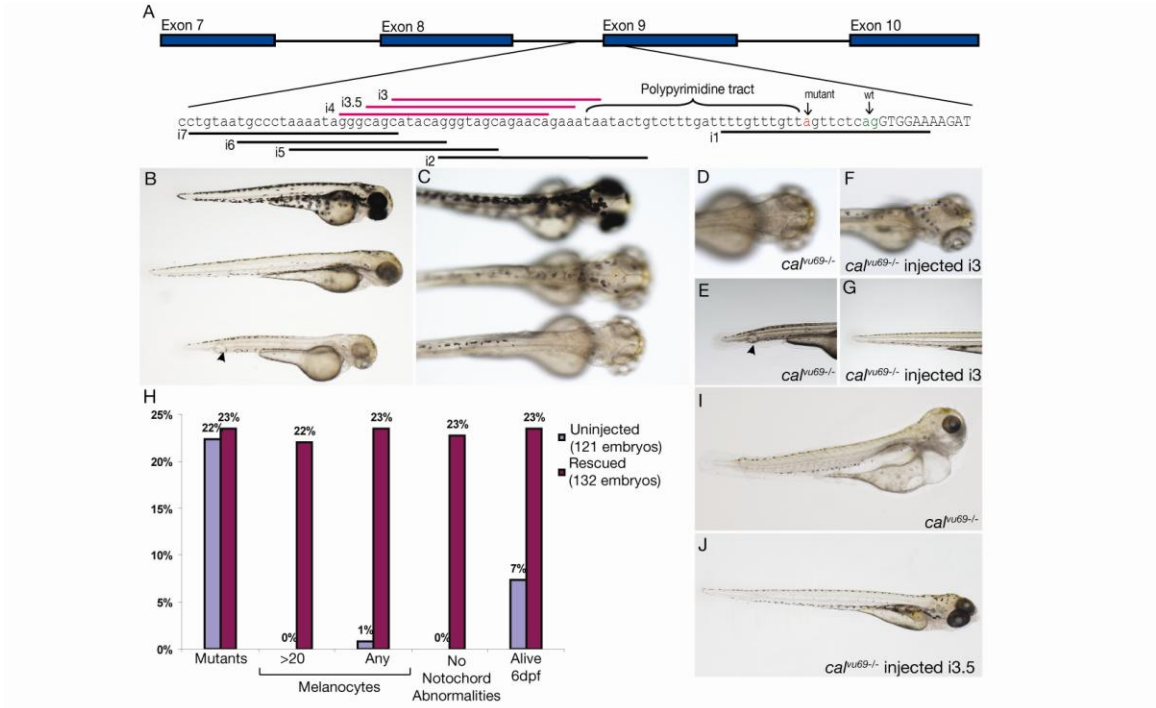


Fig. 3. Morpholinos rescue the phenotype of *cal*^{vu69} embryos.
(A) Schematic and sequence information for the design of morpholinos directed towards rescue of the mutant splicing in *cal*^{vu69}. Wild-type exon is uppercase. The mutation is highlighted in red and the wild-type splice acceptor in green. Each morpholino binding site is indicated by a line located 5' to the polypyrimidine tract. Morpholinos which cause phenotypic rescue are highlighted in pink. Morpholino i1 served as a control to demonstrate that blocking of proper splicing at this exon-exon boundary would result in the calamity phenotype (data not shown). (B) and (C) Side-by-side comparisons of 48hpf zebrafish. Top: wild-type sibling from calvu69 intercross clutch. Bottom: *cal*$^{vu69/vu69}$ mutant embryo. Middle: *cal*$^{vu69/vu69}$ embryo rescued by injection of morpholino i3. (D) and (E) Close up view of *cal*$^{vu69/vu69}$ uninjected embryo showing lack of melanin pigmentation over head region and notochord abnormalities (arrowhead). (F) and (G) Close-up view of morpholino i3 rescued *cal*$^{vu69/vu69}$ embryo showing restoration of melanin pigmentation and a notochord absent of defects. (H) Quantification of the extent of rescue. A single group of embryos derived from several clutches, half injected with rescuing morpholino (red bars; uninjected – blue bars), was scored for the presence of any or a significant number of melanocytes (>20) and the presence of notochord defects. The scale of the graph reflects the expected 25% ratio of homozygous mutants derived from the heterozygous intercross. (I) and (J) Six day post-fertilization embryos either uninjected (I) or injected (J) with rescuing morpholino i3.5. Rescued embryos maintain near normal body morphology while unrescued embryos swell, possibly due to decreased extracellular matrix integrity due to the loss of lysyl oxidase activity.

Figure 4

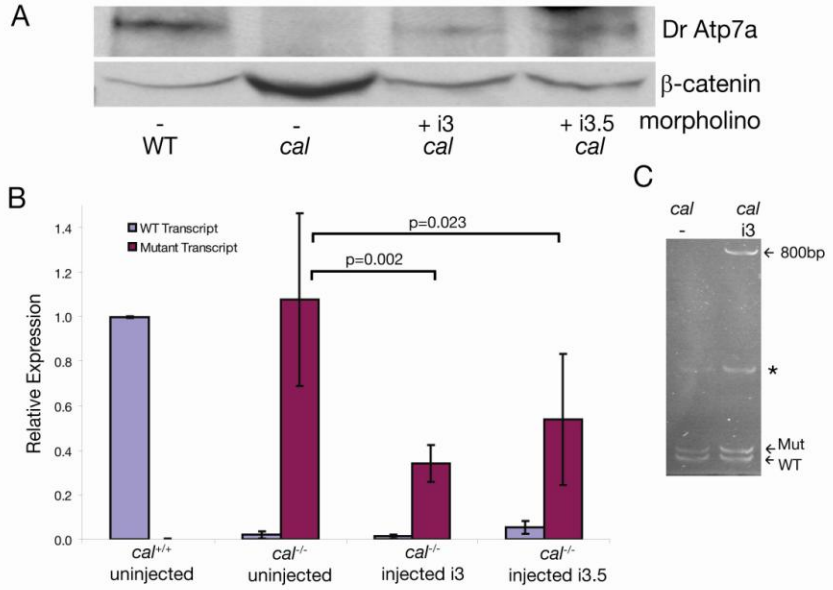
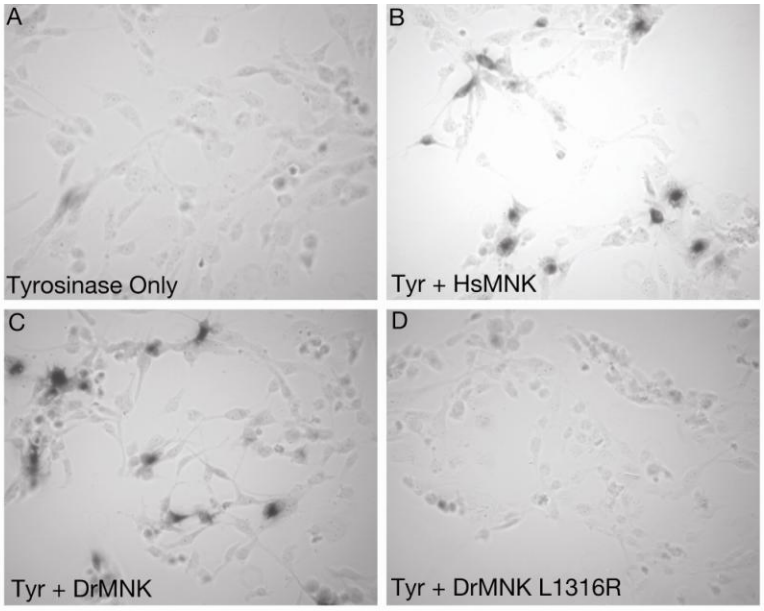


Fig. 4. Rescue morpholinos cause restoration of full-length protein without detectable changes in mRNA.

(A) Phenotypic rescue by morpholinos i3 and i3.5 is accompanied by restoration of wild-type protein. Lysates from 48hpf embryos of the indicated genotype were run on a 6% SDS-PAGE gel and blotted for zebrafish Atp7a using an antibody generated to the zebrafish protein. β-catenin was used as a loading control. Morpholino injection restored protein to 30-45% of wild-type levels in this experiment. (B) Quantitative RT-PCR using primers specific for wild-type and mutant *atp7a* transcript were used to measure the amounts of each in single embryos. Normalization of wild-type transcript was to $cal^{+/+}$ embryos and mutant transcript to $cal^{-/-}$ embryos. Error bars indicate one standard deviation around the mean. (C) Polyacrylamide gel of RT-PCR products reveals a band containing 800bp of intronic sequence caused by morpholino injection. This band was sequenced and represents use of a cryptic 3' splice site located within the intron. Also seen are mutant and wild-type transcripts in uninjected and rescued embryos. A non-specific band was present in both uninjected and injected mutant embryos (*).

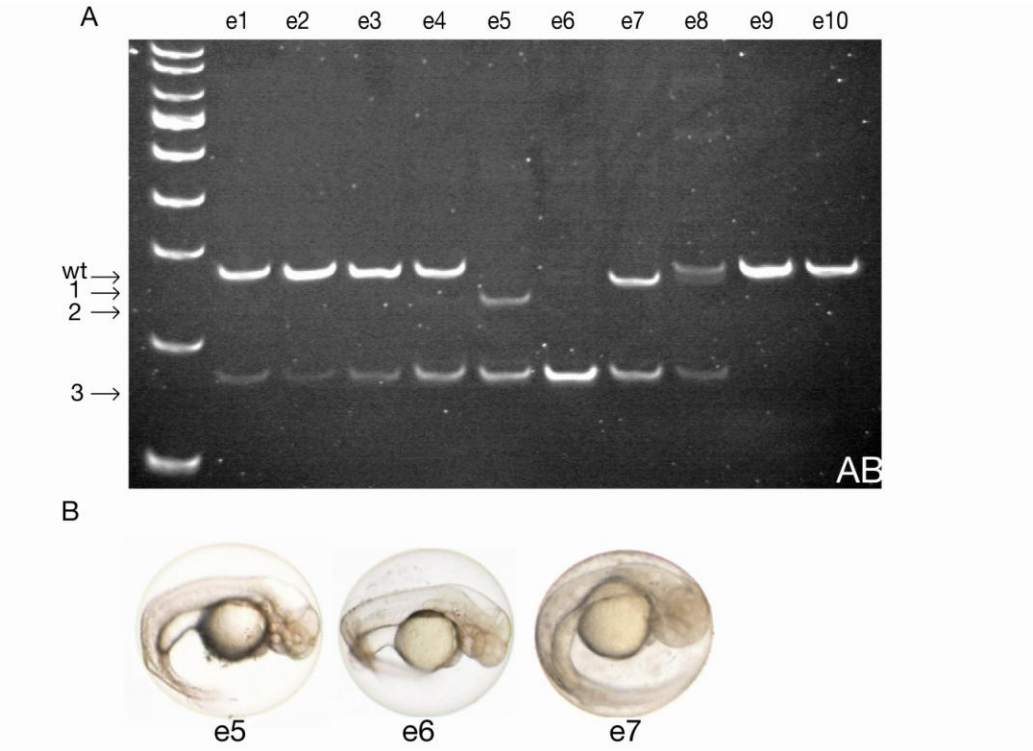
Supplemental Figure 1



Supplemental Figure 1. The point mutation L1316R results in a non-functional ATPase.
ATP7a deficient fibroblasts were transfected with the copper requiring enzyme tyrosinase (A), or tyrosinase plus human ATP7a (B), zebrafish ATP7a (Dr ATP7a) (C), or zebrafish ATP7a with L1316R point mutation (D). Transfected cells were fixed and stained with L-DOPA as previously described[1]. Cells transfected with the mutant cDNA did not produce any functional tyrosinase in this context due to deficient copper transport activity.

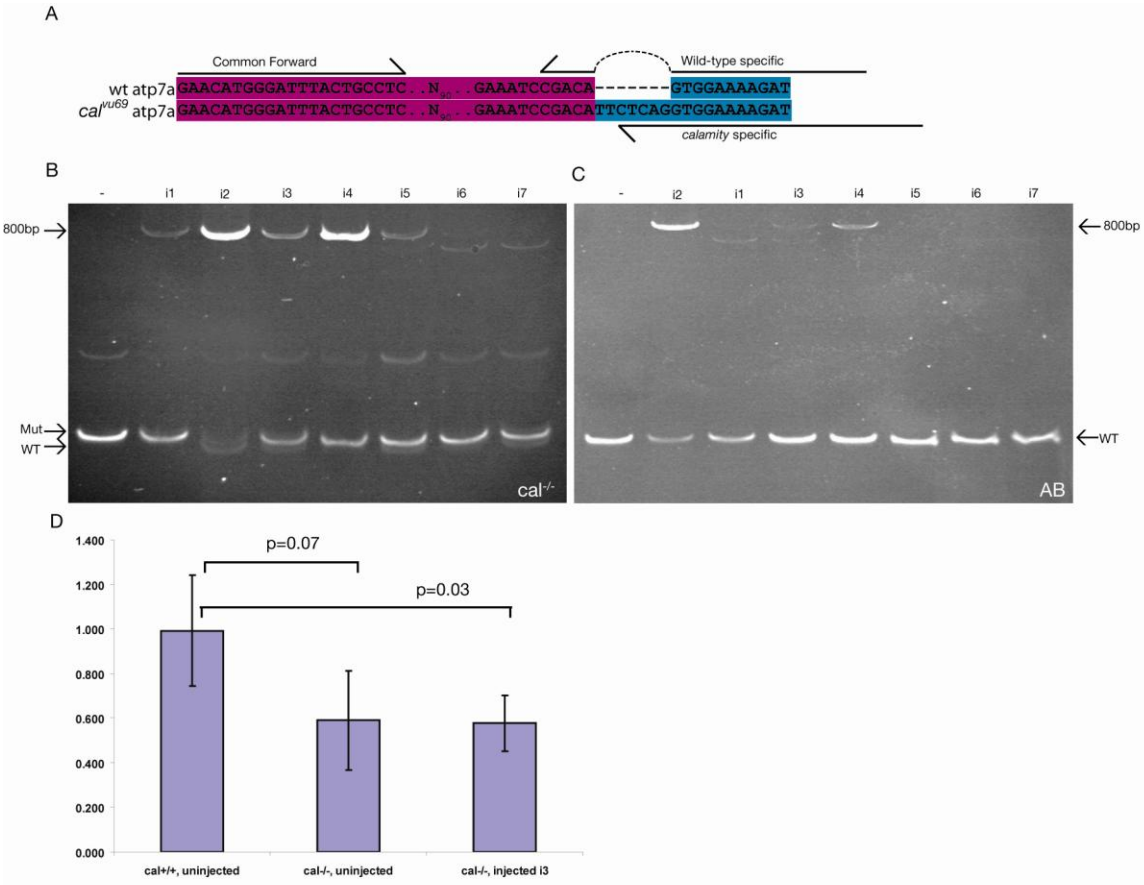
1. Petris, M. J., Strausak, D., & Mercer, J. F. (2000) Human molecular genetics 9, 2845-2851.

Supplemental Figure 2



Supplemental Figure 2 (A) Injection of the "e series" morpholinos into wild-type AB strain embryos results in a similar pattern of splicing alterations. Morpholinos e5, 6, and 7 completely abolish wild-type splicing and as a result cause a calamity phenotype in 100% of the embryos (B). Morpholino e7 demonstrates that the splice site used in gw246 is the strongest cryptic splice site even in a wild-type fish, explaining the effectiveness of the mutation in causing severe phenotypic changes.

Supplemental Figure 3



Supplemental Figure 3:
(A) A schematic indicating the primers used to differentiate between wild-type and *cal*^{vu69} splice forms in performing qPCR. A common forward primer was used in each reaction to minimize variability. The specific primers are indicated by lines overlying the sequence to which they are complementary. The dashed curved line indicates that this gap does not exist in the primer sequence. (B) and (C) Injection of morpholinos i1-7 into *cal*$^{vu69-/-}$ (B) and wild-type AB strain (C) zebrafish. In *cal-/-* embryos both wild-type and mutant transcripts are seen at baseline and no morpholino significantly altered this finding. Several morpholinos were capable of inducing the 800bp band, even in wild-type zebrafish. (D) Morpholino i2 causes a worsening of the calamity phenotype in *cal-/-* embryos, indicating that it interferes with wild-type splicing.

Chapter 4

Zebrafish mutants *calamity* and *catastrophe* define critical pathways of gene nutrient interactions in developmental copper metabolism

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Zebrafish mutants *calamity* and *catastrophe* define critical pathways of gene-nutrient interactions in developmental copper metabolism

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Running Title: Zebrafish mutants sensitive to copper deficiency

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Author Disclosures
Neither Jonathan D. Gitlin nor Erik C. Madsen have any competing interests or other disclosures concerning this manuscript.

Abstract

Nutrient availability is an important environmental variable during development that has significant effects on the metabolism, health, and viability of an organism. To understand these interactions for the nutrient copper we used a chemical genetic screen for zebrafish mutants sensitive to developmental copper deficiency. In this screen we isolated two mutants which define subtleties of copper metabolism. The first contains a viable hypomorphic allele of *atp7a* and results in a loss of pigmentation when exposed to mild nutritional copper deficiency. This mutant displays incompletely penetrant skeletal defects affected by developmental copper availability. The second carries an inactivating mutation in the vacuolar ATPase that causes punctate melanocytes and embryonic lethality. This mutant, *catastrophe*, is sensitive to copper deprivation revealing overlap between ion metabolic pathways. Together, the two mutants illustrate the utility of chemical genetic screens in zebrafish to elucidate the interaction of nutrient availability and genetic polymorphisms in cellular metabolism.

Author Summary

Copper is an essential nutrient required for multiple biologic functions. Proper uptake, transport, and excretion of copper are critical for use of this metal while reducing its inherent toxicity. While several key proteins involved in this process have been identified, there are still gaps in our understanding of copper metabolism – particularly during early development. We have used zebrafish, a genetically useful animal model system, to study genetic interactions with copper deficiency during development. We treated mutant embryonic zebrafish with a chelator which reduces the level of available copper and screened for mutants which displayed a copper deficient phenotype only in

the presence of the chelator. We identified and characterized two mutants which advance our understanding of copper metabolism during the early periods of development as well as show an interaction between copper metabolism and another fundamental pathway, that of proton transport. Our results expand our knowledge of copper metabolism and illustrate the power of this type of genetic screen in zebrafish to elucidate mechanisms of nutrient metabolism.

Introduction

Proper maternal nutrition is critical for early embryonic development. The Dutch Famine Study examined the consequences of nutrient deprivation on developmental outcome during severe food shortages near the end of the Second World War and clearly demonstrated that inadequate nutrient availability during human gestation increases the likelihood of developmental anomalies (Susser et al., 1998). From these initial observations arose the well-recognized link between maternal folate supplementation and the suppression of neural tube defects (Czeizel and Dudas, 1992). Despite overwhelming epidemiologic data indicating the benefits of folate and other nutrient supplementation we do not fully understand the genetics of predisposition to these abnormal developmental phenotypes when faced with suboptimal nutrient levels. There are several large difficulties in the study of these processes in mammals that have prevented faster progress. The first is that the genetics of mammals has been cumbersome. The second, and more important, is that development of placental animals occurs *in utero* making rapid detection of developmental phenotypes difficult. Finally, controlling the level of nutrient available to the developing embryo cannot be done with precision as it depends both on the genetics of the mother and the embryo as well as maternal nutrition.

Copper is an essential nutrient which when absent results in severe developmental abnormalities. This is most clearly illustrated by Menkes disease (OMIM #309400), a rare X-linked disorder of copper metabolism. Patients with Menkes disease have an array of symptoms including seizures, neurodegeneration, hypopigmentation, and lax skin which result from decreased copper incorporation into critical enzymes such as dopamine-β-hydroxylase and lysyl oxidase (Menkes, 1988; Menkes et al., 1962). This usually fatal disease is caused by mutations in a copper transporter, *ATP7A* (NM_000052), which resides in the secretory pathway and is responsible for transport of copper into this compartment. The Menkes gene product is also responsible for placental copper transport. While patients complete *in utero* development apparently normally, it is clear from biochemical studies at birth that there are significant defects that arise from gestational copper deficiency (Kaler et al., 2008).

In order to study the effects of developmental copper deprivation our lab has previously created a zebrafish model of severe copper deficiency (Mendelsohn et al., 2006). High doses of the cell permeable copper chelator neocuproine cause embryonic zebrafish to exhibit a Menkes-like phenotype with neurodegeneration, hypopigmentation, and connective tissue defects. Isolation and cloning of the mutant *calamity*, which shared these same characteristics, revealed a loss-of-function mutation in the zebrafish orthologue of *ATP7A* (NM_001042720). In this current study we expand this model to study the effects of induced genetic alterations on the developmental response to *mild* copper deprivation. We describe two mutants sensitive to nutritional copper deficiency that illustrate the potential power of this approach to overcome the limitations of studying gene-nutrient interactions in vertebrate organisms and that define combinations of loss-

of-function mutations of known ion homeostatic pathways that result in aberrant development.

Results

Copper Deficiency Screen

In order to elucidate the molecular genetics of copper metabolism we performed a forward genetic screen for zebrafish mutants with enhanced sensitivity to subthreshold copper deficiency. To control copper levels zebrafish embryos were treated with the cell permeable copper specific chelator neocuproine which has been previously shown to cause a copper-deficient phenotype including loss of pigmentation and notochord defects at a dose of 1 to 10 μM due to loss of cuproenzyme activity (Mendelsohn et al., 2006; Smith and McCurdy, 1952). Prior to screening, a subthreshold dose of 100 nM neocuproine was determined to cause no alteration in pigmentation in wild-type, haploid embryos. We then used this concentration of neocuproine to screen clutches of haploid embryos derived from F1 carriers of ENU-induced mutations. One half of each clutch was placed in 100 nM neocuproine at 3 hours post fertilization (hpf) and allowed to develop until 48 hpf when clutches were screened for loss of melanin pigmentation in 50% of the embryos (Figure 1A). Only those clutches which had loss of pigmentation at 100nM neocuproine but contained at least some pigmentation when untreated were scored as mutant. In this pilot screen we examined 700 F1 females and found five potential mutants. Seven hundred mutagenized haploid genomes at an estimated single locus mutation rate of $1.1x10^{-3}$ represents approximately a 65-70% coverage of the genome (Solnica-Krezel et al., 1994). Of the five potential mutants, four were confirmed

as true mutants as defined by the transmission of the neocuproine sensitive phenotype to the offspring. One of these mutants fit the "ideal" criteria (no defect in vehicle and complete loss of pigment in 100nM neocuproine in 50% of the haploid clutch) as illustrated in Figure 1A and subsequent analysis revealed important insight into the intersection of genetics with sub-optimal copper nutrition in early development. A second mutant reveals a role for proton transport in copper metabolism. The final two mutants were similar in phenotype to the first but full analysis has not been completed.

The first mutant isolated from the screen displayed normal melanin pigmentation when untreated but completely lost all melanin upon treatment with 100 nM neocuproine (Figure 1 B, C). Crossing this mutant with *calamity*^{vu69} (*cal*) which bears an inactivating mutation in the copper transport protein *atp7a* resulted in partial non-complementation. The compound heterozygote had no melanin in the developing retinal pigment epithelium (RPE) and normally distributed mild hypopigmentation over the rest of the body (Figure 1 D). Based on the partial non-complementation we tentatively assigned this mutant as an allele of *calamity*, designated *gw71*.

The second mutant has a phenotype that is independent of neocuproine. Named *catastrophe*, this mutant has normally distributed melanocytes that are small and punctate (Figure 1E). *Catastrophe* (*cto*) is homozygous lethal at about 3 days post fertilization (3 dpf). The heterozygotes have no overt phenotype. In addition, *cto* homozygotes display sensitivity to copper deficiency by losing all melanin pigmentation in 100 nM neocuproine (Figure 1F). Crossing *cto* with *cal*^{vu69} results in complete complementation (Figure 1G) including the observation that the double heterozygote is not more sensitive

to neocuproine than *cal*^{vu69} heterozygotes (data not shown). Thus, we continued our analysis on the basis that *cto* identifies a new locus involved in copper metabolism.

A hypomorphic allele of *atp7a*

Chromosomal localization using the early pressure parthenogenesis method (Johnson et al., 1995) placed the mutation in *cal*^{gw71} (referred to below as *gw71*) near the centromere of chromosome 14, the known location of *atp7a*. Combining this data with the partial non-complementation, we hypothesized that this mutant represented a hypomorphic allele of *atp7a* and confirmed this by direct sequencing of the mRNA. Mutant *atp7a* was cloned and displayed 100% identity with the published *atp7a* sequence (NM_001042720) with the exception of a single base change present in both mutant clones, T3182G, which results in a single, non-conservative amino acid substitution, I1061S (Figure S1A). This mutation is located in a region highly conserved in copper transporting ATPases and exchanges a hydrophobic amino acid for one that is polar and hydrophilic (Figure 2A). This single amino acid change results in significant depletion of the full-length protein in mutant embryos (Figure 2B).

To verify that this was the causative mutation in *gw71,* we performed an *in vitro* activity assay for the protein using wild-type and mutant *atp7a*. Fibroblasts from patients with Menkes disease which lack functional *ATP7A* were transfected with tyrosinase in combination with either wild-type or mutant zebrafish *atp7a* created via site-directed mutagenesis of the wild-type cDNA. These fibroblasts were then treated with increasing doses of neocuproine, fixed, and stained for tyrosinase activity using L-DOPA. Activity is dependent on both *atp7a* and tyrosinase cDNAs (Figure S1B and S1C). In contrast to

zebrafish mutant embryos, equal amounts of wild-type and mutant Atp7a were obtained via transfection in these fibroblasts (Figure 2D). L-DOPA staining of cells expressing mutant cDNA was only mildly reduced when compared with wild-type (Figure 2D vs. E) indicating that the mutant retains some transport function. Overnight treatment with 25 nM neocuproine resulted in complete loss of tyrosinase activity in fibroblasts transfected with mutant, but not wild-type, *atp7a* though a reduction in staining was observed with wild-type (Figure 2F, G). These data suggest that this single mutation in *atp7a* not only affects steady-state protein levels but is also capable of reducing the functional capacity of the protein, leading to sensitivity to copper deficiency.

The I1061S mutation is located in the intracellular loop which comprises the ATPase domain of the transporter (Figure 2H). Dimitriev et. al. have previously performed NMR spectroscopy on the homologous domain of the Wilson disease copper transporter, ATP7B, in the presence and absence of bound ATP and have derived from the resulting chemical shift data the residues important for ATP binding and hydrolysis (Dmitriev et al., 2006). We mapped the same region of Atp7a onto their model by sequence alignment (64% consensus, 49% identical) to better understand the potential effect of this mutation on protein function. The mutation in cal^{gw71} lies five amino acids away from a critical ATP binding residue, E1064, which is highly conserved from yeast to humans (Figure 2A and Figure S1D). While a mutation of a critical residue would be expected to significantly alter ATP binding or hydrolysis, a non-conservative mutation in the region of a critical residue might be expected to only slightly alter ATP binding/hydrolysis through minor shifts in regional structure.

gw71 mutants display post-embryonic sensitivity phenotypes

Because the *gw71* allele is homozygous viable, we were able to examine several post-embryonic roles for *atp7a*. Adult homozygous mutant zebrafish placed in varying doses of neocuproine did not display an overt sensitivity phenotype (data not shown). However, further study revealed a maternal effect of this mutation on embryonic copper metabolism. Homozygous mutant embryos derived from heterozygous females had a normal quantity and distribution of pigmentation that was partially sensitive to 100 nM neocuproine which abolished pigment in the retinal pigment epithelium (RPE) and reduced pigment over the body of the fish (Figure 3A, B). In contrast, homozygous mutant embryos derived from homozygous mutant females had no pigment in the RPE and reduced pigment over the body; treatment of these embryos with 100 nM neocuproine completely abolished pigmentation throughout the embryo (Figure 3 C, D). The effect of the mother's genotype on the embryonic phenotype indicates that though not overt, the adult homozygous mutant does have defects in copper metabolism demonstrated by a nutrient-deficient state in the offspring. Thus the sensitivity of the embryo to neocuproine is due not only to aberrant embryonic copper metabolism, as the embryos from heterozygous mothers are sensitive to copper deficiency, but also to a deficient maternal loading of copper into the egg as the phenotype is exacerbated by maternal homozygosity.

The importance of optimal copper nutrition during development is further illustrated by the presence of vertebral skeletal defects in homozygous mutants. Homozygous mutant embryos were stained at 21 dpf with alcian blue/alizarin red to reveal bone and cartilage respectively. These were compared with wild-type syngeneic

age-matched controls raised in the same manner. The wild-type fish had straight vertebral columns along the entire length with long, straight bony processes extending from each vertebra (Figure 3E). In contrast, homozygous *gw71* fish displayed variable vertebral defects, most often a significant warping of the bony structures in the caudal-most region of the column caused by irregular length of vertebrae and defects in the joint angles (Figure 3F). In addition the bony processes were also shortened and bent. Consistent with the observations in embryos that the mutation in *gw71* brings the homozygous embryo close to, but not over, a threshold for copper deficiency, the persistent skeletal defects in the juvenile fish were not fully penetrant. Whereas wild-type fish had no vertebral defects (n=26), a significant number (38%, n=60) of the *gw71* fish contained defects (Figure 3G).

Incomplete penetrance of the defect in the homozygous mutant fish could be attributed to either separate subtle genetic interactions or to variable nutrient availability. We hypothesized that if the penetrance of the defects were based on nutrient availability then reducing the nutrient levels would worsen the defects and increase the penetrance and vice versa. We thus took *gw71* mutant embryos and placed them in either normal egg water or egg water supplemented with 100 nM neocuproine or 500 nM $CuCl_2$ from 3 to 51 hpf (48 hour exposure). In addition, two separate groups of embryos were treated with neocuproine from 16 to 64 hpf and from 30 to 78 hpf to determine if there was a window of developmental time critical for the genesis of later defects. At 21 dpf the larvae were stained with alcian blue/alizarin red and scored for the presence or absence of vertebral defects (Figure 3G). Untreated wild-type embryos (not shown) or wild-type embryos treated with 100 nM neocuproine from 3-51 hpf had no perceptible skeletal defects. Thirty-eight percent of *gw71* embryos had skeletal defects and this number was

not significantly affected by treatment with 100 nM neocuproine or 500 nM $CuCl_2$ from 3-51 hpf. However, there was a 50% increase in the number of skeletal defects in *gw71* embryos treated with 100 nM neocuproine from 16 to 64 hpf. The larvae treated with 100 nM neocuproine from 30 to 78 hpf died approximately 8 dpf from an unidentified cause. These results indicate an increasing sensitivity to mild copper deprivation as the embryo develops in the first 16-72 hrs. Further experimentation with smaller, more discrete treatment times might allow the determination of any developmental window required for the effects of copper on vertebral axis formation.

In addition to the presence of vertebral skeletal defects in fully ossified skeletons, larvae at earlier stages of development displayed hyperossification of vertebrae adjacent to defects in the vertebral column (Figure 3 H, I). Normal zebrafish bone ossification begins rostrally and generally proceeds caudally with the exception of the caudal fin vertebrae (Du et al., 2001). In *gw71* this pattern is maintained (arrowhead in Figure 3 H) except for areas containing defects (arrow in 3H). The defects affected the joints between vertebrae and had differing degrees of connective tissue bulges which partially stained with alcian blue indicating the presence of some cartilaginous tissue in these defects (Figure 3H arrowhead).

catastrophe contains a defect in proton transport

Before mapping the *catastrophe* mutant it was important to determine the extent of the defect in copper metabolism. The loss of pigmentation in the mutants could result from toxicity in a "two-hit" model whereby the mutation damages melanocytes and the drug acts to further affect these already sick cells. Therefore we examined the sensitivity

of the mutant to another copper-dependent process – notochord formation. Notochord formation requires the action of the cuproenzyme lysyl oxidase and its family members. Both reduction in lysyl oxidase levels and copper chelation result in wavy, distorted notochords (Gansner et al., 2007b). Placing *cto* mutants in 2 μM neocuproine at 3 hpf resulted in wavy notochords in the mutant embryos at 24 hpf while having no effect on heterozygous or wild-type embryos (Figure 4A, B). This experiment indicates that the mutation in *cto* causes a global defect in copper metabolism and is not limited to melanocytes.

The mutation in *cto* was localized to chromosome 7 and further mapping reduced the region of interest to an approximately 1 Mbp region between markers z21519 and z43308 (Figure 4C). It was possible to assemble a nearly complete BAC contig between these markers using database BAC sequences (www.sanger.ac.uk/Projects/D_rerio/). This contig was scanned for potential genes using the FGENESH program (www.softberry.com) and comparing to the Ensembl database (www.ensembl.org). A list of candidate genes was generated from this comparison. To further refine the list, a database of zebrafish insertional mutants was scanned for mutants displaying a similar melanocyte phenotype (Amsterdam et al., 2004). Approximately 6 mutants in this database had punctate melanocytes, 5 of which had insertions in genes encoding subunits of the vacuolar (H^+) ATPase (Atp6) (NM_199620). As the critical region in *cto* contained the d subunit of the V0 complex of the vacuolar ATPase we cloned and sequenced this cDNA in the *catastrophe* mutants. A single base pair change C406T present in the mutant resulted in a premature stop codon, Q136X (Figure S2). Sequence alignment with the human sequences (NM_004691) revealed a highly conserved protein sequence (94%

identical) that most closely aligned with the d1 subunit (Figure 4D). Further database searches did not reveal a second d1 subunit in zebrafish.

The significant identity between the human and zebrafish protein sequences allowed us to use an antibody directed against human ATP6V0D1 to examine the steady state levels of protein. We hypothesized that the early stop codon would result in a significant decrease in protein levels. Indeed, in 48 hpf embryos there is a near total reduction in Atp6v0d1 protein as compared with wild-type embryos (Figure 4E). Total loss of this highly conserved and essential protein (see below) may be the cause of the *catastrophe* phenotype; however, there remains some possibility that another, tightly linked mutation may contribute to the observed phenotype. Based on significant experimentation in yeast a proposed quaternary structure for the vacuolar ATPase complex has emerged (Figure 4F) (Kane, 1995; Kane et al., 1989; Smith et al., 2003; Tomashek et al., 1997; Xu et al., 1999). In this model, the two main subcomplexes, V0 and V1 have complementary functions of proton translocation and ATP hydrolysis respectively. The complexes are connected through several stalk subunits, v1d, v0d, and v1f (not shown). Loss of these connecting subunits in yeast results in total loss of activity of the complex (Bauerle et al., 1993). Thus in *catastrophe*, the loss of the v0d subunit would be predicted to result in complete loss of proton translocation throughout the embryo.

catastrophe is sensitive to pharmacologic inhibition of proton transport

If the defect in *catastrophe* is loss of Atp6 function the heterozygotes might be sensitive to pharmacologic inhibition of this transporter. Consistent with this, wild-type

embryos placed at 24 hpf in 200 nM concanamycin A, a potent and specific inhibitor of Atp6 (Huss et al., 2002), showed no apparent phenotype at 48 hpf (Figure 5A). However, treatment of embryos heterozygous for *cto* resulted in punctate melanocytes and CNS degeneration, resembling the mutant (Figure 5B). The mutants themselves appeared qualitatively worse, with further reductions in melanocyte pigmentation and worsening of the degenerative appearance (Figure 5C).

Secretory pathway copper transport is altered in *catastrophe*

While it is apparent that loss of Atp6 results in altered cuproenzyme activity for two enzymes in the secretory pathway, it is unclear which step of global copper transport is affected in *cto* embryos. To address this we performed transplant experiments to determine the cell autonomy of the defect. Wild-type cells from *actin::GFP* transgenic zebrafish were transplanted into *cto* embryos and examined at 48 hpf for pigmented cells and GFP expression. Transplantation resulted in a few well-pigmented and stellate melanocytes over the head and body as well as clusters of pigmented retinal epithelial cells (Figure 5D). These same embryos were mosaic for GFP expression (Figure 5E). In body melanocytes the melanin obscured GFP fluorescence (Figure 5E arrowhead). In contrast, the retinal pigment epithelial melanocytes display GFP fluorescence in the central area not covered by melanin (Figure 5E arrow). From this we make two observations: First, the melanized melanocytes are derived from wild-type donor cells, and secondly, that nearby wild-type epidermal cells are not required for normal melanin pigmentation nor stellate appearance (Figure 5E arrowhead). Thus copper metabolism must not be significantly disrupted on an organismal level, as these wild-type

melanocytes in a mutant host still receive adequate copper for normal pigmentation. Also, the stellate appearance indicates that the defect that causes punctate pigment cells in *cto* is also cell-autonomous.

The transplant experiment addresses delivery of copper to each cell, but the uptake or distribution of copper within the individual cell could also be affected in *cto* embryos. We hypothesized that disruption of the transporter responsible for secretory pathway acidification would result in defects in copper metabolism in this compartment. To test this we examined the sensitivity of *cto* embryos to partial loss of Atp7a through the use of a morpholino. Previous work from our laboratory has demonstrated that melanin synthesis following loss of Atp7a is also cell-autonomous in the melanocyte indicating that knock-down of Atp7a will allow interrogation of the pathway on a cellular rather than organismal level (Mendelsohn et al., 2006). Injection of a splice morpholino previously shown to result in a copper deficient phenotype at a dose that does not cause pigmentation defects in wild-type or heterozygous embryos (Figure 5F) causes total loss of melanin pigmentation in *cto* embryos (Figure 5G). Thus *cto* embryos are sensitive to loss of the secretory pathway copper transporter, Atp7a. Embryos heterozygous for the *cto* mutation did not show sensitivity to the Atp7a morpholino indicating that near complete loss of Atp6 activity is required to sensitize to alterations in copper metabolism. At the same time, the cytochrome oxidase activity of mitochondria derived from *cto* embryos is no different from wild-type indicating that copper delivery to mitochondria is normal and that the defect in copper metabolism in *cto* embryos is limited to the secretory compartment (Figure 5H).

Subcellular morphology and melanosome formation is altered in *catastrophe*

The vacuolar ATPase has been implicated in diverse trafficking events within the cell and inhibition of this protein results in altered ion homeostasis, disrupted membrane trafficking, defective acid secretion, deficient protein degradation, and loss of protein sorting, endosomal recycling, and vesicular secretion (Dettmer et al., 2006; Hurtado-Lorenzo et al., 2006; Liegeois et al., 2007; Lin et al., 2006; Malikova et al., 2004; Taupenot et al., 2005; Tawfeek and Abou-Samra, 2004). To examine the effect of loss of this protein on cellular morphology, specifically melanocytes, we performed transmission electron microscopy focusing on the pigmented cells. Thin (500 nm) plastic sections of 48 hpf embryos stained with toluidine blue did not demonstrate any further gross defects in organismal or cellular morphology beyond those observed in the pigmented cells both of the epidermis and the retinal pigment epithelium (data not shown). Upon examination by electron microscopy in wild-type embryos both epidermal pigment cells as well as retinal pigment epithelial cells at 48 hpf display dark, uniformly round or ellipsoid melanosomes distributed throughout flat melanocytes (Figure 6A-C). In contrast, the melanocytes of *cto* embryos are rounded and contain few fully melanized melanosomes, many large vacuolated structures and small vesicles surrounded by rings of melanin pigment (Figure 6D-F). These latter structures have been identified as multi-vesicular bodies, the accumulation of which is reminiscent of early blocks in melanosome maturation found in the *cappuccino, pallid, ruby-eye 2,* and *reduced pigmentation* mice which are all models of Hermansky-Pudlak syndrome and have specific early defects in melanosome biogenesis (Nguyen et al., 2002). Thus among other abnormalities loss of proton transport results in early blocks in melanosome maturation. It is interesting to note

that there remains active tyrosinase which produces some melanin in these aberrant structures despite the loss of the proton transporting ATPase (Figure 6F).

Discussion

Genetic screen for gene-nutrient interactions

In this work we have used the power of forward genetic screens combined with the ease of *ex utero* nutrient level manipulation accessible with the zebrafish to study the relationship between specific genetic alterations, the levels of the essential nutrient copper, and their combined effects on the developmental phenotype of the embryo. From these experiments we have derived a nutrient-sensitive allele of a known copper transporter that results in a juvenile skeletal phenotype. We have also implicated the vacuolar proton pump in vertebrate copper metabolism and interconnect two ion transport proteins whose individual effects on the other would not otherwise have been appreciated.

The *ex utero* development of zebrafish provides an opportunity for manipulating the developmental levels of nutrients. Much success has been achieved in yeast using large libraries of compounds coupled with known deletion mutants to define the roles of many of the yeast proteins in cellular biology and metabolism (Hillenmeyer et al., 2008; Parsons et al., 2004). One major advantage of yeast is the ability to absolutely control the levels of different nutrients and pharmacologic compounds and to screen large numbers at a time; however, yeast lack the complexity necessary to extend such findings to multi-cellular organisms and ultimately to understand human biology for the treatment of disease. Our work shows that the zebrafish model system can fill the niche in extending

the principles of the chemical genetic screen to a vertebrate organism. Zebrafish retain the advantage of environmental exposure control while only slightly reducing the ability to screen large numbers. They also provide a system with more complex phenotypes to be examined which can then be brought back to the study of the underlying cell biology of a multi-cellular organism, particularly as the genome sequence and rapid mapping techniques improve.

Nutrient-sensitive hypomorphic allele

The first mutant which was isolated from our screen was a hypomorphic allele of *atp7a*. Animals bearing this allele have a normal pigmentation and notochord phenotype at 48 hpf but are sensitive to mild copper deficiency thus indicating that transporter function was impaired. This mutation reduced the protein levels to below the detection limits of our immunoblot demonstrating that only a fraction of wild-type protein expression is necessary to maintain a near-normal phenotype. This is consistent with our previous observations where very minor changes in Atp7a protein levels resulted in significant rescue of the *calamity* phenotype (Madsen et al., 2008). Also, the increase in severity of the cal^{vu69} allele upon incubation with neocuproine demonstrates that even in this model of severe Menkes disease, there is still residual protein function without detectable expression (Madsen et al., 2008; Mendelsohn et al., 2006). Interestingly, when the *gw71* protein was overexpressed in cell culture fibroblasts it was fully capable of loading copper into the secretory pathway as evidenced by the robust tyrosinase activity; yet, at the same time there was a clear sensitivity of this mutant transporter to copper levels.

This mutant allele is not the first hypomorphic allele of *atp7a*. A less severe form of Menkes disease, Occipital Horn Syndrome, is also caused by mutations in *atp7a*. Children with this disease have many clinical problems similar to Menkes disease; however, as this syndrome is not fatal in early life other abnormalities can be appreciated including skeletal defects such as deforming hyperostosis and kyphoscoliosis (Horn and Tumer, 2002). In this context the *gw71* mutant provides several important advances. First, within the screen itself it provides proof-of-concept that the screen design will result in the identification of critical proteins involved in copper transport and metabolism. Second, the *gw71* allele is both viable and fertile which itself provides distinct advantages. Third, this allele demonstrates that only a fraction of wild-type levels of Atp7a protein are required for near-normal pigmentation and notochord formation, a result suggested by previous experiments (Madsen et al., 2008). Fourth, this mutant expands the hierarchy of copper metabolism previously described (Mendelsohn et al., 2006). The differential effect on retinal pigment epithelial melanin versus the body pigmentation seen under a variety of genetic and environmental manipulations (Compare Figures 1D, 3B, and 3D) demonstrates an increased sensitivity of the RPE to derangements of copper metabolism. Fifth, the *gw71* mutant displays an incompletely penetrant developmental hyperostosis phenotype which is easily detected. The proximal etiology of these defects is unknown. It may be related to lysyl oxidase activity which is important for zebrafish notochord development and is sensitive to nutritional copper status (Gansner et al., 2007b). The increase in penetrance with copper chelation suggests that the variability may be due to nutritional differences. The lack of rescue observed with copper supplementation could be due to an inability of this ion to be translocated by

the mutant Atp7a protein to the proper compartment. Alternatively, lack of rescue with copper could point to residual genetic heterogeneity leading to phenotypic differences. Whichever is the case, this aspect of the mutant phenotype may provide a model to further our understanding of this poorly understood defect. The viability of this mutant would allow a modifier screen to find mutations responsible for different aspects of the copper deficient phenotype as well as to detect any genetic variability leading to the incomplete penetrance observed in the mutant.

Intersection of two ion-transporting pathways

Our second mutant contains an inactivating mutation in the vacuolar (H^+) ATPase subunit, Atp6v0d1. While abolition of this protein results in loss of proton transport into the secretory pathway, the embryo is capable of developing relatively normally to about 48 hpf when defects become visibly apparent. This lag is most likely due to the persistence of maternal protein and mRNA. At this time point the changes in melanin pigmentation patterns signal the visible presence of defects in proton transport. Grossly the melanocytes become punctate which, upon ultrastructure analysis, is shown to be a loss of mature melanosomes and a rounding of the cell body with vacuolization. The observed relationship between lack of melanosome formation and cellular morphology is not understood but may suggest a toxic effect of inappropriate melanization in the multi-vesicular bodies seen with electron microscopy or may be due to a particular sensitivity of melanocytes to loss of proton transport. As it has been shown that the vacuolar ATPase is important for vesicular trafficking and endocytosis (Dettmer et al., 2006; Hurtado-

Lorenzo et al., 2006), the distinct disruption of planar morphology in *cto* melanocytes may also be due to defects in these processes.

The sensitivity to copper deficiency of the remaining melanin implicates proton transport in the homeostasis of copper metabolism. That the notochord is equally sensitive to reduced copper demonstrates that the defect is not limited to the melanocyte, but rather that there is a universal decrease in the ability of copper to adequately reach secretory cuproenzymes. Since the effect on copper metabolism in *cto* mutants is only revealed in the context of sub-threshold copper nutrition, without a screen of this nature, this inter-relationship of two ion transport pathways in the vertebrate organism would never have been appreciated.

There are two models which could explain the defect in cuproenzyme function when proton transport is compromised. The first is that an acidic pH is important for copper incorporation into the nascent cuproproteins within the secretory pathway. The second model is that a proton gradient is required for copper transport, to balance the charge transfer across the vesicular membrane. These models are not mutually exclusive and a combination of the two could result in the final phenotype.

The data presented in this paper demonstrate the power of the zebrafish model system to examine gene-nutrient interactions as well as to delineate basic cell biologic pathways. Continuing with this methodology will provide more insight into the biology of copper metabolism in a vertebrate organism. It is easy to see how screens in zebrafish similar to the one we describe have the potential to investigate the genetics of not only copper or folate metabolism, but also that this approach could be easily extended to an array of other nutrients.

Materials and Methods

Zebrafish Maintenance: Zebrafish were maintained in the Washington University Department of Pediatrics zebrafish facility according to institutional guidelines supervised by the Division of Comparative Medicine.

Mutagenesis, Screen and Mapping: The specific alterations of these well-characterized techniques are available in the supplemental Text S1.

Immunoblot: Mutant 48 hpf embryos were identified phenotypically. Twenty to thirty embryos were manually dechorionated and de-yolked, lysed in 75 μL RIPA buffer containing 10 μL/mL Protease Inhibitor Cocktail III (Calbiochem). Unlysed material was removed by centrifugation at 1000 x g for 5 minutes. For Atp7a, 50-100 μg of lysate in Laemmli buffer with 10% β-mercaptoethanol, heating for 5 min at 65^{o}C (not fully reducing conditions) was loaded on a 6% SDS-polyacrylamide gel. The protein was transferred to nitrocellulose and blotted for Atp7a using a custom polyclonal antibody raised against a C-terminal peptide (Madsen et al., 2008). For Atp6v0d1, 30-40 μg lysate in Laemmli buffer with 10% β-ME heated to 70^{o} C for 5 minutes was loaded on a 12% SDS-polyacrylamide gel. The transferred protein was blotted for Atp6v0d1 using a mouse polyclonal raised to human recombinant protein at 1:1000 dilution (Abnova Corp). Other antibodies: Actin (Sigma) 1:5000, β-catenin (BD Biosciences) 1:1000.

Me344 Cell Culture: The Menkes patient fibroblast cell line Me344 (gift of Mick Petris) was maintained in 10% FBS/DMEM with Pen/Strep/Glut. Transfections were carried out on coverslips using Lipofectamine 2000 (Invitrogen) at a ratio of Lipo2k:DNA of 2.5 for 3 hours in Optimem (Invitrogen). The media was then replaced with 1%

FBS/DMEM/PSG. Neocuproine was added in DMSO to the indicated concentration and the cells incubated overnight.

L-DOPA Staining: Performed as previously described (Petris et al., 2000).

Alcian Blue/Alizarin Red Stain: Twenty-one dpf juvenile zebrafish were fixed overnight in 4% PFA in PBS and stained as previously described (Javidan and Schilling, 2004).

Transplantation: Approximately 50-100 cells were extracted from wild-type (AB) embryos at the 1000 cell stage and placed in mutant embryos of the same age using a micromanipulator syringe and glass needle as described previously (Westerfield, 2000).

Morpholino Injection: The *atp7a* splice morpholino e7 (TGACAACATTAACATTCATACCCTG) (Madsen et al., 2008) was injected at a dose of 965 pg/embryo at the 1 cell stage in 10% phenol red. At 48 hpf the injected embryos were scored for pigmentation and genotyped.

Cytochrome c oxidase Activity Assay: A crude mitochondrial fraction was prepared from groups of 45 embryos at 52 hpf by homogenizing in 250 mM sucrose, 10mM Tris pH 7.4 with a loose-fitting glass-glass tissue homogenizer. The homogenate was spun at 700 x g for 10 minutes. The supernatant from this spin was centrifuged at 23,000 x g for 20 min to form a pellet containing mitochondria and large vesicles. The pellet was resuspended in 150 μL of sucrose buffer with protease inhibitors and n-dodecyl-3-D-maltoside was added to 1 mM and incubated for 10 minutes at 25^{o} C. Cytochrome c oxidase activity was monitored by measuring the decrease in absorption of ferrocytochrome c at 550 nm using the protocol described for the Cytocox assay kit (Sigma, USA).

Transmission Electron Microscopy: Performed as described previously (Gansner et al., 2007b).

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Supplemental Text

Supplemental Methods:

Mutagenesis: Male AB strain zebrafish at 12 weeks of age were placed in 3mM N-ethyl-N-nitrosourea (ENU) for 1 hour in the dark and then washed several times and allowed to recover for one week. This was repeated for a total of 5 exposures. The mutagenesis rate was monitored by crossing to *nacre* and scoring for mutant progeny. A rate of 1 *nacre* in 2000 embryos was obtained by this method. These mutagenized males were then crossed to AB females to generate the F1 generation carrying a random array of ENU induced mutations.

Screen: Clutches of eggs from F1 females heterozygous for random mutations were obtained and fertilized with UV-inactivated sperm to create gynogenetic haploid offspring (Fig 1A). At 3 hours post fertilization (hpf) half of each clutch was placed in 100nM neocuproine, a dose empirically determined to be a threshold dose for pigmentation defects in wild-type AB haploid embryos in our egg water (35mg/L Crystal Sea Marinemix, Marine Enterprises Int. Baltimore, MD with 18.3MΩ deionized water). At 48 hours post fertilization clutches treated with neocuproine were scored for loss of pigmentation. If the treated half of a clutch lacked pigment, the untreated half was examined. If this half also lacked pigment the mutant was discarded; if it contained any pigment at all, it was retained for characterization. F1 females from which mutant clutches were derived were out-crossed to AB for maintenance and crossed to WIK strain males for subsequent mapping. Mutants were confirmed by transmission of the phenotype to subsequent generations.

Early Pressure Parthenogenesis and chromosomal assignment: Gynogenetic diploid mapping has been previously described (Johnson et al., 1995). Briefly, clutches of eggs from heterozygous carriers were fertilized with UV-inactivated sperm and immediately placed in a french press. At 1'20" a pressure of 8000psi was applied to the eggs and held for 5 min. The embryos were then removed from the press and raised as normal larvae. Simple sequence length polymorphisms (SSLPs, http://zebrafish.mgh.harvard.edu/) near the centromere of each chromosome were used to assign the chromosome containing the mutation, as mutants will have no recombination between the mutation and the centromere. Also distance from the centromere is estimated based on the percentage mutants in clutches derived in this manner.

Fine Mapping: Haploid embryos were obtained as above from heterozygous AB/WIK females. At 48 hours post fertilization the embryos were sorted according to phenotype, the DNA was extracted individually using proteinase K/phenol/chloroform and known SSLP's (http://zebrafish.mgh.harvard.edu/) were examined for linkage to the phenotype.

Cloning: Mutant *atp7a* was cloned and sequenced twice from a single cDNA preparation using *atp7a* specific primers. Wild-type and mutant *atp6v0d1* was similarly cloned using the following primers based on the database sequence (NM_199620).

Allele-specific PCR: Detection of the *cto*^{gw325} mutation was accomplished using allele-specific PCR with the forward primer AAGCTTCCTGGCCAATGAAGC, the mutant specific reverse primer GATGTTAACAGCCTCCATCTA, and a positive control reverse primer CCAAATCCTGTTCAGAGATGC using a 62^{o} C annealing temperature with standard Taq polymerase (Promega).

Figure 1

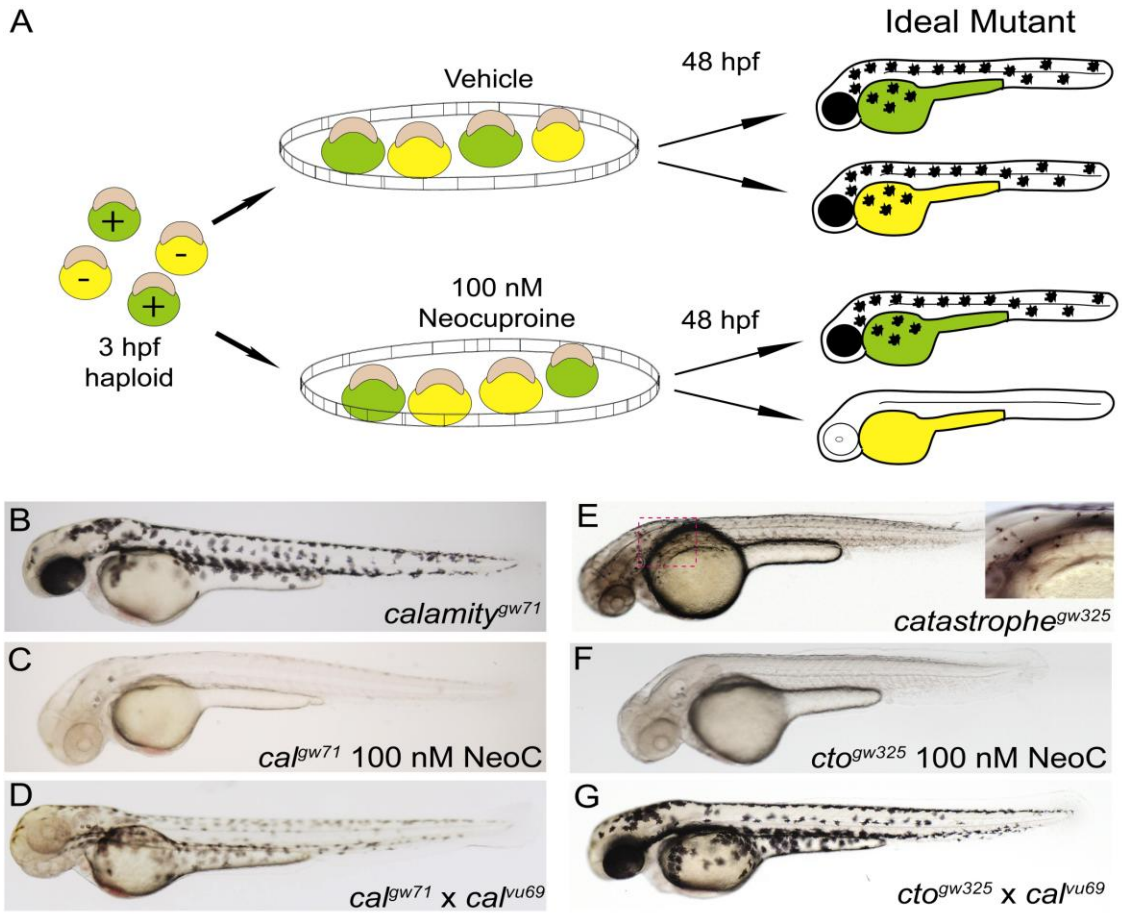


Figure 1. Chemical genetic screen for zebrafish mutants sensitive to copper deficiency.

(A) Diagram outline of the sensitivity screen. Haploid embryos are either wild-type (green) or mutant (yellow) for any given ENU-induced mutation. These embryos were placed in vehicle or 100 nM neocuproine (neoc) at 3 hpf and allowed to develop until 48 hpf when they were screened for loss of melanin pigmentation in drug only. The ideal mutant is demonstrated on the right where mutant (yellow) embryos have no pigment only upon treatment with neocuproine (B-C) The first mutant isolated has full pigmentation without neocuproine (B) and loses all pigmentation upon treatment with 100 nM neocuproine (C). (D) This mutant does not complement a known allele of the mutant calamity, establishing it as a new allele of the same gene atp7a. (E-F) The second mutant has reduced, punctate pigmentation without drug treatment (E) but loses all pigmentation upon treatment with 100 nM neocuproine (F). (G) The second mutant fully complements calamity. Therefore we have isolated a new mutant which we have called catastrophe.

Figure 2

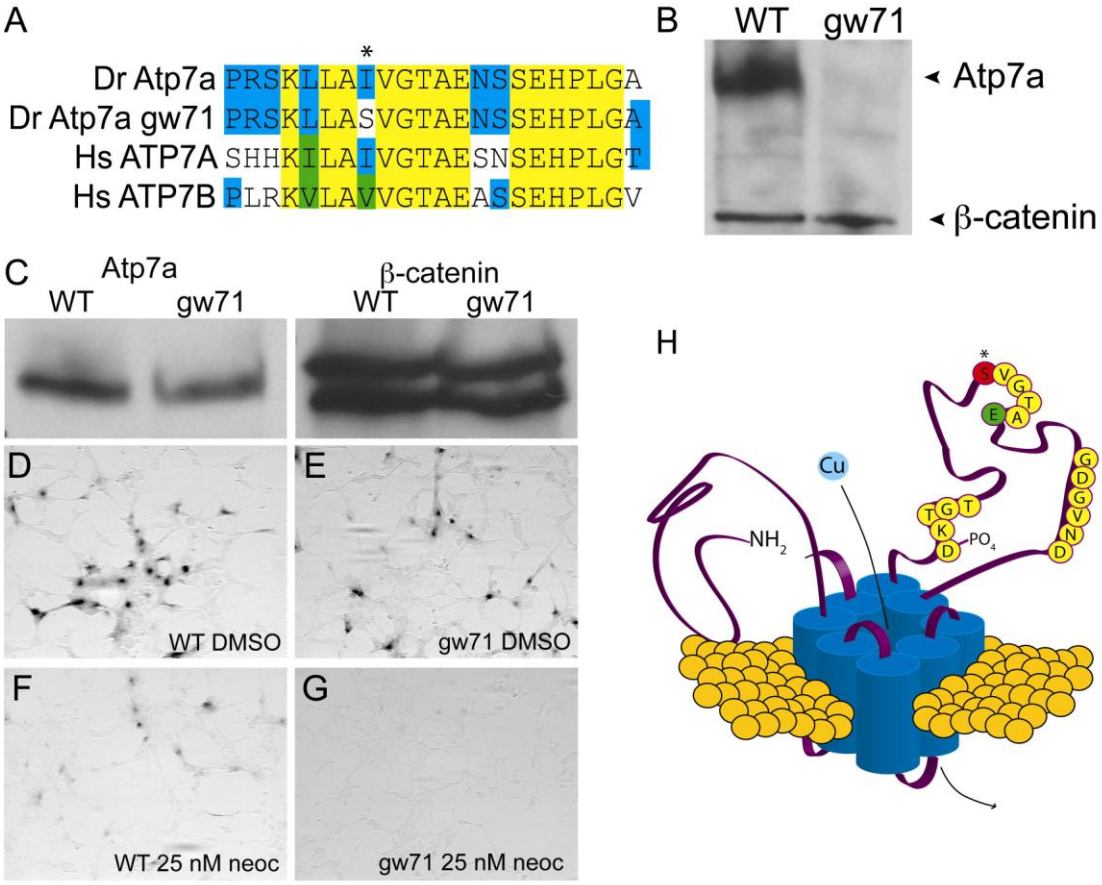


Figure 2. *calamity*^{gw71} contains a hypomorphic allele of *atp7a*
(A) The mutation in *cal*^{gw71} is a T1061S substitution in a highly conserved region of the vertebrate copper transporters and exchanges a normally hydrophobic amino acid for a hydrophilic one (asterisk). (B) This single amino acid change results in near complete loss of immunoblot-detectable protein levels. Wild-type and *gw71* mutant embryos were blotted for Atp7a using a peptide antibody to the C-terminus of the protein. β-catenin was used as a loading control. (C) Despite loss of protein in the zebrafish, transfection of an ATP7A-deficient human fibroblast cell line with either wild-type or mutant cDNA (derived from site-directed mutagensis of the wild-type) results in near equivalent expression of the zebrafish protein. β-catenin is again used as a loading control. (D-E) Both wild-type (D) and mutant (E) cDNAs are capable of producing functional protein as measured by functional tyrosinase activity in ATP7A deficient fibroblasts fixed and stained with L-DOPA. (F-G) Both wild-type (F) and mutant (G) Atp7a are sensitive to the effects of low-dose neocuproine in the above assay; however, the mutant cDNA is much more sensitive to mild copper chelation (F vs. G). (H) Model illustrating the relationship of the mutation (red, asterisk) to the known topology and functional domains of Atp7a. The mutation lies in the ATPase domain of the protein near a glutamate (green "E") required for ATP binding and hydrolysis [10].

Figure 3

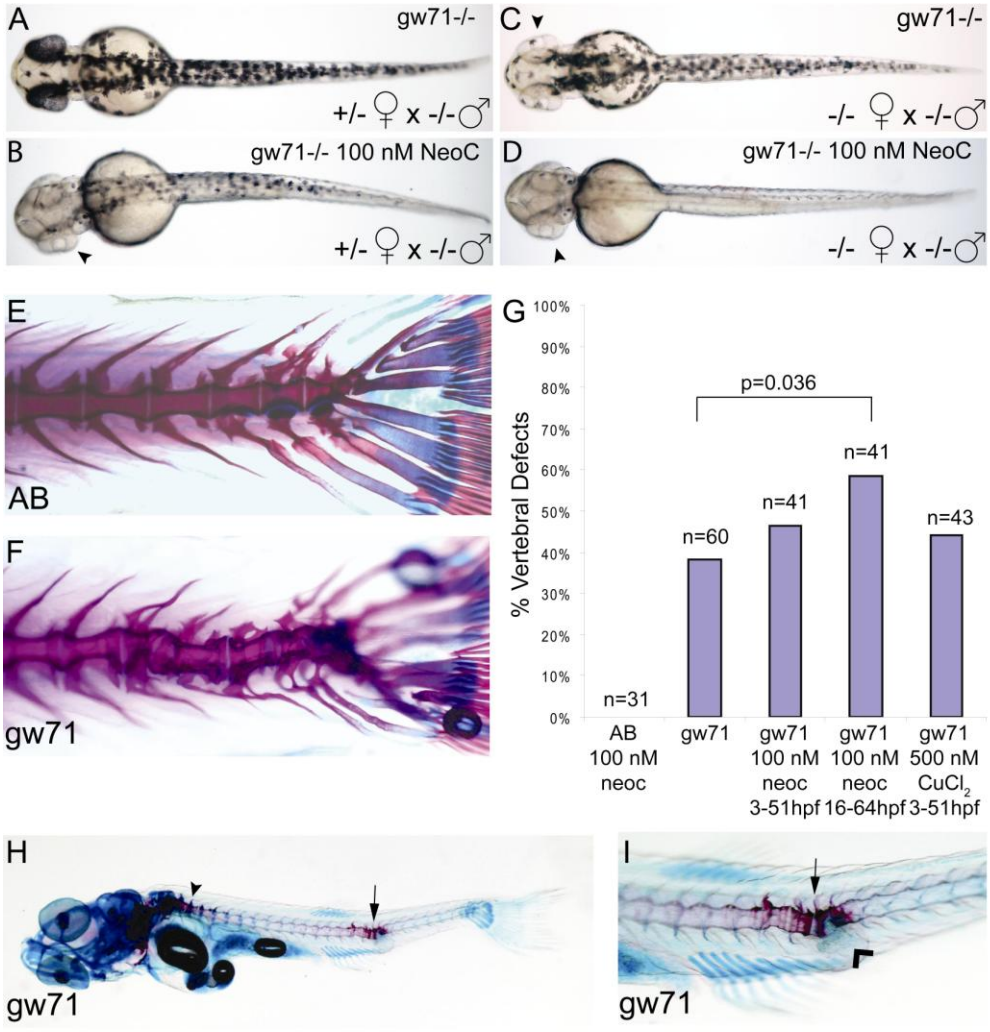


Figure 3. *calamity*^{gw71} embryos display developmental defects that are sensitive to maternal and environmental copper availability.
(A-D) Maternal effect on pigmentation in untreated *gw71* homozygous embryos. Mutant embryos derived from a heterozygous mother (A) display near normal pigmentation and have an incomplete loss of pigmentation in 100 nM neocuproine (B) most noticeable in the retina (arrowhead). Mutant embryos derived from homozygous mothers have mild hypopigmentation (C), particularly of the retina (arrowhead) and lose nearly all pigmentation in 100 nM neocuproine (D). (E-I) Partially penetrant juvenile skeletal deformities are present in *gw71* mutant fish. Wild-type (E) and *gw71* mutant (F) 21 dpf larvae were stained with alcian blue (cartilage) and alizarin red (bone) to reveal skeletal defects. In (G), embryos were untreated, treated with 100 nM neocuproine, or treated with 500 nM CuCl2 during the times indicated. The larvae at 21 dpf were scored according to absence or presence of a vertebral axis defect. A one-tailed Fisher exact probability test was used to calculate p-values. Only the indicated p-value was significant. (H) *gw71* mutants at an earlier stage of bone ossification display hyperossification at the location of the vertebral defect (arrow). Normal ossification is detected by alizarin red staining and begins rostrally (arrowhead). (I) A higher magification of the defect in (H) showing the hyperossification (arrow) and an outpouching of connective tissue which stains with alcian blue (arrowhead).

Figure 4

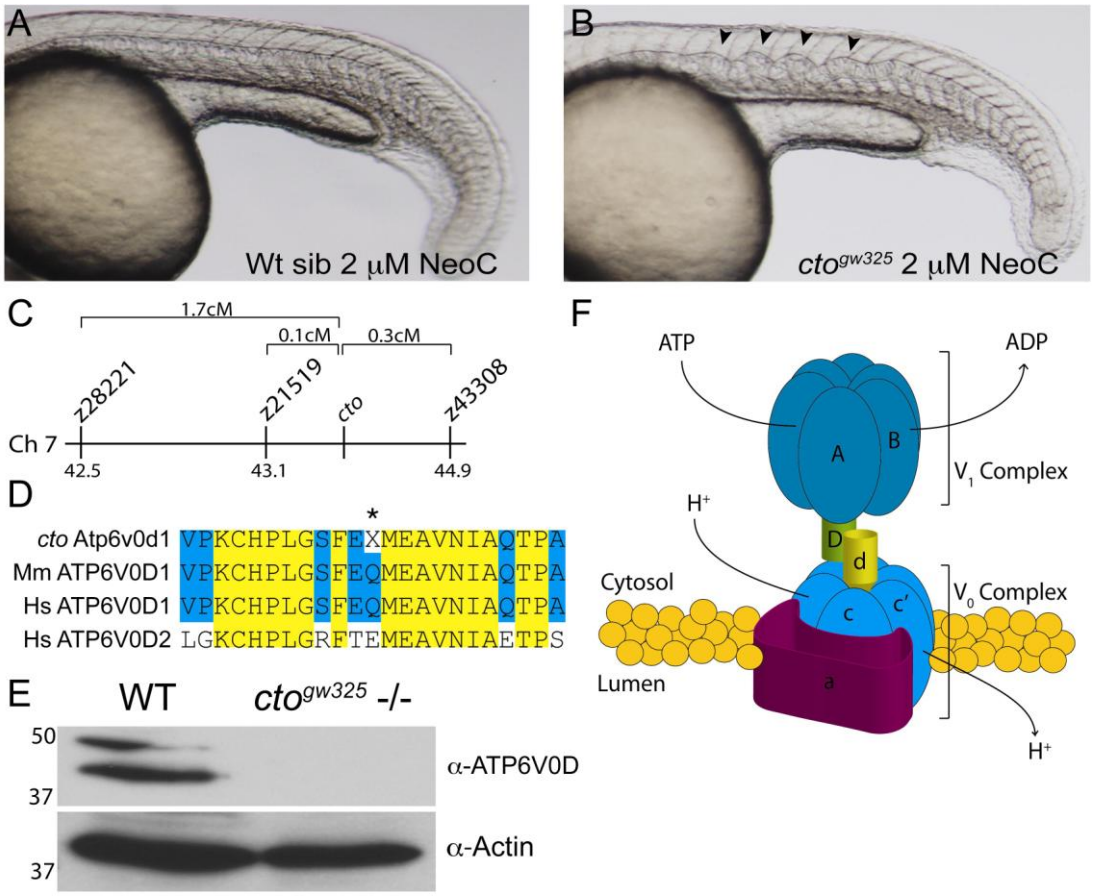


Figure 4. *catastrophe*^{gw325} contains a copper sensitive mutation in the vacuolar (H+) ATPase Atp6.

(A-B) *cto* embryos are globally sensitive to copper deficiency. Wild-type embryos (A) in 2 μM neocuproine do not display notochord defects. In contrast, *cto* embryos (B) placed in this same dose of neocuproine have significant distortion of the notochord in a pattern consistent with loss of lysyl oxidase activity [12]. (C) Meotic mapping placed the *cto* mutation between markers z21519 (43.1cM) and z43308 (44.9cM) on chromosome 7. (D) Atp6v0d1 is highly conserved between zebrafish and mammals and is easily differentiated from ATP6V0D2 present in humans. The amino acid Q136 is changed to a stop in the mutant (asterisk). (E) The mutation in *cto* abolishes expression of the full length protein. Immunoblot analysis using a C-terminal polyclonal antibody shows no recognition of the 40 kD band in 48 hpf *cto* embryos. The identity of the band at 50 kD is unknown. Actin was used as a loading control (lower panel). (F) Model of the proposed quaternary structure of Atp6. The lower-case d subunit (yellow) forms part of a connecting stalk between the V1 and V0 subunits the presence of which is required for proper formation of the entire transporter [19].

Figure 5

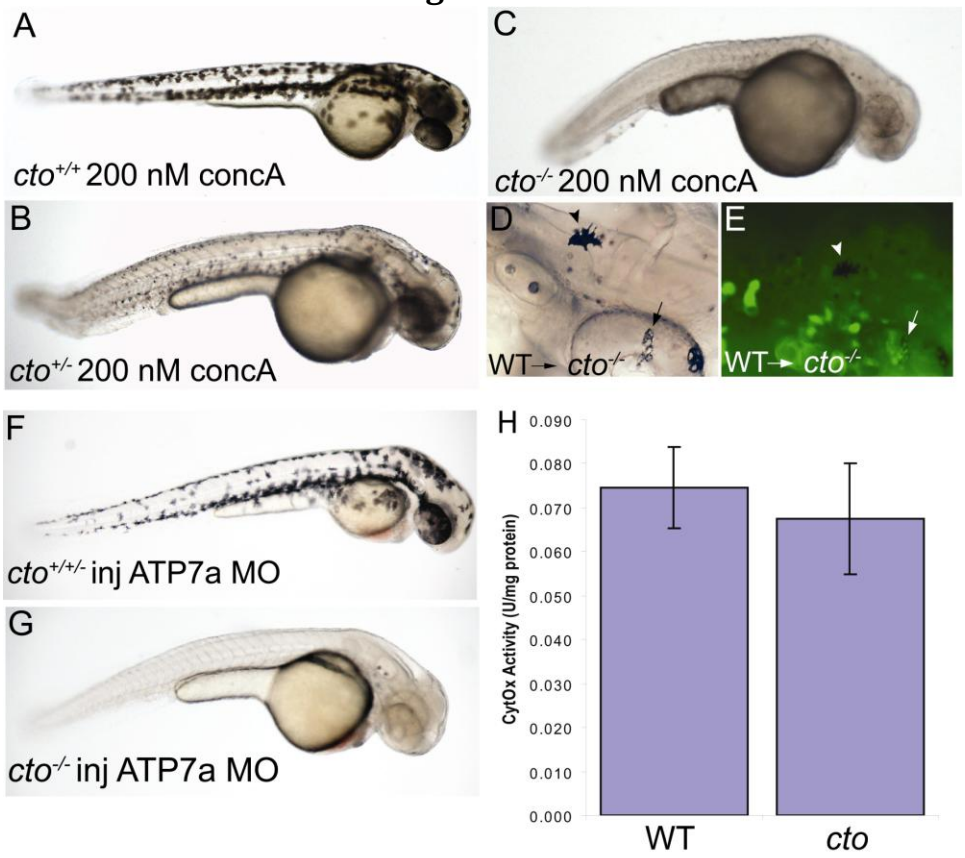


Figure 5. *cto* contains a concanamycin A sensitive, cell autonomous defect which affects secretory pathway copper transport.
(A-C) *cto* gene dosage alters sensitivity to concanamycin A (concA) an inhibitor of Atp6. Wild-type fish have no phenotypic response when incubated in 200 nM concanamycin A beginning at 24 hpf (A). Embryos heterozygous for *cto* are sensitive to this same dose of concA, resulting in punctate melanocytes (B). ConcA exacerbates the phenotype of *catastrophe* homozygotes resulting in total loss of pigmentation and increased degenerative appearance (C). (D-E) The defect in *cto* is cell autonomous both in epidermal and retinal pigment epithelial cells. Wild-type, GFP-positive cells were transplanted into *cto* mutant embryos at the 1000 cell stage and allowed to develop to 48 hpf. Robustly pigmented melanocytes with normal size and shape can be seen sparsely distributed throughout the epidermis (D, arrowhead) and retinal pigment epithelium (arrow, D). The epidermal melanocyte does not have visible GFP but is not surrounded by GFP-positive cells (E, arrowhead). The RPE cells have a central area of GFP-positivity (E, arrow) Other areas are GFP positive without melanin pigment. (F-G) *cto* homozygotes but not heterozygotes or wild-type embryos are sensitive to *atp7a* morpholino injection. At a sensitizing dose of morpholino that does not affect wild-type/heterozygotes (F), homozygous *cto* embryos lose all pigmentation (G). (H) Cytochrome c oxidase activity is not reduced in *cto* embryos. Activity was normalized to protein levels in each sample. Three independent samples were prepared from three groups of embryos and the standard deviation of the three experiments is shown.

Figure 6

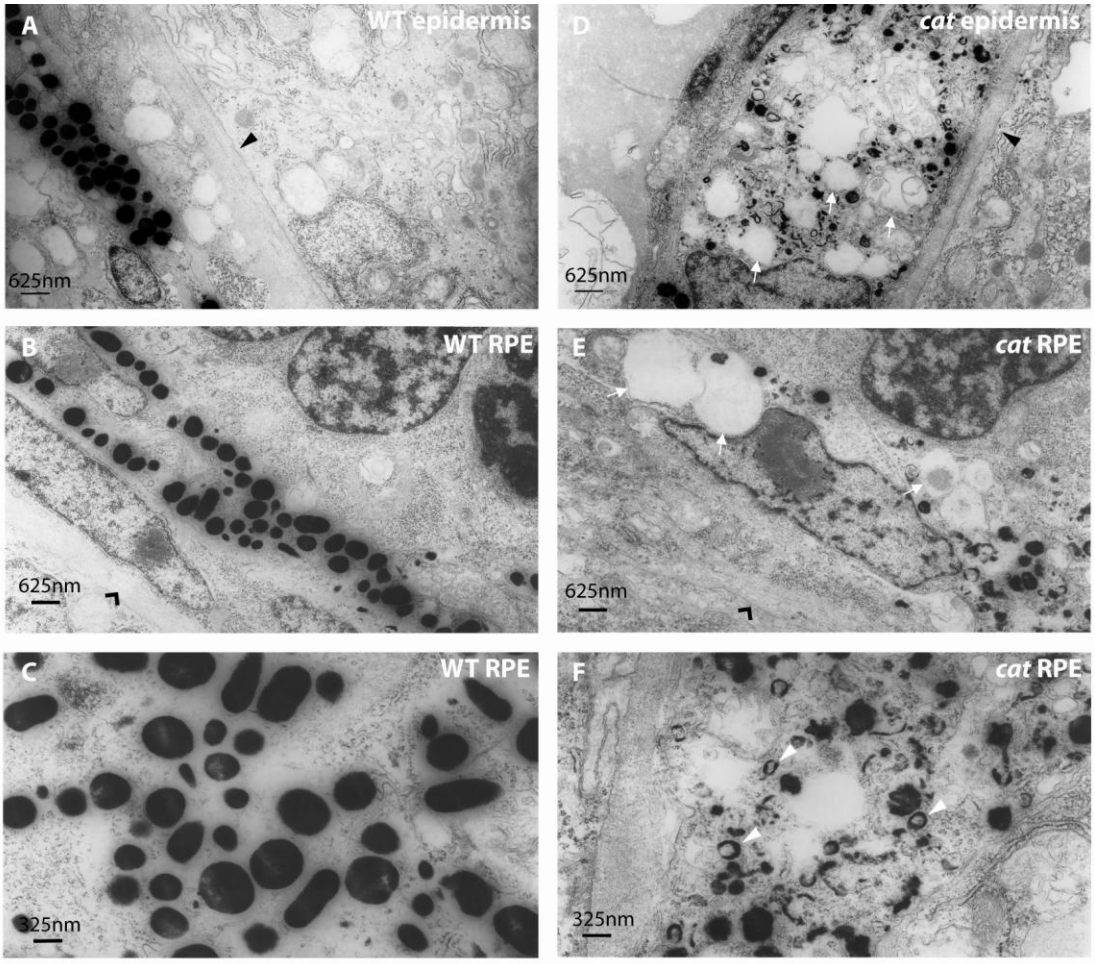
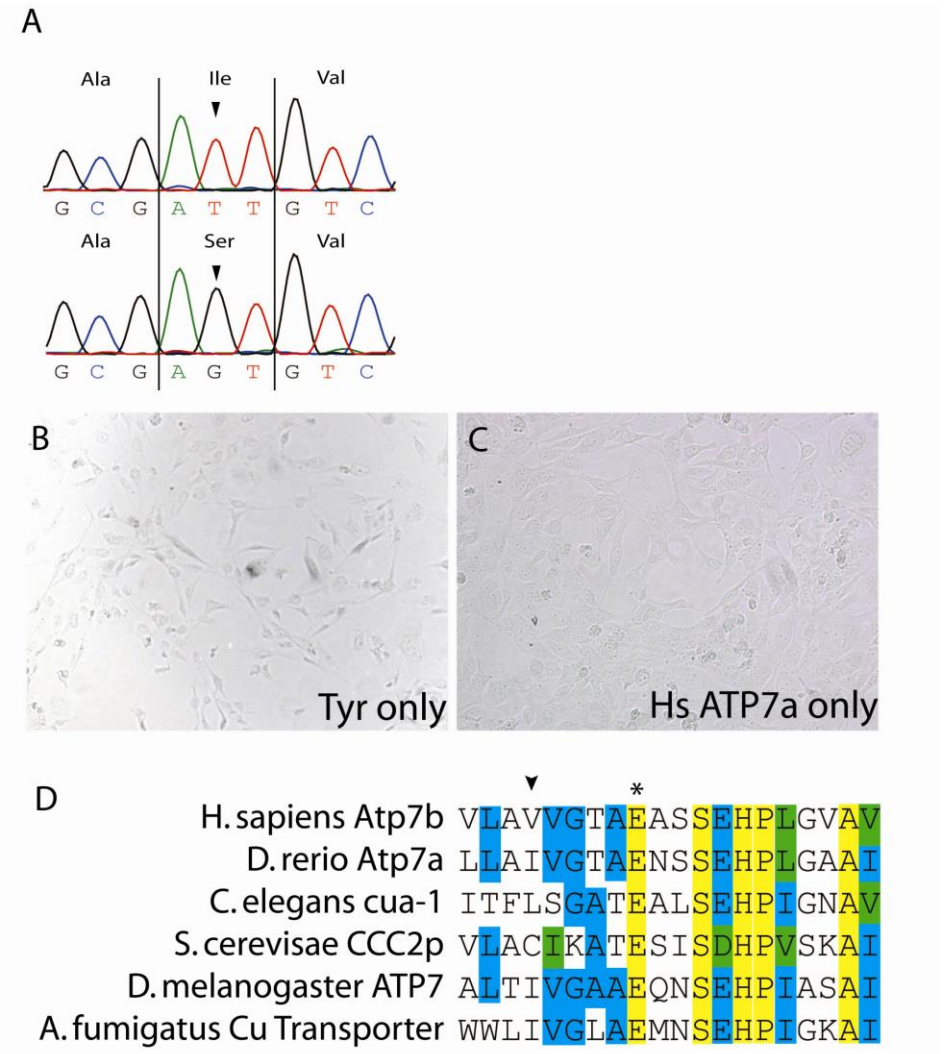


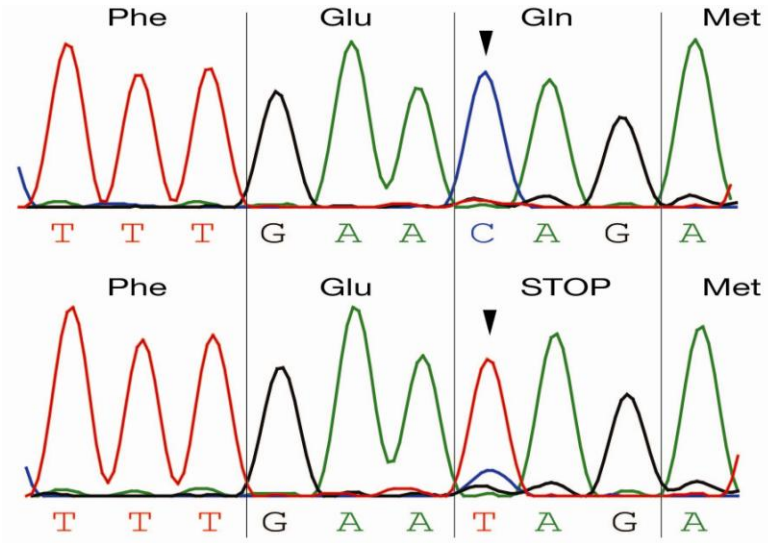
Figure 6. cat melanocytes have significant ultrastructural defects.
(A-B) Wild-type epidermal (A) and retinal pigment epithelial (RPE, B) melanocytes are elongated and thin and contain many large, densely pigmented melanosomes. The epithelial basement membrane is indicated by a black triangle in (A) and the RPE basement membrane by a black arrowhead in (B). (C) A higher magnification of wild-type melanosomes showing significant pigmentation and ellipsoid shape when cut longitudinally. (D-E) *cat* mutant epidermal (D) and RPE (E) melanocytes showing rounded, poorly pigmented cells that contain numerous large, empty vesicles (white arrows) The basement membranes are indicated as in (A and B). (F) A higher magnification of RPE melanosomes showing the diverse array of immature, poorly pigmented vesicles. The white arrowheads point to multi-lamellar, melanin filled vesicles which are identical to the melanin positive multi-vesicular bodies seen in the *cappucino* mouse (see text).

Supplemental Figure 1



(A) Sequencing of the *atp7a* cDNA in *cal*^{gw71} mutant embryos reveals a single non-synonymous nucleotide change T3182G which causes a non-conservative amino acid substitution T1061S. (B) Transfection of tyrosinase only into Me344 cells does not result in any appreciable tyrosinase activity. (C) Transfection of *atp7a* only into Me344 cells also does not result in L-DOPA oxidase activity. This activity is specific to tyrosinase expression. (D) Alignment of a small region of *atp7a* containing the mutation in *gw71* (arrowhead) and the highly conserved glutamate (asterisk) observed to be important for ATP binding/hydrolysis. This glutamate is fully conserved from fungus to humans.

Supplemental Figure 2



Sequencing of the cDNA of *atp6v0d1* which lies near the *cto* locus in revealed a single nucleotide change that creates an early stop codon.

Chapter 5

Conclusion and Future Directions

Conclusion

The studies described in this thesis have sought to elucidate the role of the critical nutrient copper in the development of the vertebrate zebrafish embryo. Severe copper deprivation caused by either nutritional deficiency or genetic deficiency results in several developmental phenotypes that phenocopy the human gentic disease, Menkes disease. Loss of cuproenzyme activity in either high-dose neocuproine treated embryos or in the mutant *calamity* results in loss of pigmentation and notochord defects. These are analagous to the hypopigmentation and basemement membrane defects observed in Menkes disease. Using this robust model of developmental copper deficiency I have sought to investigate further the genetics and cell metabolism of copper homeostasis.

Chapter 3 describes experiments which probe the possibility of early treatment of Menkes disease through correction of splice defects in zebrafish embryos which carry an inactivating mutation in *atp7a*. In human infants with Menkes disease there are subtle defects even at birth (Kaler et al., 2008) and most die by age 3. This implicates the protein in critical developmental processes. Also, it is possible that only a small amount of protein product may be necessary for restoration of function as evidenced by the disease Occipital Horn Syndrome. This latter disease is also caused by mutations in *ATP7A* but is less severe than Menkes disease and patients often survive to adulthood. If functional protein could be restored during early developmental periods, or if small amounts of functional protein could be maintained, this could offer promising therapies for severe Menkes disease.

Morpholino oligonucleotides alter splicing events via steric hindrance of the splicing machinery and have been used previously in cell culture models to restore

normal protein various diseases. This approach had not been demonstrated to rescue an *in vivo* disease phenotype. We took advantage of two splice mutations in alleles of the zebrafish mutant *calamity* (*atp7a*) to test the ability of morpholinos to functionally correct splice defects and lead to rescue of the mutant phenotype. Through the use of a series of morpholinos across an exon-intron boundary we investigated the effects of small changes in targeting sequence on the ability of the morpholino to alter splicing. The results demonstrated a set of principles of morpholino targeting to aid in the design of further molecules. The lack of functional rescue in these experiments demonstrated one of the limitations of the approach in that it is not capable of correcting point mutations which might remain in the final mRNA depending on the specific splice mutation.

The same approach for an intron-exon boundary resulted in a similar set of useful observations of targeting and the corresponding splicing effects. Three of the morpholinos used were able to rescue the phenotpe of the mutant. Complicating the analysis was the lack of observable change in the wild-type mRNA levels with morpholino injection. There were significant shifts in the splicing profile to a second upstream cryptic splice site which would also result in non-functional protein. The rescue coupled with the difficulties in identifying the mechanism could have several interpretations. The most probable, in my opinion, is that very little correct splicing is required to generate enough protein necessary for partial function. This interpretation, if correct, makes any therapy of this nature for this particular disease promising in that only a small effect must be produced to have significant phenotypic amelioration. These results also help explain the hypomorphic allele described above and discussed below.

Chapter 4 describes the central part of this thesis, a genetic investigation of novel mechanisms of copper homeostasis under conditions of limited availabilty. This screen for mutants sensitive to copper deficiency was designed to use the phenotype of loss of pigmentation to screen mutagenized zebrafish for proteins involved in copper homeostasis. The combination of subthreshold doses of the chelator neocuproine and genetic mutations increased the specificity of the screen for copper metabolic defects and removed several pigment-only mutants from the analysis (2-3 out of 700). As we expected we identified two types of mutants. The first was a hypomorphic allele of *atp7a*. This mutation demonstrated the ability of the screen to identify proteins critical for secretory-pathway copper homeostasis. If more mutants were screened we would expect to also find the zebrafish orthologue of *atox1*. This mutant also revealed a maternal effect of the mutation on embryonic pigmentation and expanded the hierarchy of copper metabolism. A developmental skeletal phenotype characterized by premature ossification of notochord defects in juvenile zebrafish mimics defects seen in both Menkes disease and Occipital Horn Syndrome and further investigation might shed light on the mechanisms of these skeletal deformities.

The second was a loss-of-function mutation in a critical subunit of the vacuolar (H^+) atpase (*atp6v0d1*). This mutant had its own distinct phenotype but was globally sensitive to copper deprivation implicating the proton gradient within the secretory compartment in the metabolism of copper. Genetic intersection with loss of *atp7a* recapitulated the neocuproine phenotype demonstrating the specificity for particular cellular compartments. Defects in intracellular morphology confirmed the role of this protein in vesicle trafficking.

Future Directions

These experiments have advanced our understanding of developmental copper metabolism and provide a foundation and tools for further investigation. There are several major ways in which this research could be expanded.

First, this was a relatively small screen of only 700 genomes. Further screening would potentially result in the identification of more interesting mutants. The two identified in this thesis demonstrated the sound nature of the screen approach and all that remains is to continue. One would expect to find more interesting intersections of copper homeostasis with other ion homeostasis as well as vesicular trafficking defects. We also expected, but have not yet found, mild mutations in tyrosinase which would affect the kinetics of copper loading into this enzyme. Such a mutant would expand our understanding of the compartments in which copper is actually transported and the mechanisms by which copper enters proteins once it is released into the secretory milieu.

Second, the identified hypomorphic allele opens another avenue for a similar approach to the same question. A modifier screen in this genetic background would be expected to find similar results as the neocuproine but might have the advantage of being more specific. It is also possible that there are mechanisms that would be sensitive to loss of copper with neocuproine but not with the weak allele, and vice versa. Thus this would be a complementary approach to continuing the original screen.

Third, the hypomorphic allele also displays prominent notochord and skeletal defects which give a model system to study the mechanisms of this process during development. Both Menkes patients and Occipital Horn Syndrome patients have skeletal deformities but it is unclear what the role of copper in these processes is. This allele

represents a tractable model for studying this process, particularly as it relates to the developing organism.

Fourth, the vacuolar atpase mutant has not been probed in depth for its effect on other phenotypes. This protein has been implicated in many cellular processes and no known knock-out exists. The *ex utero* development of the zebrafish embryo allows visualization of complex processes and the relatively normal development to 48 hpf might provide a system for evaluating the morphologic consequences of this type of severe disruption of cellular homeostasis outside of copper metabolism.

References

Ackermann, G.E., and Paw, B.H. (2003). Zebrafish: a genetic model for vertebrate organogenesis and human disorders. Front Biosci 8, d1227-1253.

Ajioka, R.S., Phillips, J.D., and Kushner, J.P. (2006). Biosynthesis of heme in mammals. Biochimica et biophysica acta 1763, 723-736.

Aller, S.G., and Unger, V.M. (2006). Projection structure of the human copper transporter CTR1 at 6-A resolution reveals a compact trimer with a novel channel-like architecture. Proc Natl Acad Sci U S A 103, 3627-3632.

Alter, J., Lou, F., Rabinowitz, A., Yin, H., Rosenfeld, J., Wilton, S.D., Partridge, T.A., and Lu, Q.L. (2006). Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat Med 12, 175-177.

Amsterdam, A., Nissen, R.M., Sun, Z., Swindell, E.C., Farrington, S., and Hopkins, N. (2004). Identification of 315 genes essential for early zebrafish development. Proc Natl Acad Sci U S A 101, 12792-12797.

Andrews, N.C. (2002). A genetic view of iron homeostasis. Seminars in hematology 39, 227-234.

Andrews, N.C., and Schmidt, P.J. (2007). Iron homeostasis. Annual review of physiology 69, 69-85.

Assaily, W., and Benchimol, S. (2006). Differential utilization of two ATP-generating pathways is regulated by p53. Cancer cell 10, 4-6.

Babcock, M., de Silva, D., Oaks, R., Davis-Kaplan, S., Jiralerspong, S., Montermini, L., Pandolfo, M., and Kaplan, J. (1997). Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin. Science (New York, N.Y 276, 1709-1712.

Bacopoulou, F., Henderson, L., and Philip, S.G. (2006). Menkes disease mimicking non-accidental injury. Archives of disease in childhood 91, 919.

Bahi-Buisson, N., Kaminska, A., Nabbout, R., Barnerias, C., Desguerre, I., De Lonlay, P., Mayer, M., Plouin, P., Dulac, O., and Chiron, C. (2006). Epilepsy in Menkes disease: analysis of clinical stages. Epilepsia 47, 380-386.

Balamurugan, K., and Schaffner, W. (2006). Copper homeostasis in eukaryotes: teetering on a tightrope. Biochimica et biophysica acta 1763, 737-746.

Barnard, R.O., Best, P.V., and Erdohazi, M. (1978). Neuropathology of Menkes' disease. Developmental medicine and child neurology 20, 586-597.

Bauerle, C., Ho, M.N., Lindorfer, M.A., and Stevens, T.H. (1993). The Saccharomyces cerevisiae VMA6 gene encodes the 36-kDa subunit of the vacuolar H(+)-ATPase membrane sector. The Journal of biological chemistry 268, 12749-12757.

Bohm, M., Pronicka, E., Karczmarewicz, E., Pronicki, M., Piekutowska-Abramczuk, D., Sykut-Cegielska, J., Mierzewska, H., Hansikova, H., Vesela, K., Tesarova, M., Houstkova, H., Houstek, J., and Zeman, J. (2006). Retrospective, multicentric study of 180 children with cytochrome C oxidase deficiency. Pediatric research 59, 21-26.

Bradbury, M.W. (1997). Transport of iron in the blood-brain-cerebrospinal fluid system. Journal of neurochemistry 69, 443-454.

Brown, D.R., Qin, K., Herms, J.W., Madlung, A., Manson, J., Strome, R., Fraser, P.E., Kruck, T., von Bohlen, A., Schulz-Schaeffer, W., Giese, A., Westaway, D., and Kretzschmar, H. (1997). The cellular prion protein binds copper in vivo. Nature 390, 684-687.

Bruno, I.G., Jin, W., and Cote, G.J. (2004). Correction of aberrant FGFR1 alternative RNA splicing through targeting of intronic regulatory elements. Hum Mol Genet 13, 2409-2420.

Bull, P.C., Thomas, G.R., Rommens, J.M., Forbes, J.R., and Cox, D.W. (1993). The Wilson disease gene is a putative copper transporting P-type ATPase similar to the Menkes gene. Nature genetics 5, 327-337.

Bush, A.I. (2000). Metals and neuroscience. Current opinion in chemical biology 4, 184-191.

Cheah, J.H., Kim, S.F., Hester, L.D., Clancy, K.W., Patterson, S.E., 3rd, Papadopoulos, V., and Snyder, S.H. (2006). NMDA receptor-nitric oxide transmission mediates neuronal iron homeostasis via the GTPase Dexras1. Neuron 51, 431-440.

Chelly, J., Tumer, Z., Tonnesen, T., Petterson, A., Ishikawa-Brush, Y., Tommerup, N., Horn, N., and Monaco, A.P. (1993). Isolation of a candidate gene for Menkes disease that encodes a potential heavy metal binding protein. Nature genetics 3, 14-19.

Chinnery, P.F., Crompton, D.E., Birchall, D., Jackson, M.J., Coulthard, A., Lombes, A., Quinn, N., Wills, A., Fletcher, N., Mottershead, J.P., Cooper, P., Kellett, M., Bates, D., and Burn, J. (2007). Clinical features and natural history of neuroferritinopathy caused by the FTL1 460InsA mutation. Brain 130, 110-119.

Christodoulou, J., Danks, D.M., Sarkar, B., Baerlocher, K.E., Casey, R., Horn, N., Tumer, Z., and Clarke, J.T. (1998). Early treatment of Menkes disease with parenteral copper-histidine: long-term follow-up of four treated patients. American journal of medical genetics 76, 154-164.

Cobine, P.A., Pierrel, F., Bestwick, M.L., and Winge, D.R. (2006a). Mitochondrial matrix copper complex used in metallation of cytochrome oxidase and superoxide dismutase. The Journal of biological chemistry 281, 36552-36559.

Cobine, P.A., Pierrel, F., and Winge, D.R. (2006b). Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. Biochimica et biophysica acta 1763, 759-772.

Craven, C.M., Alexander, J., Eldridge, M., Kushner, J.P., Bernstein, S., and Kaplan, J. (1987). Tissue distribution and clearance kinetics of non-transferrin-bound iron in the hypotransferrinemic mouse: a rodent model for hemochromatosis. Proc Natl Acad Sci U S A 84, 3457-3461.

Culotta, V.C., and Gitlin, J.D. (2001a). Disorders of copper transport. In The Molecular and Metabolic Basis of Inherited Disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, eds. (New York: McGraw-Hill), pp. 3105-3136.

Culotta, V.C., and Gitlin, J.D. (2001b). Disorders of Copper Transport. In The Molecular and Metabolic Basis of Inherited Disease. C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, eds. (New York: McGraw-Hill), pp. 3105-3136.

Culotta, V.C., Yang, M., and O'Halloran, T.V. (2006). Activation of superoxide dismutases: putting the metal to the pedal. Biochimica et biophysica acta 1763, 747-758.

Curtis, A.R., Fey, C., Morris, C.M., Bindoff, L.A., Ince, P.G., Chinnery, P.F., Coulthard, A., Jackson, M.J., Jackson, A.P., McHale, D.P., Hay, D., Barker, W.A., Markham, A.F., Bates, D., Curtis, A., and Burn, J. (2001). Mutation in the gene encoding ferritin light polypeptide causes dominant adult-onset basal ganglia disease. Nature genetics 28, 350-354.

Czeizel, A.E., and Dudas, I. (1992). Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. The New England journal of medicine 327, 1832-1835.

De Domenico, I., Ward, D.M., Musci, G., and Kaplan, J. (2006). Iron overload due to mutations in ferroportin. Haematologica 91, 92-95.

Dening, T.R. (1991). The neuropsychiatry of Wilson's disease: a review. International journal of psychiatry in medicine 21, 135-148.

Dettmer, J., Hong-Hermesdorf, A., Stierhof, Y.D., and Schumacher, K. (2006). Vacuolar H+-ATPase activity is required for endocytic and secretory trafficking in Arabidopsis. The Plant cell 18, 715-730.

Dmitriev, O., Tsivkovskii, R., Abildgaard, F., Morgan, C.T., Markley, J.L., and Lutsenko, S. (2006). Solution structure of the N-domain of Wilson disease protein:

distinct nucleotide-binding environment and effects of disease mutations. Proc Natl Acad Sci U S A 103, 5302-5307.

Doraiswamy, P.M., and Finefrock, A.E. (2004). Metals in our minds: therapeutic implications for neurodegenerative disorders. Lancet neurology 3, 431-434.

Doreulee, N., Yanovsky, Y., and Haas, H.L. (1997). Suppression of long-term potentiation in hippocampal slices by copper. Hippocampus 7, 666-669.

Du, L., Pollard, J.M., and Gatti, R.A. (2007). Correction of prototypic ATM splicing mutations and aberrant ATM function with antisense morpholino oligonucleotides. Proc Natl Acad Sci U S A 104, 6007-6012.

Du, S.J., Frenkel, V., Kindschi, G., and Zohar, Y. (2001). Visualizing normal and defective bone development in zebrafish embryos using the fluorescent chromophore calcein. Developmental biology 238, 239-246.

Durr, A., Cossee, M., Agid, Y., Campuzano, V., Mignard, C., Penet, C., Mandel, J.L., Brice, A., and Koenig, M. (1996). Clinical and genetic abnormalities in patients with Friedreich's ataxia. The New England journal of medicine 335, 1169-1175.

El Meskini, R., Cline, L.B., Eipper, B.A., and Ronnett, G.V. (2005). The developmentally regulated expression of Menkes protein ATP7A suggests a role in axon extension and synaptogenesis. Developmental neuroscience 27, 333-348.

Emre, S., Atillasoy, E.O., Ozdemir, S., Schilsky, M., Rathna Varma, C.V., Thung, S.N., Sternlieb, I., Guy, S.R., Sheiner, P.A., Schwartz, M.E., and Miller, C.M. (2001). Orthotopic liver transplantation for Wilson's disease: a single-center experience. Transplantation 72, 1232-1236.

Everett, C.M., Matharu, M., and Gawler, J. (2006). Neuropathy progressing to myeloneuropathy 20 years after partial gastrectomy. Neurology 66, 1451.

Ferenci, P. (2004). Pathophysiology and clinical features of Wilson disease. Metabolic brain disease 19, 229-239.

Gaeta, A., and Hider, R.C. (2005). The crucial role of metal ions in neurodegeneration: the basis for a promising therapeutic strategy. British journal of pharmacology 146, 1041-1059.

Gansner, J.M., Mendelsohn, B.A., Hultman, K.A., Johnson, S.L., and Gitlin, J.D. (2007a). Essential role of lysyl oxidases in notochord development. Dev Biol.

Gansner, J.M., Mendelsohn, B.A., Hultman, K.A., Johnson, S.L., and Gitlin, J.D. (2007b). Essential role of lysyl oxidases in notochord development. Developmental biology 307, 202-213.

Gebauer, F., Corona, D.F., Preiss, T., Becker, P.B., and Hentze, M.W. (1999). Translational control of dosage compensation in Drosophila by Sex-lethal: cooperative silencing via the 5' and 3' UTRs of msl-2 mRNA is independent of the poly(A) tail. The EMBO journal 18, 6146-6154.

Gebski, B.L., Mann, C.J., Fletcher, S., and Wilton, S.D. (2003). Morpholino antisense oligonucleotide induced dystrophin exon 23 skipping in mdx mouse muscle. Hum Mol Genet 12, 1801-1811.

Geller, T.J., Pan, Y., and Martin, D.S. (1997). Early neuroradiologic evidence of degeneration in Menkes' disease. Pediatric neurology 17, 255-258.

Gitlin, J.D. (1998). Aceruloplasminemia. Pediatric research 44, 271-276.

Gitlin, J.D. (2003). Wilson disease. Gastroenterology 125, 1868-1877.

Godwin, S.C., Shawker, T., Chang, B., and Kaler, S.G. (2006). Brachial artery aneurysms in Menkes disease. The Journal of pediatrics 149, 412-415.

Gollan, J.L., and Deller, D.J. (1973). Studies on the nature and excretion of biliary copper in man. Clinical science 44, 9-15.

Gollan, J.L., and Gollan, T.J. (1998). Wilson disease in 1998: genetic, diagnostic and therapeutic aspects. Journal of hepatology 28 Suppl 1, 28-36.

Grange, D.K., Kaler, S.G., Albers, G.M., Petterchak, J.A., Thorpe, C.M., and DeMello, D.E. (2005). Severe bilateral panlobular emphysema and pulmonary arterial hypoplasia: unusual manifestations of Menkes disease. Am J Med Genet A 139A, 151-155.

Gregory, A., and Hayflick, S.J. (2005). Neurodegeneration with brain iron accumulation. Folia neuropathologica / Association of Polish Neuropathologists and Medical Research Centre, Polish Academy of Sciences 43, 286-296.

Guo, H.H., Choe, J., and Loeb, L.A. (2004). Protein tolerance to random amino acid change. Proc Natl Acad Sci U S A 101, 9205-9210.

Haacke, E.M., Cheng, N.Y., House, M.J., Liu, Q., Neelavalli, J., Ogg, R.J., Khan, A., Ayaz, M., Kirsch, W., and Obenaus, A. (2005). Imaging iron stores in the brain using magnetic resonance imaging. Magnetic resonance imaging 23, 1-25.

Hamza, I., Faisst, A., Prohaska, J., Chen, J., Gruss, P., and Gitlin, J.D. (2001). The metallochaperone Atox1 plays a critical role in perinatal copper homeostasis. Proc Natl Acad Sci U S A 98, 6848-6852.

Hamza, I., and Gitlin, J.D. (2002). Copper chaperones for cytochrome c oxidase and human disease. Journal of bioenergetics and biomembranes 34, 381-388.

Hamza, I., Prohaska, J., and Gitlin, J.D. (2003). Essential role for Atox1 in the copper-mediated intracellular trafficking of the Menkes ATPase. Proc Natl Acad Sci U S A 100, 1215-1220.

Hardingham, G.E., and Bading, H. (2003). The Yin and Yang of NMDA receptor signalling. Trends in neurosciences 26, 81-89.

Harless, W., Crowell, E., and Abraham, J. (2006). Anemia and neutropenia associated with copper deficiency of unclear etiology. American journal of hematology 81, 546-549.

Harris, Z.L., Durley, A.P., Man, T.K., and Gitlin, J.D. (1999). Targeted gene disruption reveals an essential role for ceruloplasmin in cellular iron efflux. Proc Natl Acad Sci U S A 96, 10812-10817.

Harris, Z.L., Klomp, L.W., and Gitlin, J.D. (1998). Aceruloplasminemia: an inherited neurodegenerative disease with impairment of iron homeostasis. The American journal of clinical nutrition 67, 972S-977S.

Harris, Z.L., Takahashi, Y., Miyajima, H., Serizawa, M., MacGillivray, R.T., and Gitlin, J.D. (1995). Aceruloplasminemia: molecular characterization of this disorder of iron metabolism. Proc Natl Acad Sci U S A 92, 2539-2543.

Hartter, D.E., and Barnea, A. (1988). Evidence for release of copper in the brain: depolarization-induced release of newly taken-up 67copper. Synapse (New York, N.Y 2, 412-415.

Hayflick, S.J., Westaway, S.K., Levinson, B., Zhou, B., Johnson, M.A., Ching, K.H., and Gitschier, J. (2003). Genetic, clinical, and radiographic delineation of Hallervorden-Spatz syndrome. The New England journal of medicine 348, 33-40.

Hellman, N.E., and Gitlin, J.D. (2002). Ceruloplasmin metabolism and function. Annual review of nutrition 22, 439-458.

Hentze, M.W., Muckenthaler, M.U., and Andrews, N.C. (2004). Balancing acts: molecular control of mammalian iron metabolism. Cell 117, 285-297.

Hillenmeyer, M.E., Fung, E., Wildenhain, J., Pierce, S.E., Hoon, S., Lee, W., Proctor, M., St Onge, R.P., Tyers, M., Koller, D., Altman, R.B., Davis, R.W., Nislow, C., and Giaever, G. (2008). The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science (New York, N.Y 320, 362-365.

Horn, N., and Tumer, Z. (2002). Menkes Disease and the Occipital Horn Syndrome. In Connective Tissue and Its Heritable Disorders. P.M. Royce, and B. Steinmann, eds. (New York: Wiley-Liss, Inc.), pp. 651-685.

Horvath, R., Freisinger, P., Rubio, R., Merl, T., Bax, R., Mayr, J.A., Shawan, Muller-Hocker, J., Pongratz, D., Moller, L.B., Horn, N., and Jaksch, M. (2005). Congenital

cataract, muscular hypotonia, developmental delay and sensorineural hearing loss associated with a defect in copper metabolism. Journal of inherited metabolic disease 28, 479-492.

Hurtado-Lorenzo, A., Skinner, M., El Annan, J., Futai, M., Sun-Wada, G.H., Bourgoin, S., Casanova, J., Wildeman, A., Bechoua, S., Ausiello, D.A., Brown, D., and Marshansky, V. (2006). V-ATPase interacts with ARNO and Arf6 in early endosomes and regulates the protein degradative pathway. Nature cell biology 8, 124-136.

Huss, M., Ingenhorst, G., Konig, S., Gassel, M., Drose, S., Zeeck, A., Altendorf, K., and Wieczorek, H. (2002). Concanamycin A, the specific inhibitor of V-ATPases, binds to the V(o) subunit c. The Journal of biological chemistry 277, 40544-40548.

Javidan, Y., and Schilling, T.F. (2004). Development of cartilage and bone. Methods in cell biology 76, 415-436.

Jeong, S.Y., and David, S. (2003). Glycosylphosphatidylinositol-anchored ceruloplasmin is required for iron efflux from cells in the central nervous system. The Journal of biological chemistry 278, 27144-27148.

Jeong, S.Y., and David, S. (2006). Age-related changes in iron homeostasis and cell death in the cerebellum of ceruloplasmin-deficient mice. J Neurosci 26, 9810-9819.

Johnson, M.A., Kuo, Y.M., Westaway, S.K., Parker, S.M., Ching, K.H., Gitschier, J., and Hayflick, S.J. (2004). Mitochondrial localization of human PANK2 and hypotheses of secondary iron accumulation in pantothenate kinase-associated neurodegeneration. Annals of the New York Academy of Sciences 1012, 282-298.

Johnson, S.L., Africa, D., Horne, S., and Postlethwait, J.H. (1995). Half-tetrad analysis in zebrafish: mapping the ros mutation and the centromere of linkage group I. Genetics 139, 1727-1735.

Kaler, S.G. (1998). Metabolic and molecular bases of Menkes disease and occipital horn syndrome. Pediatr Dev Pathol 1, 85-98.

Kaler, S.G., Buist, N.R., Holmes, C.S., Goldstein, D.S., Miller, R.C., and Gahl, W.A. (1995). Early copper therapy in classic Menkes disease patients with a novel splicing mutation. Annals of neurology 38, 921-928.

Kaler, S.G., Holmes, C.S., Goldstein, D.S., Tang, J., Godwin, S.C., Donsante, A., Liew, C.J., Sato, S., and Patronas, N. (2008). Neonatal diagnosis and treatment of Menkes disease. The New England journal of medicine 358, 605-614.

Kane, P.M. (1995). Disassembly and reassembly of the yeast vacuolar H(+)-ATPase in vivo. The Journal of biological chemistry 270, 17025-17032.

Kane, P.M., Yamashiro, C.T., and Stevens, T.H. (1989). Biochemical characterization of the yeast vacuolar H(+)-ATPase. The Journal of biological chemistry 264, 19236-19244.

Kaneko, K., Yoshida, K., Arima, K., Ohara, S., Miyajima, H., Kato, T., Ohta, M., and Ikeda, S.I. (2002). Astrocytic deformity and globular structures are characteristic of the brains of patients with aceruloplasminemia. Journal of neuropathology and experimental neurology 61, 1069-1077.

Kardos, J., Kovacs, I., Hajos, F., Kalman, M., and Simonyi, M. (1989). Nerve endings from rat brain tissue release copper upon depolarization. A possible role in regulating neuronal excitability. Neuroscience letters 103, 139-144.

Keen, C.L., Uriu-Hare, J.Y., Hawk, S.N., Jankowski, M.A., Daston, G.P., Kwik-Uribe, C.L., and Rucker, R.B. (1998). Effect of copper deficiency on prenatal development and pregnancy outcome. The American journal of clinical nutrition 67, 1003S-1011S.

Kelly, E.J., and Palmiter, R.D. (1996). A murine model of Menkes disease reveals a physiological function of metallothionein. Nature genetics 13, 219-222.

Klomp, L.W., Farhangrazi, Z.S., Dugan, L.L., and Gitlin, J.D. (1996). Ceruloplasmin gene expression in the murine central nervous system. The Journal of clinical investigation 98, 207-215.

Klomp, L.W., and Gitlin, J.D. (1996). Expression of the ceruloplasmin gene in the human retina and brain: implications for a pathogenic model in aceruloplasminemia. Human molecular genetics 5, 1989-1996.

Kodama, H., Murata, Y., and Kobayashi, M. (1999). Clinical manifestations and treatment of Menkes disease and its variants. Pediatr Int 41, 423-429.

Kono, S., and Miyajima, H. (2006). Molecular and pathological basis of aceruloplasminemia. Biological research 39, 15-23.

Koury, M.J., and Ponka, P. (2004). New insights into erythropoiesis: the roles of folate, vitamin B12, and iron. Annual review of nutrition 24, 105-131.

Kozma, M., Szerdahelyi, P., and Kasa, P. (1981). Histochemical detection of zinc and copper in various neurons of the central nervous system. Acta histochemica 69, 12-17.

Kruse, J.P., and Gu, W. (2006). p53 aerobics: the major tumor suppressor fuels your workout. Cell metabolism 4, 1-3.

Kumar, N., Ahlskog, J.E., Klein, C.J., and Port, J.D. (2006). Imaging features of copper deficiency myelopathy: a study of 25 cases. Neuroradiology 48, 78-83.

Kumar, N., Gross, J.B., Jr., and Ahlskog, J.E. (2004). Copper deficiency myelopathy produces a clinical picture like subacute combined degeneration. Neurology 63, 33-39.

Kuo, Y.M., Gybina, A.A., Pyatskowit, J.W., Gitschier, J., and Prohaska, J.R. (2006). Copper transport protein (Ctr1) levels in mice are tissue specific and dependent on copper status. The Journal of nutrition 136, 21-26.

Kuo, Y.M., Zhou, B., Cosco, D., and Gitschier, J. (2001). The copper transporter CTR1 provides an essential function in mammalian embryonic development. Proc Natl Acad Sci U S A 98, 6836-6841.

LaVaute, T., Smith, S., Cooperman, S., Iwai, K., Land, W., Meyron-Holtz, E., Drake, S.K., Miller, G., Abu-Asab, M., Tsokos, M., Switzer, R., 3rd, Grinberg, A., Love, P., Tresser, N., and Rouault, T.A. (2001). Targeted deletion of the gene encoding iron regulatory protein-2 causes misregulation of iron metabolism and neurodegenerative disease in mice. Nature genetics 27, 209-214.

Leary, S.C., Cobine, P.A., Kaufman, B.A., Guercin, G.H., Mattman, A., Palaty, J., Lockitch, G., Winge, D.R., Rustin, P., Horvath, R., and Shoubridge, E.A. (2007). The human cytochrome c oxidase assembly factors SCO1 and SCO2 have regulatory roles in the maintenance of cellular copper homeostasis. Cell metabolism 5, 9-20.

Leary, S.C., Mattman, A., Wai, T., Koehn, D.C., Clarke, L.A., Chan, S., Lomax, B., Eydoux, P., Vallance, H.D., and Shoubridge, E.A. (2006). A hemizygous SCO2 mutation in an early onset rapidly progressive, fatal cardiomyopathy. Molecular genetics and metabolism 89, 129-133.

Lee, D.W., Andersen, J.K., and Kaur, D. (2006). Iron dysregulation and neurodegeneration: the molecular connection. Molecular interventions 6, 89-97.

Lee, J., Prohaska, J.R., and Thiele, D.J. (2001). Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. Proc Natl Acad Sci U S A 98, 6842-6847.

Leventer, R.J., Kornberg, A.J., Phelan, E.M., and Kean, M.J. (1997). Early magnetic resonance imaging findings in Menkes' disease. Journal of child neurology 12, 222-224.

Levi, S., Cozzi, A., and Arosio, P. (2005). Neuroferritinopathy: a neurodegenerative disorder associated with L-ferritin mutation. Best practice & research 18, 265-276.

Liegeois, S., Benedetto, A., Michaux, G., Belliard, G., and Labouesse, M. (2007). Genes required for osmoregulation and apical secretion in Caenorhabditis elegans. Genetics 175, 709-724.

Lill, R., and Muhlenhoff, U. (2006). Iron-sulfur protein biogenesis in eukaryotes: components and mechanisms. Annual review of cell and developmental biology 22, 457-486.

Lin, L.Y., Horng, J.L., Kunkel, J.G., and Hwang, P.P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. American journal of physiology 290, C371-378.

Liu, P.C., Chen, Y.W., Centeno, J.A., Quezado, M., Lem, K., and Kaler, S.G. (2005). Downregulation of myelination, energy, and translational genes in Menkes disease brain. Molecular genetics and metabolism 85, 291-300.

Llanos, R.M., Ke, B.X., Wright, M., Deal, Y., Monty, F., Kramer, D.R., and Mercer, J.F. (2006). Correction of a mouse model of Menkes disease by the human Menkes gene. Biochim Biophys Acta 1762, 485-493.

Logan, J.I., Harveyson, K.B., Wisdom, G.B., Hughes, A.E., and Archbold, G.P. (1994). Hereditary caeruloplasmin deficiency, dementia and diabetes mellitus. Qjm 87, 663-670.

Lozoff, B., Beard, J., Connor, J., Barbara, F., Georgieff, M., and Schallert, T. (2006). Long-lasting neural and behavioral effects of iron deficiency in infancy. Nutrition reviews 64, S34-43; discussion S72-91.

Lu, Q.L., Rabinowitz, A., Chen, Y.C., Yokota, T., Yin, H., Alter, J., Jadoon, A., Bou-Gharios, G., and Partridge, T. (2005). Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc Natl Acad Sci U S A 102, 198-203.

Lu, W., Man, H., Ju, W., Trimble, W.S., MacDonald, J.F., and Wang, Y.T. (2001). Activation of synaptic NMDA receptors induces membrane insertion of new AMPA receptors and LTP in cultured hippocampal neurons. Neuron 29, 243-254.

Lutsenko, S., Barnes, N.L., Bartee, M.Y., and Dmitriev, O.Y. (2007). Function and regulation of human copper-transporting ATPases. Physiol Rev 87, 1011-1046.

Lutsenko, S., and Petris, M.J. (2003). Function and regulation of the mammalian copper-transporting ATPases: insights from biochemical and cell biological approaches. The Journal of membrane biology 191, 1-12.

Maciel, P., Cruz, V.T., Constante, M., Iniesta, I., Costa, M.C., Gallati, S., Sousa, N., Sequeiros, J., Coutinho, P., and Santos, M.M. (2005). Neuroferritinopathy: missense mutation in FTL causing early-onset bilateral pallidal involvement. Neurology 65, 603-605.

Madsen, E.C., Morcos, P.A., Mendelsohn, B.A., and Gitlin, J.D. (2008). In vivo correction of a Menkes disease model using antisense oligonucleotides. Proc Natl Acad Sci U S A 105, 3909-3914.

Malikova, M., Shi, J., and Kandror, K.V. (2004). V-type ATPase is involved in biogenesis of GLUT4 vesicles. American journal of physiology 287, E547-552.

Mancuso, M., Davidzon, G., Kurlan, R.M., Tawil, R., Bonilla, E., Di Mauro, S., and Powers, J.M. (2005). Hereditary ferritinopathy: a novel mutation, its cellular pathology, and pathogenetic insights. Journal of neuropathology and experimental neurology 64, 280-294.

Matoba, S., Kang, J.G., Patino, W.D., Wragg, A., Boehm, M., Gavrilova, O., Hurley, P.J., Bunz, F., and Hwang, P.M. (2006). p53 regulates mitochondrial respiration. Science (New York, N.Y 312, 1650-1653.

Mendelsohn, B.A., Yin, C., Johnson, S.L., Wilm, T.P., Solnica-Krezel, L., and Gitlin, J.D. (2006). Atp7a determines a hierarchy of copper metabolism essential for notochord development. Cell Metab 4, 155-162.

Menkes, J.H. (1988). Kinky hair disease: twenty five years later. Brain & development 10, 77-79.

Menkes, J.H., Alter, M., Steigleder, G.K., Weakley, D.R., and Sung, J.H. (1962). A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. Pediatrics 29, 764-779.

Mercer, J.F. (1998). Menkes syndrome and animal models. The American journal of clinical nutrition 67, 1022S-1028S.

Mercer, J.F., Livingston, J., Hall, B., Paynter, J.A., Begy, C., Chandrasekharappa, S., Lockhart, P., Grimes, A., Bhave, M., Siemieniak, D., and et al. (1993). Isolation of a partial candidate gene for Menkes disease by positional cloning. Nature genetics 3, 20-25.

Merchant, S.S., and Sagasti, A. (2006). Precious metal economy. Cell metabolism 4, 99-101.

Miyajima, H., Nishimura, Y., Mizoguchi, K., Sakamoto, M., Shimizu, T., and Honda, N. (1987). Familial apoceruloplasmin deficiency associated with blepharospasm and retinal degeneration. Neurology 37, 761-767.

Moller, L.B., Tumer, Z., Lund, C., Petersen, C., Cole, T., Hanusch, R., Seidel, J., Jensen, L.R., and Horn, N. (2000). Similar splice-site mutations of the ATP7A gene lead to different phenotypes: classical Menkes disease or occipital horn syndrome. American journal of human genetics 66, 1211-1220.

Moos, T., and Morgan, E.H. (2004). The metabolism of neuronal iron and its pathogenic role in neurological disease: review. Annals of the New York Academy of Sciences 1012, 14-26.

Moos, T., Skjoerringe, T., Gosk, S., and Morgan, E.H. (2006). Brain capillary endothelial cells mediate iron transport into the brain by segregating iron from transferrin without the involvement of divalent metal transporter 1. Journal of neurochemistry 98, 1946-1958.

Morgan, N.V., Westaway, S.K., Morton, J.E., Gregory, A., Gissen, P., Sonek, S., Cangul, H., Coryell, J., Canham, N., Nardocci, N., Zorzi, G., Pasha, S., Rodriguez, D., Desguerre, I., Mubaidin, A., Bertini, E., Trembath, R.C., Simonati, A., Schanen, C., Johnson, C.A., Levinson, B., Woods, C.G., Wilmot, B., Kramer, P., Gitschier, J., Maher, E.R., and

Hayflick, S.J. (2006). PLA2G6, encoding a phospholipase A2, is mutated in neurodegenerative disorders with high brain iron. Nature genetics 38, 752-754.

Morita, H., Ikeda, S., Yamamoto, K., Morita, S., Yoshida, K., Nomoto, S., Kato, M., and Yanagisawa, N. (1995). Hereditary ceruloplasmin deficiency with hemosiderosis: a clinicopathological study of a Japanese family. Annals of neurology 37, 646-656.

Moulton, H.M., Fletcher, S., Neuman, B.W., McClorey, G., Stein, D.A., Abes, S., Wilton, S.D., Buchmeier, M.J., Lebleu, B., and Iversen, P.L. (2007). Cell-penetrating peptide-morpholino conjugates alter pre-mRNA splicing of DMD (Duchenne muscular dystrophy) and inhibit murine coronavirus replication in vivo. Biochem Soc Trans 35, 826-828.

Munakata, M., Sakamoto, O., Kitamura, T., Ishitobi, M., Yokoyama, H., Haginoya, K., Togashi, N., Tamura, H., Higano, S., Takahashi, S., Ohura, T., Kobayashi, Y., Onuma, A., and Iinuma, K. (2005). The effects of copper-histidine therapy on brain metabolism in a patient with Menkes disease: a proton magnetic resonance spectroscopic study. Brain & development 27, 297-300.

Nemeth, E., and Ganz, T. (2006). Regulation of iron metabolism by hepcidin. Annual review of nutrition 26, 323-342.

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T., and Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science (New York, N.Y 306, 2090-2093.

Nguyen, T., Novak, E.K., Kermani, M., Fluhr, J., Peters, L.L., Swank, R.T., and Wei, M.L. (2002). Melanosome morphologies in murine models of hermansky-pudlak syndrome reflect blocks in organelle development. The Journal of investigative dermatology 119, 1156-1164.

Niciu, M.J., Ma, X.M., El Meskini, R., Ronnett, G.V., Mains, R.E., and Eipper, B.A. (2006). Developmental changes in the expression of ATP7A during a critical period in postnatal neurodevelopment. Neuroscience 139, 947-964.

Nittis, T., and Gitlin, J.D. (2002). The copper-iron connection: hereditary aceruloplasminemia. Seminars in hematology 39, 282-289.

Norgate, M., Lee, E., Southon, A., Farlow, A., Batterham, P., Camakaris, J., and Burke, R. (2006). Essential roles in development and pigmentation for the Drosophila copper transporter DmATP7. Mol Biol Cell 17, 475-484.

Nose, Y., Kim, B.E., and Thiele, D.J. (2006a). Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell metabolism 4, 235-244.

Nose, Y., Rees, E.M., and Thiele, D.J. (2006b). Structure of the Ctr1 copper trans'PORE'ter reveals novel architecture. Trends in biochemical sciences 31, 604-607.

O'Halloran, T.V., and Culotta, V.C. (2000). Metallochaperones, an intracellular shuttle service for metal ions. The Journal of biological chemistry 275, 25057-25060.

Oder, W., Grimm, G., Kollegger, H., Ferenci, P., Schneider, B., and Deecke, L. (1991). Neurological and neuropsychiatric spectrum of Wilson's disease: a prospective study of 45 cases. Journal of neurology 238, 281-287.

Oide, T., Yoshida, K., Kaneko, K., Ohta, M., and Arima, K. (2006). Iron overload and antioxidative role of perivascular astrocytes in aceruloplasminemia. Neuropathology and applied neurobiology 32, 170-176.

Okeda, R., Gei, S., Chen, I., Okaniwa, M., Shinomiya, M., and Matsubara, O. (1991). Menkes' kinky hair disease: morphological and immunohistochemical comparison of two autopsied patients. Acta neuropathologica 81, 450-457.

Olivares, J.L., Bueno, I., Gallati, S., and Ramos, F.J. (2006). Late-onset treatment in Menkes disease: is there a correlation between genotype and response to therapy? Clinical genetics 69, 363-366.

Parsons, A.B., Brost, R.L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G.W., Kane, P.M., Hughes, T.R., and Boone, C. (2004). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. Nature biotechnology 22, 62-69.

Patel, B.N., Dunn, R.J., and David, S. (2000). Alternative RNA splicing generates a glycosylphosphatidylinositol-anchored form of ceruloplasmin in mammalian brain. The Journal of biological chemistry 275, 4305-4310.

Patel, B.N., Dunn, R.J., Jeong, S.Y., Zhu, Q., Julien, J.P., and David, S. (2002). Ceruloplasmin regulates iron levels in the CNS and prevents free radical injury. J Neurosci 22, 6578-6586.

Pena, M.M., Lee, J., and Thiele, D.J. (1999). A delicate balance: homeostatic control of copper uptake and distribution. The Journal of nutrition 129, 1251-1260.

Petris, M.J., Mercer, J.F., Culvenor, J.G., Lockhart, P., Gleeson, P.A., and Camakaris, J. (1996). Ligand-regulated transport of the Menkes copper P-type ATPase efflux pump from the Golgi apparatus to the plasma membrane: a novel mechanism of regulated trafficking. Embo J 15, 6084-6095.

Petris, M.J., Strausak, D., and Mercer, J.F. (2000). The Menkes copper transporter is required for the activation of tyrosinase. Human molecular genetics 9, 2845-2851.

Pietrangelo, A. (2006a). Hereditary hemochromatosis. Biochimica et biophysica acta 1763, 700-710.

Pietrangelo, A. (2006b). Hereditary hemochromatosis. Annual review of nutrition 26, 251-270.

Ponka, P. (1999). Cellular iron metabolism. Kidney international 69, S2-11.

Ponka, P. (2004). Hereditary causes of disturbed iron homeostasis in the central nervous system. Annals of the New York Academy of Sciences 1012, 267-281.

Prodan, C.I., Bottomley, S.S., Holland, N.R., and Lind, S.E. (2006). Relapsing hypocupraemic myelopathy requiring high-dose oral copper replacement. Journal of neurology, neurosurgery, and psychiatry 77, 1092-1093.

Prohaska, J.R., and Brokate, B. (2002). The timing of perinatal copper deficiency in mice influences offspring survival. The Journal of nutrition 132, 3142-3145.

Puccio, H., Simon, D., Cossee, M., Criqui-Filipe, P., Tiziano, F., Melki, J., Hindelang, C., Matyas, R., Rustin, P., and Koenig, M. (2001). Mouse models for Friedreich ataxia exhibit cardiomyopathy, sensory nerve defect and Fe-S enzyme deficiency followed by intramitochondrial iron deposits. Nature genetics 27, 181-186.

Rae, T.D., Schmidt, P.J., Pufahl, R.A., Culotta, V.C., and O'Halloran, T.V. (1999). Undetectable intracellular free copper: the requirement of a copper chaperone for superoxide dismutase. Science (New York, N.Y 284, 805-808.

Rouault, T.A. (2006). The role of iron regulatory proteins in mammalian iron homeostasis and disease. Nature chemical biology 2, 406-414.

Rouault, T.A., and Tong, W.H. (2005). Iron-sulphur cluster biogenesis and mitochondrial iron homeostasis. Nature reviews 6, 345-351.

Sato, M., Ohtomo, K., Daimon, T., Sugiyama, T., and Iijima, K. (1994). Localization of copper to afferent terminals in rat locus ceruleus, in contrast to mitochondrial copper in cerebellum. J Histochem Cytochem 42, 1585-1591.

Schilsky, M.L., and Fink, S. (2006). Inherited metabolic liver disease. Current opinion in gastroenterology 22, 215-222.

Schlief, M.L., Craig, A.M., and Gitlin, J.D. (2005). NMDA receptor activation mediates copper homeostasis in hippocampal neurons. J Neurosci 25, 239-246.

Schlief, M.L., and Gitlin, J.D. (2006). Copper homeostasis in the CNS: a novel link between the NMDA receptor and copper homeostasis in the hippocampus. Molecular neurobiology 33, 81-90.

Schlief, M.L., West, T., Craig, A.M., Holtzman, D.M., and Gitlin, J.D. (2006). Role of the Menkes copper-transporting ATPase in NMDA receptor-mediated neuronal toxicity. Proc Natl Acad Sci U S A 103, 14919-14924.

Schumacher, G., Platz, K.P., Mueller, A.R., Neuhaus, R., Luck, W., Langrehr, J.M., Settmacher, U., Steinmueller, T., Becker, M., and Neuhaus, P. (2001). Liver transplantation in neurologic Wilson's disease. Transplantation proceedings 33, 1518-1519.

Shaw, G.C., Cope, J.J., Li, L., Corson, K., Hersey, C., Ackermann, G.E., Gwynn, B., Lambert, A.J., Wingert, R.A., Traver, D., Trede, N.S., Barut, B.A., Zhou, Y., Minet, E., Donovan, A., Brownlie, A., Balzan, R., Weiss, M.J., Peters, L.L., Kaplan, J., Zon, L.I., and Paw, B.H. (2006). Mitoferrin is essential for erythroid iron assimilation. Nature 440, 96-100.

Sheela, S.R., Latha, M., Liu, P., Lem, K., and Kaler, S.G. (2005). Copper-replacement treatment for symptomatic Menkes disease: ethical considerations. Clin Genet 68, 278-283.

Sickmier, E.A., Frato, K.E., Shen, H., Paranawithana, S.R., Green, M.R., and Kielkopf, C.L. (2006). Structural basis for polypyrimidine tract recognition by the essential pre-mRNA splicing factor U2AF65. Mol Cell 23, 49-59.

Smith, A.N., Lovering, R.C., Futai, M., Takeda, J., Brown, D., and Karet, F.E. (2003). Revised nomenclature for mammalian vacuolar-type H+ -ATPase subunit genes. Molecular cell 12, 801-803.

Smith, G.F., and McCurdy, W.H. (1952). 2,9-Dimethyl-1,10-phenanthroline. Analytical Chemistry 24, 371-373.

Solnica-Krezel, L., Schier, A.F., and Driever, W. (1994). Efficient recovery of ENU-induced mutations from the zebrafish germline. Genetics 136, 1401-1420.

Sparks, D.L., and Schreurs, B.G. (2003). Trace amounts of copper in water induce beta-amyloid plaques and learning deficits in a rabbit model of Alzheimer's disease. Proc Natl Acad Sci U S A 100, 11065-11069.

Susser, E., Hoek, H.W., and Brown, A. (1998). Neurodevelopmental disorders after prenatal famine: The story of the Dutch Famine Study. American journal of epidemiology 147, 213-216.

Suwanmanee, T., Sierakowska, H., Fucharoen, S., and Kole, R. (2002). Repair of a splicing defect in erythroid cells from patients with beta-thalassemia/HbE disorder. Mol Ther 6, 718-726.

Tan, J.C., Burns, D.L., and Jones, H.R. (2006). Severe ataxia, myelopathy, and peripheral neuropathy due to acquired copper deficiency in a patient with history of gastrectomy. Jpen 30, 446-450.

Tang, J., Robertson, S., Lem, K.E., Godwin, S.C., and Kaler, S.G. (2006). Functional copper transport explains neurologic sparing in occipital horn syndrome. Genet Med 8, 711-718.

Tanzi, R.E., Petrukhin, K., Chernov, I., Pellequer, J.L., Wasco, W., Ross, B., Romano, D.M., Parano, E., Pavone, L., Brzustowicz, L.M., and et al. (1993). The Wilson disease gene is a copper transporting ATPase with homology to the Menkes disease gene. Nature genetics 5, 344-350.

Tao, T.Y., and Gitlin, J.D. (2003). Hepatic copper metabolism: insights from genetic disease. Hepatology (Baltimore, Md 37, 1241-1247.

Taupenot, L., Harper, K.L., and O'Connor, D.T. (2005). Role of H+-ATPase-mediated acidification in sorting and release of the regulated secretory protein chromogranin A: evidence for a vesiculogenic function. The Journal of biological chemistry 280, 3885-3897.

Tawfeek, H.A., and Abou-Samra, A.B. (2004). Important role for the V-type H(+)-ATPase and the Golgi apparatus in the recycling of PTH/PTHrP receptor. American journal of physiology 286, E704-710.

Thomas, M., and Jankovic, J. (2004). Neurodegenerative disease and iron storage in the brain. Current opinion in neurology 17, 437-442.

Tomashek, J.J., Graham, L.A., Hutchins, M.U., Stevens, T.H., and Klionsky, D.J. (1997). V1-situated stalk subunits of the yeast vacuolar proton-translocating ATPase. The Journal of biological chemistry 272, 26787-26793.

Trombley, P.Q., and Shepherd, G.M. (1996). Differential modulation by zinc and copper of amino acid receptors from rat olfactory bulb neurons. Journal of neurophysiology 76, 2536-2546.

Ugarte, M., Aguado, C., Desviat, L.R., Sanchez-Alcudia, R., Rincon, A., and Perez, B. (2007). Propionic and Methylmalonic Acidemia: Antisense Therapeutics for Intronic Variations Causing Aberrantly Spliced Messenger RNA. Am J Hum Genet 81.

Vidal, R., Ghetti, B., Takao, M., Brefel-Courbon, C., Uro-Coste, E., Glazier, B.S., Siani, V., Benson, M.D., Calvas, P., Miravalle, L., Rascol, O., and Delisle, M.B. (2004). Intracellular ferritin accumulation in neural and extraneural tissue characterizes a neurodegenerative disease associated with a mutation in the ferritin light polypeptide gene. Journal of neuropathology and experimental neurology 63, 363-380.

Vlachova, V., Zemkova, H., and Vyklicky, L., Jr. (1996). Copper modulation of NMDA responses in mouse and rat cultured hippocampal neurons. The European journal of neuroscience 8, 2257-2264.

Voncken, M., Ioannou, P., and Delatycki, M.B. (2004). Friedreich ataxia-update on pathogenesis and possible therapies. Neurogenetics 5, 1-8.

Vulpe, C., Levinson, B., Whitney, S., Packman, S., and Gitschier, J. (1993). Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase. Nature genetics 3, 7-13.

Waggoner, D.J., Bartnikas, T.B., and Gitlin, J.D. (1999). The role of copper in neurodegenerative disease. Neurobiology of disease 6, 221-230.

Wang, G.S., and Cooper, T.A. (2007). Splicing in disease: disruption of the splicing code and the decoding machinery. Nat Rev Genet 8, 749-761.

Weihl, C.C., and Lopate, G. (2006). Motor neuron disease associated with copper deficiency. Muscle & nerve 34, 789-793.

Weiser, T., and Wienrich, M. (1996). The effects of copper ions on glutamate receptors in cultured rat cortical neurons. Brain research 742, 211-218.

Westerfield, M. (2000). The Zebrafish Book: A guide for the laboratory use of zebrafish (*Danio rerio*). (Eugene, OR: University of Oregon Press).

Willis, M.S., Monaghan, S.A., Miller, M.L., McKenna, R.W., Perkins, W.D., Levinson, B.S., Bhushan, V., and Kroft, S.H. (2005). Zinc-induced copper deficiency: a report of three cases initially recognized on bone marrow examination. American journal of clinical pathology 123, 125-131.

Wong, P.C., Waggoner, D., Subramaniam, J.R., Tessarollo, L., Bartnikas, T.B., Culotta, V.C., Price, D.L., Rothstein, J., and Gitlin, J.D. (2000). Copper chaperone for superoxide dismutase is essential to activate mammalian Cu/Zn superoxide dismutase. Proc Natl Acad Sci U S A 97, 2886-2891.

Wood, M., Yin, H., and McClorey, G. (2007). Modulating the expression of disease genes with RNA-based therapy. PLoS Genet 3, e109.

Wu, L.J., Leenders, A.G., Cooperman, S., Meyron-Holtz, E., Smith, S., Land, W., Tsai, R.Y., Berger, U.V., Sheng, Z.H., and Rouault, T.A. (2004). Expression of the iron transporter ferroportin in synaptic vesicles and the blood-brain barrier. Brain research 1001, 108-117.

Xu, T., Vasilyeva, E., and Forgac, M. (1999). Subunit interactions in the clathrin-coated vesicle vacuolar (H(+))-ATPase complex. The Journal of biological chemistry 274, 28909-28915.

Yamaguchi, Y., Heiny, M.E., and Gitlin, J.D. (1993). Isolation and characterization of a human liver cDNA as a candidate gene for Wilson disease. Biochemical and biophysical research communications 197, 271-277.

Yeo, G., Hoon, S., Venkatesh, B., and Burge, C.B. (2004). Variation in sequence and organization of splicing regulatory elements in vertebrate genes. Proc Natl Acad Sci U S A 101, 15700-15705.

Yoshida, K., Furihata, K., Takeda, S., Nakamura, A., Yamamoto, K., Morita, H., Hiyamuta, S., Ikeda, S., Shimizu, N., and Yanagisawa, N. (1995). A mutation in the ceruloplasmin gene is associated with systemic hemosiderosis in humans. Nature genetics 9, 267-272.

Zecca, L., Youdim, M.B., Riederer, P., Connor, J.R., and Crichton, R.R. (2004). Iron, brain ageing and neurodegenerative disorders. Nat Rev Neurosci 5, 863-873.

Zhou, B., Westaway, S.K., Levinson, B., Johnson, M.A., Gitschier, J., and Hayflick, S.J. (2001). A novel pantothenate kinase gene (PANK2) is defective in Hallervorden-Spatz syndrome. Nature genetics 28, 345-349.

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Daniell K, Stewart M, **Madsen E**, Le M, Handl H, Brooks N, Kiakos K, Hartley JA, Lee M. (2005) Design, synthesis, and biological evaluation of achiral analogs of duocarmycin SA. *Bioorg Med Chem Lett*. **15**(1):177-80.

E.R. Lacy, **E.C. Madsen**, M. Lee and W.D. Wilson, 2003, Polyamide Dimer Stacking in the Minor Groove and Recognition of T.G Mismatched Base Pairs of DNA. In **Small Molecule DNA and RNA Binders: From Synthesis to Nucleic**

Acid Complexes, Vol. 2, M. Demeunynck, C. Bailly and W.D. Wilson Eds., Wiley-VCH, New York. p. 384-413.
Madsen E, Gitlin JD. Copper deficiency. *Curr Opin Gastroenterol*. (2007) **23**(2):187-92.

Madsen E, Gitlin JD. (2007) Copper and iron disorders of the brain. *Annu Rev Neurosci*. **30**:317-37.

Madsen E.C., Morcos P.A., Mendelsohn B.A., Gitlin J.D. (2008) In vivo correction of a Menkes disease model using antisense oligonucleotides. *Proc Natl Acad Sci U S A*. **105**(10):3909-14.

Gansner, J.M., **Madsen, E.C.,** Mecham, R.P., Gitlin, J.D. (2008) Essential role for fibrillin-2 in zebrafish notochord and vascular morphogenesis. *Dev. Dyn.* **237**(10):2844-61.

Madsen, E.C., and Gitlin, J.D. (2008) Zebrafish mutants *calamity* and *catastrophe* define critical pathways of gene-nutrient interactions in developmental copper metabolism. *PLoS Genetics 4(11): e1000261 doi:10.1371/journal.pgen.1000261*

Posters and Presentations:

Madsen, E., Price, C., Toth, J., Lingerfelt, B., Hubbard III, R.B., Handl, H., Hudson, S., Kiakos, K., Hartley, J., Bowen, P.J. and Lee, M.. (2001) Oral Presentation. *Molecular Recognition of DNA Sequence-Specific Alkylation by Conjugates of Hairpin Polyamide-Duocarmycin Analogs*. 2001 Southeast ACS Meeting, Savannah, GA.

Madsen, E. and Lee, M. (2001) Oral Presentation. Design of a Novel *Heterodimeric Model for DNA Sequence Specific Recognition*. Third Beckman Foundation Symposium, Irvine, CA.

Madsen, E., Toth, J., Price, C., Lingerfelt, B., Handl, H., Hudson, S., Kiakos, K., Hartley, J. and Lee, M.. (2002). Poster. *Molecular Recognition of DNA Sequence-Specific Alkylation by Conjugates of Hairpin Polyamide-Duocarmycin Analogs*. 2002 Spring National ACS Meeting, Orlando, FL.

Madsen, E.C. and Gitlin, J.D. (2007) Oral Presentation. *A Small Molecule Screen Identifies Novel Mutants In Copper Homeostasis.* 5th European Zebrafish Genetics and Development Meeting, Amsterdam, Netherlands.

Madsen, E.C., Morcos, P., and Gitlin, J.D. (2007) Poster. *Morpholino-Targeted Correction Of Genetic Diseases Due To Splice Mutations.* 5th European Zebrafish Genetics and Development Meeting, Amsterdam, Netherlands. **Poster Prize Winner.**

Madsen, E.C. and Gitlin, J.D. (2008) Oral Presentation. *A Small Molecule Screen Identifies Novel Mutants In Copper Homeostasis.* Department of Developmental Biology Inaugural Symposium, Washington University School of Medicine, Saint Louis, MO.