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Golgi-Dependent Mechanisms of Cellular Copper Homeostasis

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Golgi-Dependent Mechanisms of Cellular Copper Homeostasis

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A.B., Princeton University, 2010

Advisor: Victor Faundez, MD PhD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Biochemistry, Cell, & Developmental Biology
2017

Abstract

Golgi-Dependent Mechanisms of Cellular Copper Homeostasis

By Heather Skye Comstra

Copper is required for diverse cellular processes including pigment production, neuropeptide synthesis and mitochondrial function, yet possesses the capacity to inflict oxidative damage to cells. Cells possess a network of chaperones and transporters that maintain appropriate copper levels both for its provision to cuproenzymes and to avoid oxidative damage. Mutations in copper-binding proteins strongly associate with neuropathologies, and neurodegenerative diseases such as Alzheimer's and Parkinson's disease exhibit altered copper homeostasis. The cellular trafficking and regulation of copper has traditionally been described as occurring in a discrete, organelle-specific manner, yet emerging research supports a model of copper-sensing and communication taking place between organelles.

I hypothesize that genetic defects in molecules required for the subcellular localization of copper transporters will impair neuronal tissue viability. I define an interaction network for the copper transporter ATP7A and find it is enriched in genes associated with neuropathologies. Among these genes are those encoding subunits of the conserved oligomeric Golgi (COG) complex, a multimeric tethering complex required for retrograde intra-Golgi traffic. I present biochemical and genetic evidence of an interaction between ATP7A and COG, and establish a role for the COG complex in copper homeostasis at multiple cellular compartments. I find that COG null cells display decreased ATP7A levels and perturbed surface expression of both ATP7A and the copper importer CTR1. Further, both copper content and levels of copper-sensitive transcripts are altered in COG null cells. Reduced copper content, measured by inductively coupled plasma mass spectrometry, and impaired mitochondrial function, assayed by the activity of mitochondrial reductases, can be rescued by the addition of copper in conjunction with an ionophore. Finally, ATP7A and COG synthetically interact in *Drosophila melanogaster* to influence viability and the development of the neuromuscular junction.

These data support a model of global cellular copper homeostasis, and the altered copper homeostasis observed in COG null cells suggests a mechanism to account for neurodevelopmental phenotypes in patients bearing COG mutations. The ATP7A interactome offers avenues for future work to elucidate copper homeostasis mechanisms, and the observed interaction between copper homeostasis and mitochondrial function in this work illuminates a possible mechanism in the etiology of copper-related neuropathologies.

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Acknowledgements

I owe a great deal of thanks to many people that supported me during my dissertation work and writing. It would not have possible without you.

Thank you to my mentor Victor for finding something to celebrate everyday, whether it is a successful, or even not so successful, experiment or an exciting new book; you have taught me to find beauty in unexpected places. Thank you for encouraging me to pursue my interest in teaching and for modeling how to be a great educator. I look forward to many more impromptu visits to your office to discuss science, politics, books, news, and everything else.

Thank you to the Faundez lab members, past and present. It has been the greatest joy to work alongside Drs. Stephanie Zlatic and Avanti Gokhale. Thank you both for training me and for being incredible role models of women in science. Your dedication to and enthusiasm for science are inspiring. Thank you to former lab members Drs. Pearl Ryder and Ariana Mullin for laying the groundwork for my dissertation project, and thank you to Dr. Erica Werner for your assistance and training in all things mitochondrial.

Drs. Michael Koval, Andrew Kowalczyk, Steven L'Hernault and Win Sale – You have been instrumental not only to my dissertation, but to my graduate career. Thank you for your commitment, time, insight, and support. Thank you Dr. L'Hernault for the training and encouragement to set out on this path, and Dr. Koval for helping me continue onwards.

Thank you to my family, both by birth and by choice, for all of the love and support you have offered. Mom – Thank you for encouraging my love of science and for supporting me every step of the way, even when that meant moving across the country at fourteen. You fostered my independent spirit but were, and still are, always ready with a shoulder to cry on, a story to make me laugh, and biscuits to make it all better. You are a model of strength, confidence, and compassion, and I couldn't be happier being told I'm just like my mom. Dad – Thank you for all of the afternoon phone calls and long weekend visits; you made Texas feel much closer than it is. You also provided me with one of the greatest gifts - a love of books and reading that has sustained my educational pursuits and offered countless hours of happiness. Sean – I feel very lucky to have a little brother I look up to so much. I admire your willingness to bring an open heart and an open mind to every situation. You are the most generous person I know, and I can never thank you enough for all that you've done for me.

And finally, thank you to Sachin for celebrating every accomplishment, for making the failures not seem so bad, for always knowing how to make me smile, for introducing yourself on my first day of graduate school, and for being by my side every day since.

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CHAPTER 1
GENERAL INTRODUCTION

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Overview

There is over fifty years of research substantiating the role of trace metals in the pathogenesis of neurodegenerative disease. Both Alzheimer's and Parkinson's disease display altered metal content, particularly in the brain, along with oxidative stress that is tentatively attributed to the redox activity of unbound metals. While there is convincing correlative evidence that metals such as zinc and copper function in the pathogenesis of neurodegenerative disease, there is a dearth of information regarding the mechanistic underpinnings. In order to better understand this relationship, we look to genes involved in cellular copper homeostasis, mutations in which are often causative of neuropathologies ranging from neurodevelopmental disorders to neurodegeneration. By better understanding mechanisms of copper regulation, we hope to uncover novel mechanisms tying aberrant regulation to neurodegeneration.

In approaching this question, we focused on the Golgi-localized copper transporter ATP7A for three primary reasons: it is required not only for loading copper in cuproenzymes that traverse the Golgi complex but also in maintaining total cellular copper content, we have limited understanding of its trafficking mechanisms, and the severe neurodegeneration observed in patients with ATP7A loss of function mutations cannot be accounted for by our current understanding of ATP7A molecular interactions and ascribed functions. Our strategy was to first define the ATP7A interactome using a proteomics approach and to then further characterize candidates prioritized by bioinformatic tools. Our aim was to broaden our

understanding of ATP7A regulatory mechanisms and their influence in copper homeostasis in general. This approach yielded the identification of five hundred and forty one interaction partners for ATP7A, many identified for the first time. We found that this interactome was enriched in proteins encoding genes associated with neurodevelopmental and neurodegenerative disorders. We concentrated on a novel ATP7A interactor, the conserved oligomeric Golgi (COG) complex, an eight-subunit complex required for retrograde trafficking of Golgi resident enzymes. To an even greater extent than ATP7A, there are many unanswered questions regarding the COG complex. It too shares severe, early onset neurodevelopmental phenotypes in patients with COG subunit mutations, the etiology of which is not explained. Our work points to aberrant copper homeostasis as a possible contributing factor. We found that COG null cells exhibit copper deficiency and decreased mitochondrial function, both of which can be alleviated by the addition of a copper ionophore. Using *Drosophila melanogaster*, we also confirmed a genetic interaction between ATP7A and the COG complex. While these findings prompt further questions, particularly regarding a mechanistic understanding of the COG-ATP7A interaction, these data support our assertion that investigating the interactomes of copper regulatory genes can yield fruitful information to better understand how copper influences the progression of neurodegenerative disease.

Organization of the Introduction

In this introduction, I will first discuss basic concepts of copper homeostasis at the cellular level, including the molecules responsible for binding copper and moving

this metal across membranes and in between different subcellular compartments, and how these molecules are regulated. Following is a description of the phenotypic consequences of copper imbalances with a particular emphasis on the nervous system, culminating in a discussion of the indirect evidence supporting the role of copper in neurodegenerative disorders. These foundations set up the unresolved question I address in this dissertation, namely, how genetic defects in a membrane bound copper transporter, ATP7A, cause neurological phenotypes.

Section 1: Copper Transport

The ability of copper to transition between oxidation states is a valuable asset for many catalytic reactions [1]. However, along with this property comes the capacity to induce oxidative damage [2]. Consequently, levels of both intracellular and plasma copper are maintained at extremely low concentrations for cellular viability. For example, in *Saccharomyces cerevisiae*, the total intracellular copper concentration is thought to be $1.3x10^6$ ions per cell, approximately 70 μM, but there exists less than one free copper ion per cell [3, 4]. The metal is essentially always bound to a series of transporters, chaperones, and cuproenzymes. Below is an overview of the key copper trafficking pathways in the cell.

1.1 Uptake

Just as intracellular copper is always bound to a series of metal-binding proteins, copper is mobilized to assorted tissue types by proteins present in the blood plasma. This role is fulfilled chiefly by ceruloplasmin, which accounts for 50-90% of the

copper in circulation, along with a small pool bound by albumin and transcuprein [5] [6] [7]. Some redundancy exists among these copper-binding proteins as deletion of ceruloplasmin does not produce disruptions in copper distribution [8]. It was recently shown that ceruloplasmin-bound copper circulating in the blood plasma is transferred via direct protein interaction to copper transporters on the plasma membrane [9]. This direct method of transfer is a recurring theme, underscoring a global mechanism of sequestering copper to prevent potential damage caused by unbound redox active copper.

The primary mechanism of intracellular copper uptake occurs via the human copper transporter 1 (Ctr1), a molecule that in this dissertation I demonstrate is part of a copper homeostasis regulatory interactome [10]. The Ctr1 transporter exclusively binds copper and in HEK293 cells accounts for at least 70% of copper uptake. While Ctr1 is thought to account for the majority of copper uptake, observations in CTR1 KO cells suggest that other uptake mechanisms exist as well [11]. Auxiliary import mechanisms likely vary by cell type to meet the demands of the extracellular environment. For example, the divalent metal transporter 1 (Dmt1), primarily known as an iron transporter, will import copper in both HEK293 cells and duodenal enterocytes, particularly under conditions of iron deficiency in the case of the latter cell type [12, 13]. Work performed in human umbilical vein endothelial cells revealed that siRNA silencing of either Ctr1 or Dmt1 did not slow the rate of copper uptake, but elimination of both transporters led to a complete lack of copper uptake [11]. In the same study, prolonged (24 hour) exposure to excess copper

correlated with decreased Ctr1 protein levels. Ctr1 is known to undergo reversible endocytosis when copper levels are elevated, and selected studies suggest it may be degraded after endocytosis as well [14, 15]. These findings illustrate the tissue complexity of copper transport mechanisms.

1.2 Delivery to and activity of copper varies by cell compartment

Once transporters at the plasma membrane have taken up copper, it is transferred to one of three known cytosolic chaperones that deliver the metal to the appropriate subcellular compartment; in mammalian cells, these chaperones are ATOX1, copper chaperone for superoxide dismutase-1 (CCS), and COX17, which deliver copper to the Golgi complex, cytosol, and mitochondria, respectively [16, 17] [18, 19] (Figure 1). There is conflicting evidence as to whether an intermediate carrier exists between Ctr1 and these chaperones. A 2013 study demonstrated that reducing copper chaperone levels in HEK293 cells bore no effect on the rate of copper entry, suggesting copper could be imported without direct interaction with chaperones, while impairing glutathione (GSH) production significantly reduced copper import and could be rescued by the addition of exogenous GSH [20]. However, it was recently demonstrated by in vitro NMR dynamics that transfer of copper from Ctr1 to Atox1 occurs via a direct interaction between the two proteins [21].

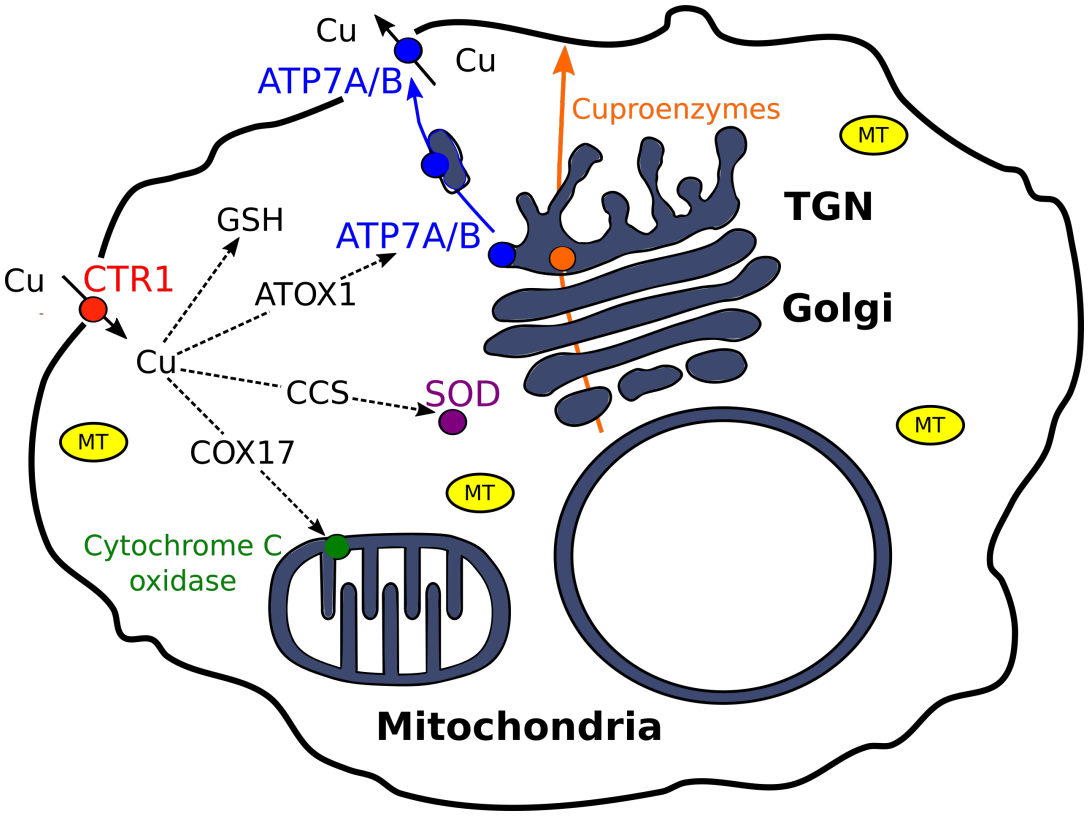


Figure 1. Overview of cellular copper trafficking

Copper enters the cell at the plasma membrane, primarily via the copper transporter CTR1 (red circle). CTR1 then transfers copper to three cytosolic copper chaperones, ATOX1, CCS, and COX17, which deliver the metal to the appropriate compartment. There is also evidence that CTR1 transfers copper to glutathione (GSH) as an intermediary. Of the copper chaperones, ATOX1 provides copper to the Golgi-resident transporters, ATP7A and ATP7B (blue circles). These P-type ATPases pump the copper into the lumen of the Golgi, where it is bound by holoenzymes that traverse the biosynthetic pathway and require copper as a cofactor (orange circle and arrow). Under steady state conditions, ATP7A and B are localized to the Golgi complex but translocate to the plasma membrane to pump copper out of the cell if the intracellular copper levels become elevated. CCS, the aptly named copper chaperone for SOD1, provides copper to the cytosolic superoxide dismutase 1 (purple circle). Finally, COX17 is thought to be responsible for the majority of copper provision to the mitochondria, where the metal is required as a cofactor for cytochrome c oxidase (green circle). In addition to these copper-specific transporters and chaperones, metallothioneins (yellow circles labeled MT) possess general metal-binding properties, and their protein expression is modified as necessary to buffer excess copper. Figure modified from Polishchuk and Lutsenko 2013.

After copper has been delivered to the appropriate compartment, the Golgi complex, cytoplasm, or mitochondria, it is incorporated into cuproenzymes that require the metal as a cofactor. Mutations in membrane transporters or cytoplasmic metallochaperones that regulate the incorporation of copper into apoenzymes lead to decreased function of corresponding cuproenzymes, and often to disruptions in cellular copper homeostasis, suggesting both local and global copper sensing [22-25]. There are three primary pathways that copper must traverse to maintain copper homeostasis: the secretory pathway by way of membrane bound transporters ATP7A and ATP7B present in the Golgi complex, delivery to the cytosol, primarily to the soluble cuproenzyme superoxide dismutase 1 (SOD1), and incorporation into the mitochondrial membrane-bound cytochrome c oxidase (COX) [16, 17] [18, 19]. These pathways have traditionally been considered discrete entities, but evidence from our work and that of others increasingly suggests the presence of global copper homeostasis mechanisms. In the next sections I describe essential cuproenzymes whose function is required in different cellular compartments.

1.2.1 Cytoplasm: SOD1

The enzyme superoxide dismutase 1 is ubiquitously expressed in all tissues and, as the name suggests, plays an important role in removing superoxide species via the generation of oxygen and hydrogen peroxide [26]. It forms a homodimer and requires both copper and zinc for catalytic activity and structural integrity, respectively [27]. SOD1 localizes primarily to the cytoplasm but is also found in

nuclei, lysosomes and the intermembrane space of the mitochondria [28-32]. Delivery to and incorporation of copper into SOD1 is carried out by copper-specific metallochaperone for SOD1 (CCS) [33]. Like other copper chaperones, CCS contains an MXCXXC metal-binding domain that facilitates passage of copper to the binding site in SOD1[33]. CCS selectively targets SOD1 for copper transfer, and mutations in either protein phenocopy to a large extent [34]. However, low, residual SOD1 activity remains in the absence of CCS, suggesting that SOD1 may be capable of acquiring copper from other sources. Candidate chaperones include metallothioneins, which have been shown to interact with and deliver copper to SOD1 in vitro, and glutathione-bound copper (Cu-GSH), which is an established mechanism of copper delivery to SOD1 in *Caenorhabditis elegans* and *Saccharomyces cerevisiae* [35-37].

1.2.2 Mitochondria: cytochrome c oxidase (COX)

Of the three respiratory complexes that make up the electron transport chain, cytochrome c oxidase (COX) is the terminal member of the chain and the only one that requires copper as a cofactor [38, 39]. COX is the only copper-dependent enzyme in the mitochondria, with the exception of a small pool of SOD1 that exists in the intermembrane space [32]. Incorporation of copper into COX requires multiple copper-binding proteins, the most upstream being COX17, a soluble copper chaperone that delivers copper to the mitochondria from CTR1 and is required for the assembly and function of COX [40, 41]. Two COX subunits, Cox1 and Cox2, require copper as a cofactor. Insertion of the metal is dependent on the copper-

binding integral membrane proteins COX11, for Cox1, and SCO1 and SCO2, for Cox2, all of which are thought to be supplied with copper by COX17 [17, 42-45] (Figure 2). While the requirement for SCO1, SCO2 and COX11 in copper transfer is long established, there is a growing list of soluble accessory proteins present in the intermembrane space that are required for COX function. COX 19, COX23, and PET191 are relatively newly identified proteins required for COX function, all of which localize to the intermembrane space and cytosol and share a twin-Cx_9C copper-binding motif with COX 17 [46-48].

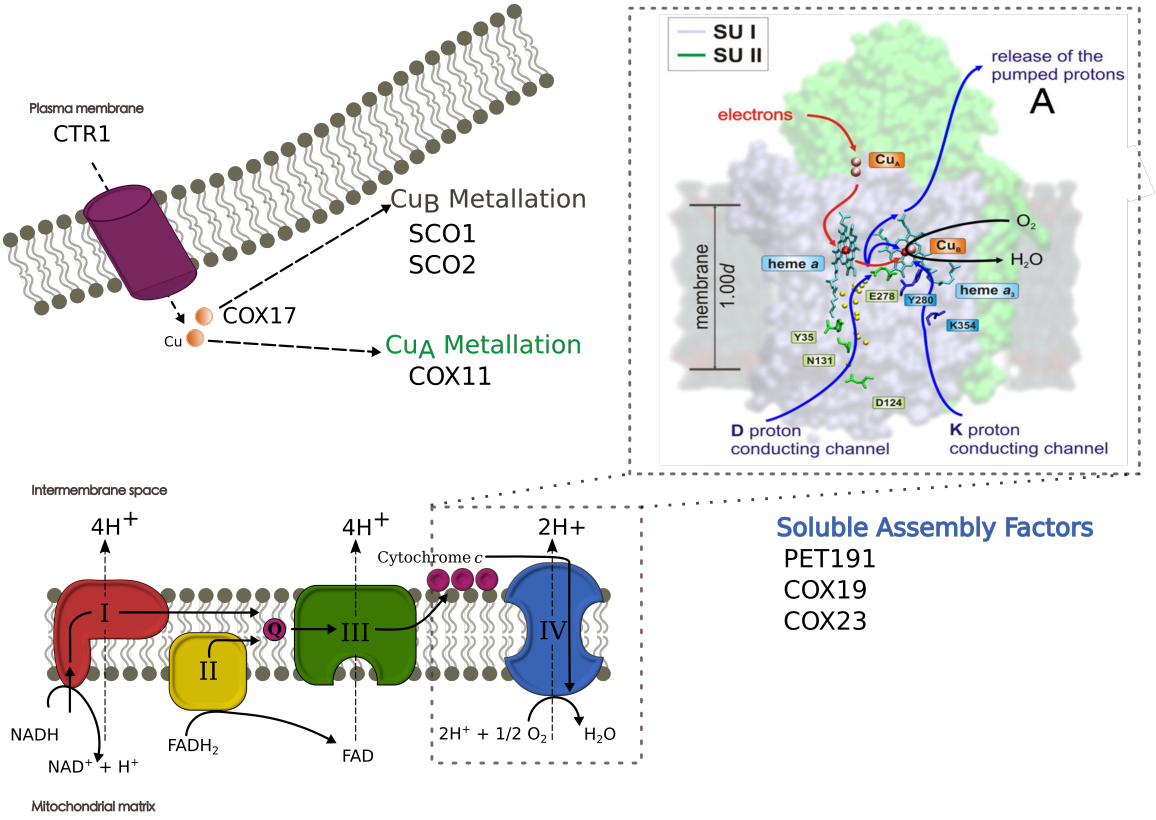


Figure 2. Mitochondrial copper handling

In the mitochondria, cytochrome *c* oxidase (COX) is the fourth component of the electron transport chain and requires copper as a cofactor. Depicted here are seven of the proteins required for the

copper loading and assembly of COX. After entering the cell via CTR1, copper is bound by the chaperone COX17 for transport to the mitochondria. At the inner membrane, COX17 provides copper to three membrane-bound chaperones: SCO1, SCO2, and COX11. SCO1 and SCO2 are required for copper loading of subunit 1 of COX, at the site indicated as Cu_B. Similarly COX11 is required for copper loading of subunit 2, at the site Cu_A. Appropriate copper delivery and binding are necessary for the catalytic activity of COX. Additional assembly factors including PET191, COX19, and COX23 are also required for COX function, but further understanding of the function of these factors is still under investigation. Figure modified from Belevich et al. 2010.

Interestingly, both SCO1 and SCO2 appear to have a role in cellular copper homeostasis that is distinct from COX subunit assembly; induction of genetic loss of either metallochaperone triggers global cellular copper deficiency due to increased copper export [49]. In SCO1 and SCO2 deficient patient cells, a fluorescent copper sensor was used to demonstrate that maintenance of the mitochondrial copper supply is prioritized when cellular copper content is compromised [50]. Recent work has shed light on the mechanism responsible for the role of SCO1 and SCO2 in cellular copper homeostasis. One study observed a correlation between total copper content and the redox state of the SCO1 copper-binding motif, CxxxC with copper deficiency leading to oxidation of the cysteines and excess leading to reduction. Alteration of the SCO1 redox state is though to be downstream of SCO2, which possesses oxidoreductase activity [51]. One model suggests that the COX17 homolog, COX19, present in the mitochondria and cytosol, acts as an intermediary between SCO1 and ATP7A, which is responsible for the copper efflux observed in SCO1 and SCO2 deficient cells [52] (Figure 3). This is an important precedent for the work I will present in Chapter 2, which points to another instance of multi-compartment copper regulatory mechanisms.

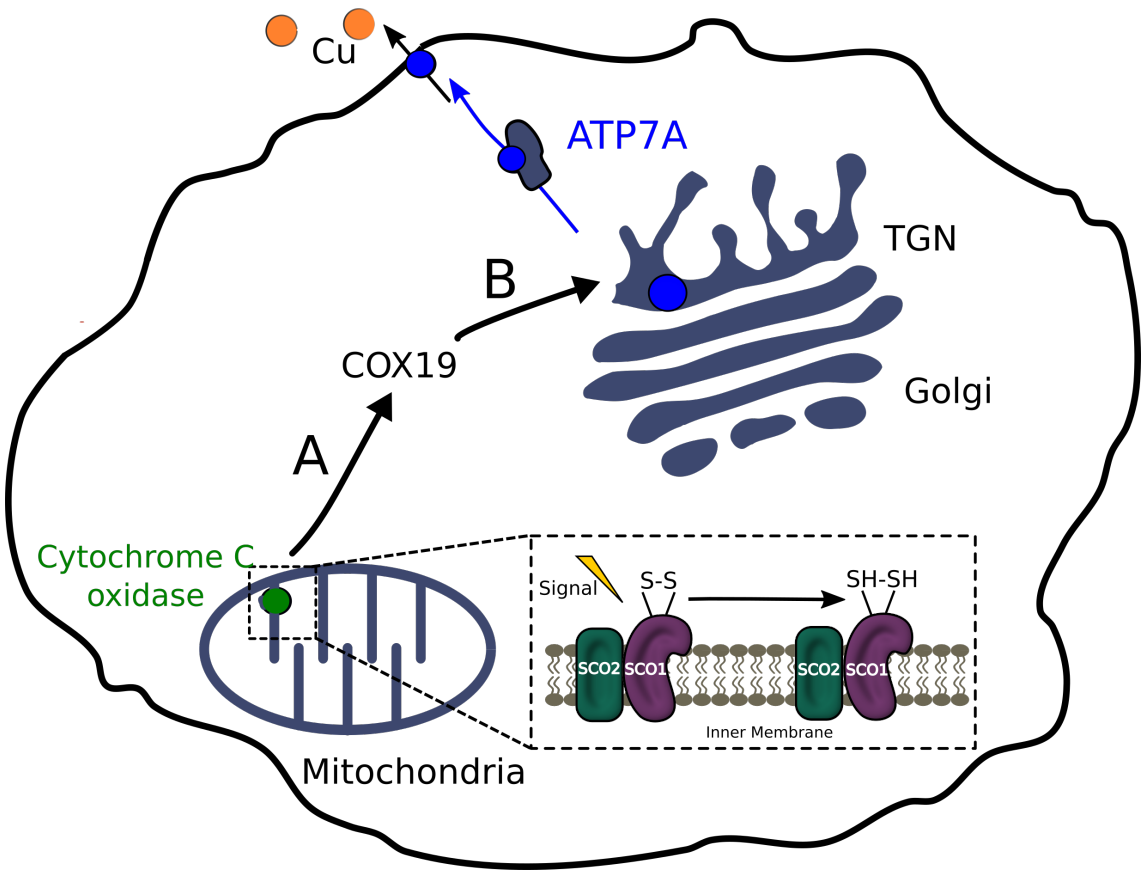


Figure 3. Regulation of cellular copper homeostasis via multiple cell compartments

While often studied in a cellular compartment specific manner, there is an increasing interest in global copper homeostasis mechanisms. One such mechanism depicted here demonstrates copper sensing by mitochondrial proteins that is then relayed to a copper transporter at the Golgi complex. SCO1 contains a copper-binding domain, CxxC, which varies in its oxidation state. While not definitively demonstrated, SCO2 is thought to act as an oxidoreductase regulating the oxidation state of SCO1. When intracellular copper levels are elevated, the ratio of reduced to oxidized SCO1 cysteine is increased. This change in redox state correlates with the translocation of ATP7A to the plasma membrane, where it exports copper from the cell. While the mechanism underlying the relay of this signal is not yet understood, it does require the presence of cytosolic copper binding factor COX19, a homolog of the mitochondrial copper chaperone COX17. This suggests a model in which the redox state of mitochondrially localized proteins is relayed to COX19 (A), which then signals this change to ATP7A at the Golgi complex (B). Figure modified from Leary 2010.

SCO1 may also play a role in regulating the expression of the copper transporter CTR1. One study found that, via unknown mechanisms, SCO1 deficiency leads to degradation of CTR1. Mice rendered conditionally null for SCO1 in the liver present with copper deficiency, which can be rescued by inhibition of the proteasome, demonstrating post-translational regulation of CTR1 levels by SCO1 [53].

Collectively these are important results that have received little attention. These findings illustrate a concept I elaborate in this dissertation of mechanisms controlling copper homeostasis encompassing organelles beyond the subcellular localization of a mutated protein.

1.2.3 Golgi: ATP7A & ATP7B

Delivery of copper from the plasma membrane to the Golgi complex is mediated by the metallochaperone Atox1 [23]. This cytosolic chaperone contains a single conserved MXCXXC metal-binding motif and functions by transferring copper from its own metal binding domains to the cytosolic N-terminal metal binding domains of ATP7A and ATP7B [54, 55]. While residues essential for transfer have been identified, the mechanism by which this transfer occurs has not been characterized [56]. Further, deletion of Atox1 in mammals results in intracellular copper retention, suggesting that the chaperone also plays a role in copper homeostasis [23].

The structure and function of ATP7A and ATP7B are similar; they are both P_{1B}-type ATPases and share ~60% identity. This family of proteins transports metals across membranes and its members share a conserved structure. ATP7A and ATP7B both contain eight transmembrane domains, six MXCXXC metal binding motifs at the N-terminus, and a transmembrane CPX metal binding motif. The remainder of their catalytic units can be characterized as N-, A-, or P- domains. The N-domain contains the ATP-binding site, the A-domain contains phosphatase activity, and the P-domain contains the phosphorylation domain with a conserved aspartic acid residue that is auto-phosphorylated during copper transport (Figure 4). (Structural features reviewed in [57]).

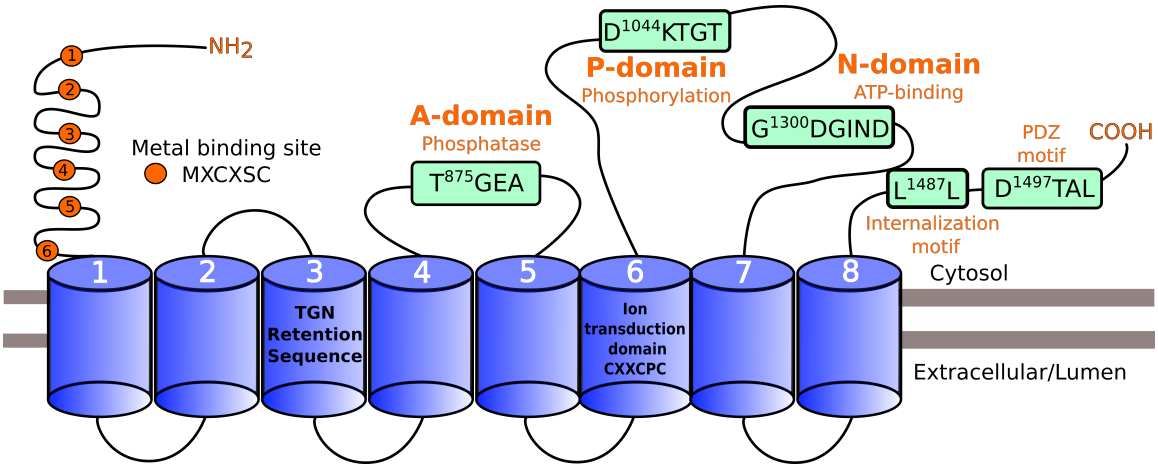


Figure 4. ATP7A Structure

ATP7A is an eight pass transmembrane protein that possesses the conserved architecture of a P-type ATPase. Near the N-terminus, the protein contains eight metal binding sites with the sequence MXCXSC. There are two transmembrane sequences important for function and localization: the third transmembrane domain contains a sequence required for retention at the TGN, while the sixth transmembrane domain contains the region through which copper is shuttled from the cytosol to the lumen of the Golgi. The cytosolic regions of the protein contain three domains required for ATPase activity, termed the A-, P-, and N-domain, required for phosphatase, phosphorylation and ATP-binding activity, respectively. At the C-terminus, a dileucine motif is required for endocytosis and a

PDZ domain, while not fully characterized, is required for appropriate plasma membrane localization in some polarized cell types. Figure modified from Telianidis et al. 2013.

Both transporters are required for supplying copper to ER-derived cuproenzymes and exporting copper at the plasma membrane, and the two differ primarily in their tissue specificity. ATP7A is expressed ubiquitously aside from the liver, and ATP7B is highly expressed in liver and at much lower levels in other tissue types. Under steady state conditions, both transporters localize to the TGN, but when intracellular copper levels are elevated, they will translocate to, or in close proximity to, the plasma membrane to export copper from the cell. (Reviewed in Lutsenko 2007 [58]).

1.2.4 Extracellular and cytosolic copper binding

While the general flow of copper upon reaching the cell membrane passes from CTR1 to one of the three copper metallochaperones described above and then on to the appropriate subcellular compartment, there are also general copper buffering mechanisms in the cytosol. Metallothioneins, small (~7kDa), metal-binding proteins not specific for copper, and the small molecule glutathione both appear to play an important role in maintaining copper homeostasis [20, 59, 60]. The particulars of metallothionein function are still unknown, but important pieces of evidence point to a role in copper binding [61, 62]. Synthesis of metallothioneins is at least in some part transcriptionally regulated; metallothioneins mRNA production is stimulated by the binding of metal responsive transcription factor MTF-1 to metal responsive elements in the promoter of metallothioneins, while MTF-1 itself is activated by

elevated metal content [61-63]. Much of the work studying metallothionein function has focused on zinc, but while the mechanism of copper response is not well understood, studies suggest that metallothioneins protein levels are responsive to copper content. For example, elevated metallothioneins levels are detected in the liver of patients with Wilson's disease, characterized by the accumulation of excess copper, suggesting a function in compensatory copper binding [64]. Conversely, a study performed in mice fed a copper-deficient diet provides evidence that metallothioneins bind copper under these conditions of chronic deficiency and may redistribute the metal based on cellular prioritization [65]. In addition to changes in metal content, MTF-1 activity is also upregulated under conditions of oxidative stress, leading to increased transcription of metallothioneins-1 [66]. There is also evidence in mammalian and *Drosophila melanogaster* systems that both glutathione (GSH) and glutaredoxin1 (GRX1) play a role in copper homeostasis, possibly by binding to and regulating the activity of ATP7A [67-69].

1.3 Copper sensing and response mechanisms

There are many open questions regarding how copper sensing occurs both on a cellular level and by individual copper-handling proteins; for example what mechanism triggers the mobilization of ATP7A/B to the surface when intracellular copper levels are elevated, and what degree of coordinated regulation exists between cell compartments to maintain ideal copper levels? In the following sections, I describe three putative regulatory mechanisms of copper responsive

protein expression including post-translational, transcriptional, and redox regulation (Figure 5).

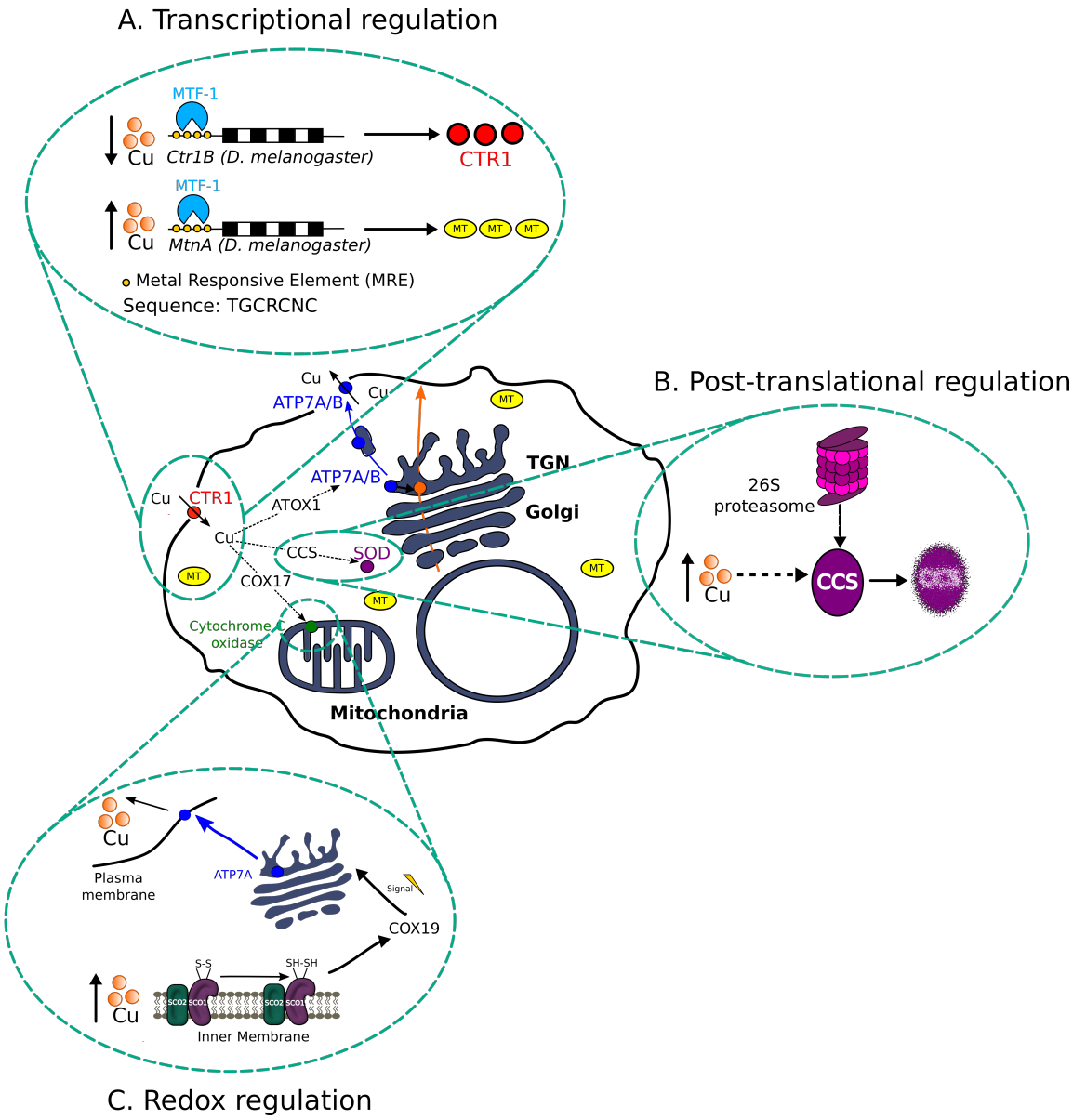


Figure 5. Cellular copper regulatory mechanisms

There exists a wide range of regulatory mechanisms to detect and respond to intracellular copper levels. (A) Work in *Drosophila* demonstrates that the activity of metal transcription factor 1 (MTF-1) varies depending on copper status. Under low copper conditions the transcription factor binds metal responsive elements, sequence TGCRCNC where R is adenine or guanine and N is any nucleotide, in

the promoter of the copper transporter Ctr1B. MTF-1 binding increases transcription and consequently protein levels of CTR1, allowing for an increase in copper import. Contrastingly, under elevated copper conditions, MTF-1 binds to metal responsive elements in the promoter of metallothioneins genes, increasing metallothioneins production to buffer excess copper. (B) Elevated copper levels lead to degradation of the copper transporter CCS by the 26S proteasome. (C) SCO1 redox state is copper sensitive. Elevated copper levels correspond with an increase in reduced cysteines, which in turn relays a signal to the Golgi complex. This ultimately leads to an increase in ATP7A at the plasma membrane and in copper export. COX19 is required for the relay of copper status signaling from the mitochondria to the Golgi complex. Figure 5C modified from Leary 2010.

1.3.1 Post-translational regulation

Regulation of copper transport to SOD1 by CCS appears to occur post-translationally. Support for this mechanism is drawn from the relationship observed between levels of CCS and intracellular copper, which are inversely related; CCS protein expression increases during copper deficit and decreases during copper excess while mRNA levels remain unchanged [70, 71]. The regulation of CCS protein levels occurs via modulation of the activity of the 26S proteasome and, while the precise mechanism is unclear, degradation may be triggered by a conformational change induced by copper binding to CCS, rendering the protein less stable [72].

1.3.2 Redox regulation

The activity and expression of at least a subset of copper homeostasis genes are dependent on redox state. Further, these redox-sensitive reactions allow for coordination of copper responses in multiple cellular compartments [52, 53]. One interesting example discussed briefly in Section 1.2.2 provides evidence of a link between the redox state of mitochondrially localized chaperones, SCO1, SCO2 and

COX 19, and the localization of ATP7A to either the Golgi complex or plasma membrane. The observation that mutations in either SCO1 or SCO2, both required for copper loading of cytochrome c oxidase in the mitochondria, led to not only dysfunction of COX but also reduced cellular copper content, suggested to researchers that the SCO proteins play a role in maintaining copper homeostasis [52]. This study revealed that the redox state of SCO1, in particular its CxxxC motif, plays a key role in maintaining appropriate cellular copper content; they found that reduced cysteines were overrepresented in copper-deficient cells, while oxidized cysteines were more common under normal copper conditions. Further, the copper deficiency observed in *SCO1* patient cells could be rescued by knocking down ATP7A, suggesting that ATP7A copper efflux is responsible for this copper deficiency. They also found that copper chaperone COX19 is required to relay the redox signal from the mitochondria to ATP7A [52]. The concept of multicompartment regulation of copper homeostasis has not been formulated as a general principle of copper homeostasis. Later in this dissertation, I will discuss further evidence for a connection between mitochondrial and Golgi copper homeostasis mechanisms.

1.3.3 Transcriptional regulation

Section 1.2.4 addressed the gene expression of metallothioneins in response to changes in metal content and oxidative stress, and other copper binding proteins undergo transcriptional regulation as well [61] [73]. Transcriptional regulation of copper homeostasis genes is a well-conserved mechanism that can be observed in

both prokaryotes and eukaryotes. In *Escherichia coli*, at least four transcription factors are responsive to copper levels and regulate the expression of a suite of copper regulatory genes [73]. In *Drosophila melanogaster*, the transcription factor MTF-1, a homolog of the mammalian transcription factor of the same name, is responsive to both copper deficiency and excess and is required to stimulate expression of the appropriate protein as copper status varies [74]. Genes for both metallothioneins and the copper importer Ctr1B, a larval isoform of the Ctr1 homolog Ctr1A, contain upstream metal responsive elements that bind MTF-1[73]. MTF-1 binds the metal responsive elements of either metallothioneins or Ctr1B under conditions of copper excess or deficiency, respectively [75]. In mammals, the copper chaperone to the Golgi complex, ATOX1, may act as a transcription factor for cyclin D1, *Ccnd1*. A 2008 study provided in silico evidence that ATOX1 may homodimerize in a copper-deficient fashion and bind the promoter of *Ccnd1*. This in turn would promote cellular proliferation in the presence of excess copper, a hypothesis that is bolstered by the observation that Atox1-/- mouse embryonic fibroblasts do not exhibit increased proliferation in the presence of copper observed in controls [76] [77].

1.4 Summary

The multitude of copper transporters and chaperones, many of which are highly conserved, underscores the vital role of this metal in cellular function and in turn the mechanisms that have evolved to mitigate its reactivity. Copper is carefully handled, passing from copper-binding proteins in the plasma, to surface

transporters, to copper chaperones, and ultimately incorporated into cuproenzymes. The cell has diverse mechanisms to sense and response to changing copper content and can regulate the levels of copper-binding proteins transcriptionally, post-translationally, and by modification of redox state. The handling of copper has historically been considered in a compartment specific fashion, with its regulation at the mitochondria, Golgi complex, cytoplasm, and plasma membrane considered as discrete pathways. As novel copper-binding proteins are encountered and existing proteins are further characterized, the complexity of copper homeostasis comes to light, and it becomes clear that maintaining this homeostasis occurs on a cellular level. In the following section I will highlight the evidence that imbalances in copper homeostasis contribute to the development of neurodegeneration.

Section 2: Copper Homeostasis and Neurodegenerative Disease

Mutations in the copper handling proteins described in the previous section perturb copper balance, both at a cellular and systemic level. Many of the resulting phenotypes correlate with loss of the corresponding cuproenzyme function, but interestingly, many of these mutations are also highly predictive of neurodegeneration, the genesis of which cannot yet be explained. There is also increasing evidence that copper homeostasis is altered in canonical neurodegenerative diseases, including Alzheimer's and Parkinson's disease. Below I will discuss our current understanding of the neuronal phenotypes downstream of copper imbalance.

2.1 Copper in the nervous system

It is well established that copper, among other metals, plays an important role in the brain. In non-pathological states, copper transporters and copper itself are concentrated both in the brain as a whole, accounting for 7-10% of total copper, and in particular brain regions, including the *substantia nigra* and cerebellum [78, 79]. The blood brain barrier is thought to be the main site of copper entry to the brain, and copper import and export via the blood brain barrier is regulated by CTR1, ATP7A and, to a lesser extent, ATP7B [79-84]. While many of the cuproproteins discussed in Section 1 such as cytochrome c oxidase and SOD1 are ubiquitously expressed, there are cuproenzymes that have particular importance in the nervous system [85]. Most notably, these include peptidylglycine α-amidating monooxygenase (PAM) and dopamine-β-hydroxylase (DBH), both of which are dependent on ATP7A for provision of copper and are required for the production of a subset of neuropeptides and neurotransmitters [86, 87].

2.2 Genetic and environmental factors, such as metals, influence the development and progression of neuropathologies

Both genetic and environmental factors influence the development of neuropathologies. For example, single gene mutations are responsible for only ~10% of Parkinson's cases, while the remaining 90% are influenced by both a susceptible genetic background and exposure to environmental risk factors [88]. The known breadth of environmental risk factors is evolving quickly and, in the case of Parkinson's disease for example, ranges from accidental ingestion of drug

synthesis byproducts, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to consumption of β-N-methylamino-L-alanine (BMAA), a natural product of cyanobacteria that can concentrate in seafood [89, 90]. Epidemiological studies have demonstrated that prolonged exposure, often occupational, to pesticides and metals including manganese and copper influences the development of Parkinson's disease [91-94]. The contribution of transition metal homeostasis to neurodegenerative disease has been long studied but remains poorly understood. As early as 1958, Hallgren and Sourander observed elevated iron levels in the *substantia nigra* of Parkinson's patients [95]. This study laid the groundwork for future investigations into the role of metals in human health, and particularly in the brain. However, over the past sixty years a detailed mechanistic understanding of the relationship between metal homeostasis and neuropathologies has not yet emerged. Mutations in metal binding proteins, such as chaperones and transporters, are often associated with neuropathologies, a representative sample of which will be described in following sections, along with irregular levels of intracellular and/or extracellular metal content. In turn, the observation that metal content is often perturbed in patients with neurodegenerative diseases has led some to posit that metal homeostasis may play a causative role in the development of diseases such as Parkinson's and Alzheimer's. Below I describe observations concerning one such metal, copper, and the current evidence for its role in neuropathologies.

2.3 Genetic defects in copper binding proteins provide direct evidence of metal homeostasis mechanisms in neurodegenerative diseases

Mutations in copper binding proteins have long been associated with a loss of downstream cuproenzyme function. As discussed in Section 1, copper homeostasis genes are localized to particular cellular compartments, and thus the exploration of phenotypes and corresponding disease mechanisms has often been limited to the cell compartment with the highest expression of the relevant copper homeostasis genes. However, recent work, along with the work presented in this dissertation, increasingly provides evidence for global copper homeostasis mechanisms. Given this emerging understanding of global copper homeostasis, the etiology of copper homeostasis gene related disease and copper-related neurodegeneration is likely more complex than loss of cuproenzyme function. Strikingly, mutations in copper binding proteins are often accompanied by neuropathologies, underscoring the possibility that copper may play an active role in the development of neurodegeneration[96]. Mutations leading to loss of function of the copper transporters ATP7A and ATP7B provide models for pathological copper deficiency and excess, respectively, and both are associated with early onset neurodegeneration.

2.3.1 ATP7A: Menkes disease

Section three will discuss Menkes disease in great detail but, briefly, this childhood neurodegenerative disease results from mutations in the copper transporter ATP7A that lead to complete loss or greatly reduced levels of ATP7A protein expression. Allelic variants that produce partial loss of function cause milder disorders including occipital horn syndrome and a variant of spinal muscular atrophy

(SMAX3) [97, 98]. In this dissertation, I will concentrate on the molecular function of ATP7A and the phenotypes associated with its deficiency.

2.3.2 ATP7B: Wilson's disease

Deficiency of the ATP7B transporter results in Wilson's disease, which is a manifestation of excess copper; the disease has a later onset than Menkes and becomes symptomatic in adolescence or adulthood (OMIM 606882) [99-101]. ATP7B is highly expressed in the liver and, similar to the copper-responsive trafficking of ATP7A, translocates to vesicles adjacent to the plasma membrane to excrete copper when intracellular levels become elevated [102]. When ATP7B is absent or does not localize properly, hepatocytes can no longer remove excess copper into bile for excretion [103]. This copper then enters circulation and is deposited in multiple organs, but it is in the brain and liver where the metal exerts particularly detrimental effects in the form of oxidative damage [104-106]. The neurodegeneration and resulting seizures experienced by Wilson's patients are thought to be due to oxidative stress in the brain [104]. These patients also commonly express psychiatric symptoms whose origin is unknown. In fact, the most common early sign of Wilson's disease is psychosis. These behavioral symptoms are not thought to be due solely to neurodegeneration as they can be alleviated by therapeutic copper chelation [85].

2.3.3 SOD1: Amyotrophic lateral sclerosis

Of the 10% of amyotrophic lateral sclerosis (ALS) cases that are inherited, 20% are a result of mutations in the gene encoding superoxide dismutase 1 (SOD1), a

cytosolic cuproenzyme important for the removal of superoxide species (OMIM 147450) [107]. Over 100 ALS causative SOD1 mutations have been identified, a large proportion of which are dominantly inherited missense mutations [108]. The role of copper in the pathogenesis of ALS has been contentious at times. In 2000, one group found that deletion of the copper chaperone upstream of SOD1 (CCS), which they hypothesized should exacerbate phenotypes associated with loss of SOD1, did not in fact affect the onset and progression of ALS symptoms in an SOD1 mouse model [109]. This led to the suggestion that mutations in SOD1 act independent of its copper-bound status. However, recent work affirms a role for copper in the pathogenesis of ALS caused by mutant SOD1 [110, 111]. Delivery of copper using a vehicle permeable to the CNS in an SOD1 mutant mouse model of ALS led to significant increases in both the amount of copper-loaded SOD1, as well as in the lifespan of the mice [112]. Similarly, overexpression of the copper regulatory protein metallothionein-I in an SOD1 mouse model led to a significant extension of lifespan and reduction in phenotypes such as muscle atrophy and motor neuron death [110]. These findings suggest that more than one copper-dependent mechanism is engaged in maintaining the levels of copper loaded SOD1.

2.3.4 ATOX1

Many of the phenotypes observed in ATOX1 null mice mirror those of Menkes disease: failure to thrive, lax skin, hypopigmentation and seizures, reflecting the requirement for ATOX1 to provide copper to the secretory pathway by way of ATP7A/B (OMIM 602270) [23, 54, 113]. Embryonic fibroblasts from ATOX1$^{-/-}$ mice

also exhibit an increased propensity to accumulate copper, due to decreased efflux as opposed to increased influx, indicating a role for ATOX1 in maintaining copper homeostasis [23]

2.3.5 SCO1

As described in Section 1.2.2, SCO1 and SCO2 are required for the assembly of cytochrome c oxidase (COX) and may also play a distinct role in copper homeostasis. These copper chaperones are just two of at least thirty proteins required for the assembly and function of COX, mutations in at least four of which are associated with a spectrum of respiratory chain deficiencies [41, 114]. While mutations in SCO2, which shares 40% homology with SCO1, manifest in patients as phenotypes related to muscle and cardiac function, patients harboring SCO2 mutations are unique in presenting with severe neurological symptoms (OMIM 603644)[43].

2.4 Indirect role of metal homeostasis mechanisms in neurodegenerative diseases

Loss of function mutations in copper homeostasis genes often lead to neurodegeneration, so it follows that neurodegenerative disease may be accompanied by disruptions in copper homeostasis. Indeed, a 2011 study employed inductively coupled plasma mass spectrometry (ICP-MS) to measure biological metal content in the cerebrospinal fluid (CSF) of patients with amyotrophic lateral sclerosis (ALS), Parkinson's disease, and Alzheimer's disease. In all three patient cohorts, the researchers detected significantly elevated copper content as compared

to controls [115]. Even earlier, a 1987 study found that copper concentration in the CSF of Parkinson's patients correlated with both disease severity and progression of the disease ([116]. While the contribution of heavy metals, as well as transition metals such as copper, to the pathogenesis of neurodegenerative disease is rather well accepted, the mechanisms that underlie this connection are ripe for investigation.

2.4.1 Alzheimer's disease

The accumulation of amyloid-β (Aβ) plaques in the neocortex is a hallmark of Alzheimer's disease (AD), and much work has been conducted investigating the contribution of the plaques to neuropathogenesis [117, 118]. There is a relationship between the deposition of Aβ and the presence of oxidative damage in the cell and tissue, but the causal relationship between the two is controversial. Both copper and iron are enriched in Aβ plaques, and it is thought that these metals may contribute to the generation of ROS, particularly via the production of H_2O_2 as a precursor to hydroxyl radical formation. Both metals appear to preferentially bind to the longer, self-aggregating form of Aβ peptide associated with greater pathogenicity [119-124]. In vitro assays have demonstrated that, not only does the addition of copper in the presence of pathogenic Aβ peptides lead to the generation of H_2O_2 species, but also the addition of a copper-chelating agent abrogates this reaction [124]. The addition of copper chelators in vitro also facilitates the dissolution of Aβ plaques, lending credence to the role of copper in plaque formation and maintenance [125]. In animal models, trace amounts of copper, 10% of EPA approved levels, in the

water of rabbits provided with food containing excess cholesterol was sufficient to induce the formation of Aβ plaques. These rabbits also exhibited deficits in memory and learning tasks as compared to controls [126]. One interesting possibility is that the propensity of Aβ peptide monomers to bind copper with high affinity is a protective mechanism to prevent oxidative damage of cellular components, and it is only when the concentration of copper ions relative to Aβ is too high that Aβ aggregates [121, 127-129].

Amyloid precursor protein (APP), which is proteolytically cleaved to produce Aβ peptide, contains a putative copper-binding site at its N-terminus, and APP knockout mice display elevated copper levels, specifically in the cerebral cortex and liver [130]. The authors of the above study propose that APP may be a modulator of copper homeostasis, and thus Alzheimer's disease may indeed be a consequence of altered copper homeostasis. Reciprocally, altering copper levels can regulate both the localization and gene expression of APP. The addition of excess, 25-150 μM, copper to neuroblastoma cells induces the exocytosis and reduces the endocytosis of APP; hence copper binding to APP stimulates its trafficking and may in turn regulate its function [131]. Another study investigated the effect of copper depletion on APP expression using fibroblasts overexpressing ATP7A, which leads to net efflux of copper. APP expression was significantly decreased in copper depleted fibroblasts, and a copper regulatory region was identified in the promoter of APP [132]. The documented association between copper and two of the proteins most

strongly correlated with the development of Alzheimer's disease is suggestive of a role for copper homeostasis in the pathogenesis of AD.

2.4.2 Parkinson's disease

Analogous to the development of Aβ plaques in Alzheimer's disease, the aggregation of α-synuclein (AS) into insoluble Lewy bodies appears to be important in the generation of Parkinson's disease. Structural studies demonstrate that α-synuclein contains two copper binding domains, and that copper may enhance aggregation [133].

Deletion mutations in the ubiquitin protein ligase PARK2 (Parkin) gene are associated with a recessive form of early onset Parkinson's disease but do not exhibit complete penetrance [134-136]. To test the contribution of environmental factors in a genetically susceptible background, one study generated neuroprogenitor cells from fibroblast-derived iPSCs obtained from two male siblings with PARK2 loss-of-function mutations alongside control cells. The neuroprogenitor cells were exposed to elevated copper, cadmium, manganese, and mercury, and the cell lines with PARK2 mutations exhibited increased cytoxicity, mitochondrial fragmentation and ROS generation as compared to control cells in the presence of copper and cadmium, but not manganese or mercury [137]. These results provide a compelling example of the interplay between environmental exposure and genetic background.

2.4.3 Prion diseases

Like Alzheimer's and Parkinson's disease, prion diseases are characterized by increased oxidative stress, aggregation of protein products, and neuronal loss. In this case the generation and accumulation of a misfolded variant, PRP^{Sc} (scrapie isoform) of normal prion protein, PRP^{C} (cellular isoform) is thought to be responsible for the development of prion diseases [138]. Like APP, PRP^{C} contains copper binding domains and may play a role in copper homeostasis [139-141]. Relevant to the development of prion disease, binding of copper to PRP^{C} destabilizes the non-pathogenic conformation and may contribute to misfolding and formation of PRP^{Sc} [142].

2.5 Summary of current models for the role of copper in the development of neuropathologies

The prevailing model to account for the contribution of copper to neurodegenerative disease is one of metal-induced oxidative stress. The same biochemical properties of transition and heavy metals that allow them to participate in redox reactions also give rise to the generation of reactive oxygen species (ROS). Increased metal content is thus thought to induce a state of oxidative stress and that in turn leads to neurodegeneration [93]. If one accepts the assertion that unregulated metal content contributes to this oxidative damage, the question then becomes why metals become dysregulated and whether the mechanisms upstream are unique to each manifestation of neurodegenerative disease or shared among

them. Fundamentally, we are compelled to explore genes that control copper homeostasis and how they are regulated.

Section 3: Utilizing ATP7A as an Entry Point to Study Copper-Related Neurodegeneration

Thus far I have described the existing evidence for a connection between copper homeostasis and neurodegeneration. The observations that support this relationship are enticing, but there are many unanswered questions to be addressed. For example, there is little to no mechanistic understanding regarding the role of copper in the development of neuropathologies, even for those diseases where the genetic defect affects a copper-binding molecule. It is also unknown to what degree the genesis of copper-associated neurodegeneration in various disorders is related. To explore possible connections between copper and neurodegeneration, we view ATP7A as an entry point to expand our understanding of copper regulatory mechanisms. We begin by looking for novel interaction partners, focusing on genes encoding proteins associated with neurodevelopmental and neurodegenerative disorders. In the following sections, I will illustrate why we view ATP7A as an excellent candidate to further explore the link between copper homeostasis and neuropathologies.

3.1 ATP7A expression and localization

3.1.1 CNS

ATP7A expression varies by development stage and by tissue type (Figure 6). Studies in mice have shown that ATP7A protein expression is highest in the weeks before and after birth and then declines [143]. ATP7A is ubiquitously expressed, albeit at very low concentrations in the liver where ATP7B is predominant, but its abundance varies by cell type, particularly in the central nervous system. Neurons in the cortex, hippocampus, olfactory bulb, cerebellum and hypothalamus all express ATP7A, along with non-neuronal cells including astrocytes, microglia and oligodendrocytes [85, 143-145]. ATP7A is a primary mechanism by which copper passes the blood brain barrier, as evidenced by copper deficiency in the brain in Menkes patients and model systems (Figure 7). Passage of copper from astrocytes to neurons via ATP7A also appears to be an important delivery system, and a Menkes mouse model displays elevated copper in astrocytes (Figure 7) [146].

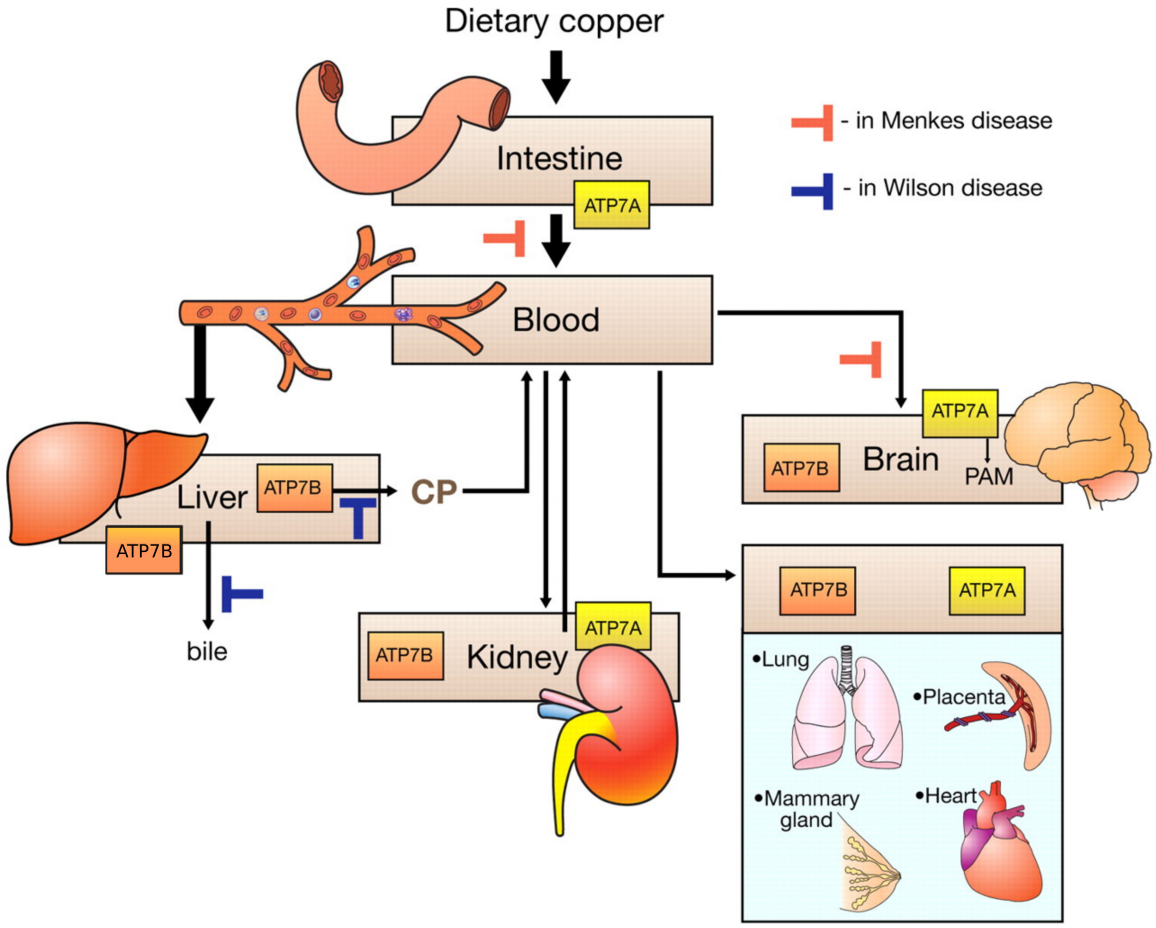


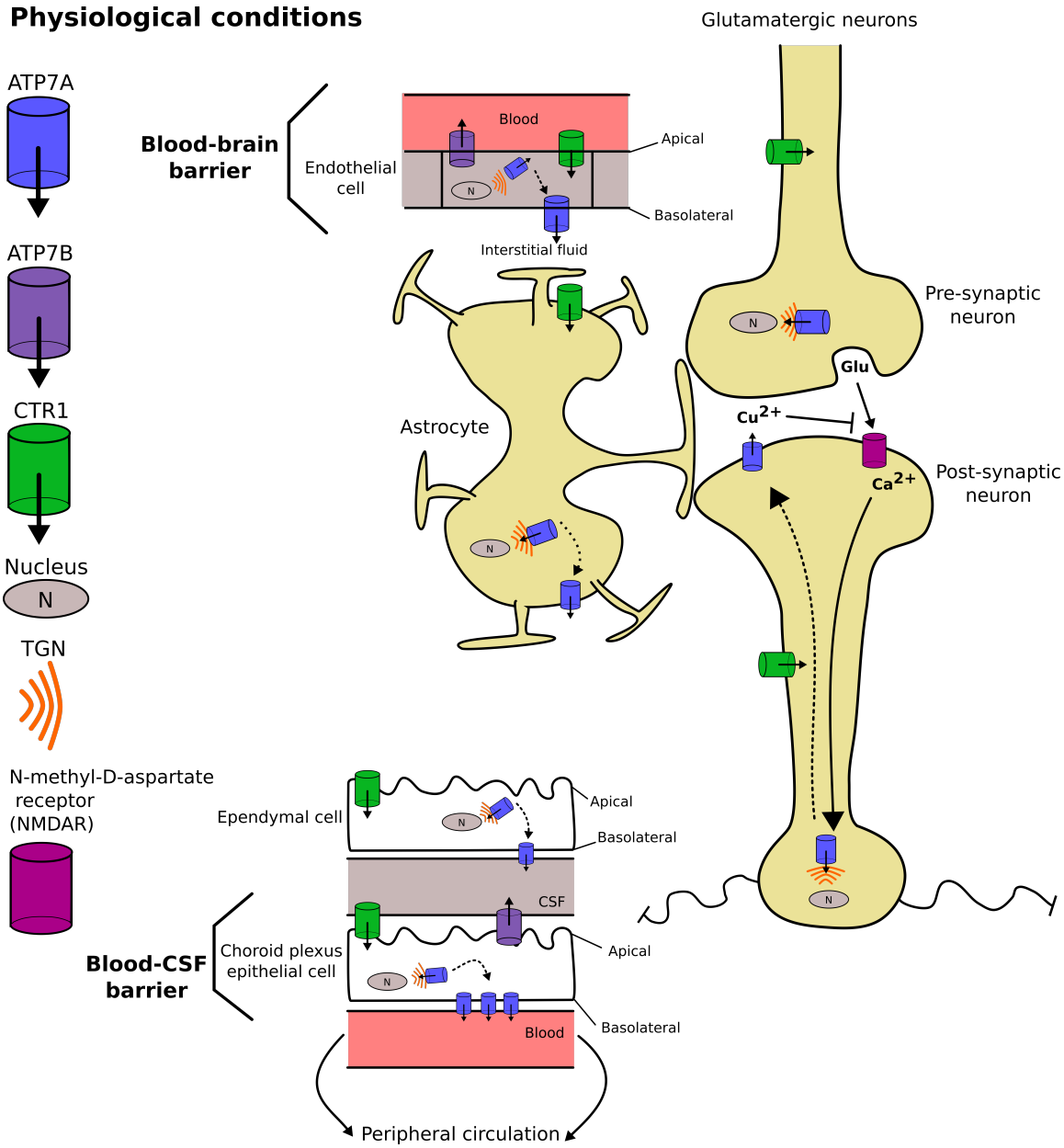
Figure 6. Copper distribution and transport by tissue type

Expression of ATP7A and ATP7B varies by tissue type. While both transporters are present in most cell types including the brain, kidney and heart, some tissue types rely exclusively or predominantly on one transporter than the other. For example while both ATP7A and ATP7B are expressed in the intestine, ATP7A primarily regulates the transport of copper from enterocytes of the intestine to circulation and cannot be compensated for by ATP7B. In the liver ATP7B is expressed at significantly higher levels than ATP7A and is required for exporting copper into ceruloplasmin for circulation and into the bile for excretion. While both transporters are expressed in the brain, ATP7A appears to be the primary mechanism by which copper is transported across the blood brain barrier. Orange and blue T-bars indicate the pathways that are blocked in Menkes and Wilson's disease, respectively and

are predictive of where copper accumulates or is deficient in specific tissue types under disease conditions. Figure from Lutsenko et al. 2007.

Section 3.2 presents the argument that the neuronal phenotypes observed in Menkes patients cannot be accounted for solely by loss of cuproenzyme function. While long accepted, this view is being challenged by recent results, including a 2015 study that shed light on the mechanism underlying ATP7A-related neuropathologies [147]. In this report, researchers specifically eliminated ATP7A in neural and glial progenitor cells, generating a mouse model referred to as the *ATP7A*^{Nes} mouse, and compared the resulting defects to those observed in ATP7A deficient mice, also known as mottled-brindle or *mo-br* mice. *ATP7A*^{Nes} mice retain ATP7A expression in the choroid plexus and are thus capable of transporting copper across the blood brain barrier. Intriguingly, they found that the *ATP7A*^{Nes} mice exhibited significantly milder neuronal phenotypes than the *mo-br* mice [147]. Like the *mo-br* mice, *ATP7A*^{Nes} mice have significantly reduced ATP7A expression in the brain and decreased function of ATP7A-depedent cuproenzymes as measured by DBH function, hence they modeled neuronal and glial ATP7A deficiency. The principal difference between these mouse models is the copper content in neuronal and glial cells, which is reduced in the *mo-br* mice due to the lack of ATP7A in the choroid plexus, while it is slightly elevated above WT in the *ATP7A*^{Nes} mice. These observations suggest that it is copper deficiency, rather than ATP7A deficiency per se, that primarily contributes to the neuropathologies of Menkes patients.

The same research group generated a mouse model to investigate the cellular mechanisms underlying a disorder caused by missense mutations in ATP7A, X-linked spinal muscular atrophy type 3 (SMAX3)[148]. Patients with these mutations do not exhibit complete loss of ATP7A function and systemic copper deficiency as observed in Menkes patients, rather ATP7A dysfunction is confined to the motor neurons. By specifically deleting ATP7A in the motor neurons, researchers were able to effectively model SMAX3 as these mice displayed the clinical features associated with the disease, including muscle atrophy and loss of motor neuron cell bodies. They found that the characteristic loss of motor neuron cell bodies is downstream of denervation of the neuromuscular junction and accumulation of copper in the motor neurons, along with a corresponding copper deficit in the spine [97]. While the ATP7A missense mutations causative of SMAX3 result in a global reduction of ATP7A function on the order of 20-40%, the clinical effects are confined to the motor neuron [148]. One possibility to account for this cell-type specificity is that ATP7A regulatory mechanisms and interaction partners vary by cell type.


Physiological conditions
Glutamatergic neurons
ATP7A
ATP7B
CTR1
Nucleus
N
TGN
N-methyl-D-aspartate receptor (NMDAR)
Blood-brain barrier
Endothelial cell
Blood
Apical
Basolateral
Interstitial fluid
Astrocyte
Pre-synaptic neuron
Glu
Cu2+
Ca2+
Post-synaptic neuron
Ependymal cell
CSF
Blood-CSF barrier
Choroid plexus epithelial cell
Peripheral circulation

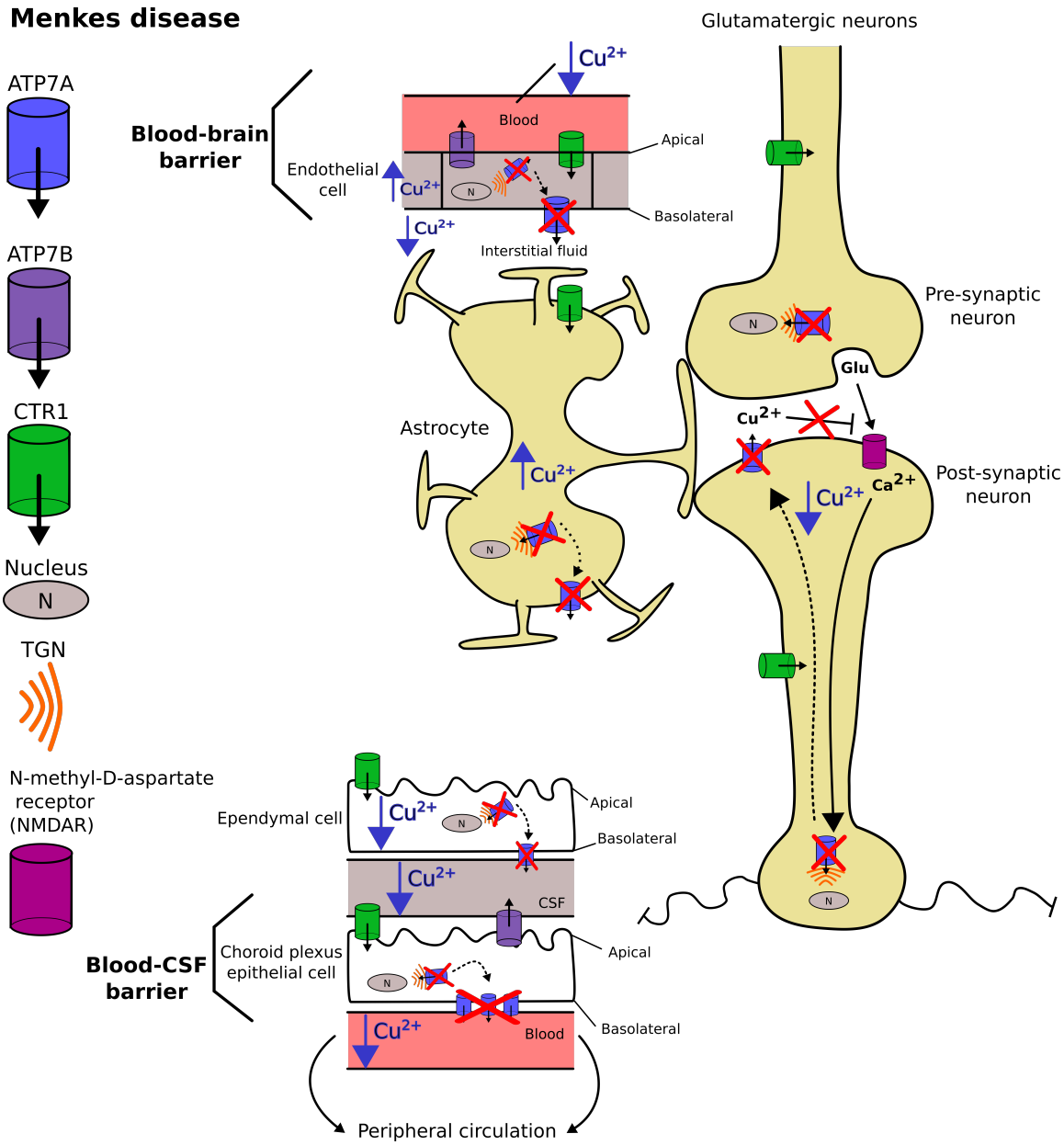


Figure 7. Copper transport into and within the brain

The copper transporters ATP7A, ATP7B and CTR1 regulate copper content and transport in the brain. (Physiological conditions) Under normal conditions, CTR1 is responsible for copper import into cells as in other cell types. At the blood brain barrier, ATP7A is expressed in endothelial cells and is required to transport copper across the barrier. At the blood-CSF barrier, ATP7A is highly expressed in the choroid plexus where it can remove excess copper from the brain into the blood. In both cell types, ATP7A is localized to the basolateral membrane, while ATP7B is localized to the apical membrane. Across the blood brain barrier, astrocytes take up copper via CTR1 and facilitate its transport into neurons. Here the role of ATP7A is depicted in a glutamatergic neuron. Activation of

NMDAR stimulates the trafficking of ATP7A to the cell surface where it exports copper from the cell. This released copper is thought to act in an inhibitory fashion on NMDAR, terminating the calcium influx and prevent excitotoxicity. (Menkes disease) In the absence of ATP7A, there is reduced transport of copper across both the blood brain barrier and the blood-CSF barrier. Copper accumulates in the endothelial cells of the blood brain barrier and exhibits reduced transport across the membrane. Any copper taken up by astrocytes accumulates in these cells, and transport into neurons is deficient. Figure modified from Telianidis et al. 2013.

3.2 Molecular Basis of Neurodegeneration and Neurodevelopmental Defects in Menkes Disease

The following section comprises a review titled "Molecular Basis of Neurodegeneration and Neurodevelopmental Defects in Menkes Disease", published in September 2015 in *Neurobiology of Disease* [96]. It provides a framework of our reasoning for pursuing ATP7A as an entry point to investigate copper-associated neurodegeneration. The work examines the current understanding of pathogenesis mechanisms that result from a range of mutations in ATP7A and underscores the deficiencies of the "oligoenzymatic hypothesis" to account for neurodegeneration in Menkes patients.

3.2.1 Introduction

Genetic defects in the trans-Golgi copper-transporter P-ATPase, ATP7A, cause three distinct X-linked recessive disorders: occipital horn syndrome (OMIM 304150), spinal muscular atrophy, distal, X-linked 3 (SMAX3, OMIM 300489), and Menkes disease (OMIM 309400)[149]. More than 350 different mutations affecting the ATP7A gene have been described [150, 151]. These disease-associated mutations

are quite heterogeneous in their genomic location and the type of DNA defect and, unlike other genetic disorders, there are not recurrent genetic defects that account for a significant number of cases [151]. Milder mutations in ATP7A result in occipital horn syndrome in which connective tissue and bone abnormalities predominate and patients lack the severe neurological phenotypes of Menkes disease [98, 152]. Yet another ATP7A-related disease is SMAX3, in which missense mutations not severe enough to perturbate systemic copper status cause a non-demyelinating spinomuscular atrophy [148, 153]. At the far end of the spectrum is Menkes disease in which the most severe loss-of-function mutations result in a multisystemic metabolic disorder of copper deficiency. Here we focus in Menkes disease, first described in 1962 in a single family that in two generations accumulated five male infants affected by intellectual disability, failure to thrive, prominent neurological manifestations, neurodegeneration, epilepsy, and 'peculiar white hair' [154]. Menkes disease is a rare affliction with an incidence of 1/140,000 to 1/300,000 [155, 156]. Although this disease has been studied for more than 50 years and its metabolic foundations are known [149, 157], we contend that the pathogenic mechanisms underlying neurodegeneration and neurodevelopmental defects remain poorly understood. In this review, we explore neuropathogenic hypotheses and argue that some of the classic ideas invoked to explain Menkes disease phenotypes, although logical, remain speculative and inadequate. We propose an updated modified hypothesis in light of newer findings to account for the neurological manifestations of ATP7A loss-of-function mutations.

Our interest in Menkes disease pathogenesis extends beyond this genetic disorder. Because the neurological symptoms associated with Menkes disease are common to other neuropsychiatric disorders of childhood and adulthood [149, 157], it is increasingly recognized that Menkes disease studies may shed light into the mechanisms of other prevalent disorders. Menkes pathogenesis mechanisms can thus be a tool to understand: a) neuronal mechanisms where copper participates either as a micronutrient or a toxicant; b) pathways of neuronal cell death triggered by altered metabolic homeostasis; c) mechanisms that cells use to respond to neurotoxic anticancer agents such as platinum compounds, which bind to ATP7A [158-161]; d) regulatory mechanisms of key receptors and channels involved in neurotransmission and neurodegeneration. These include N-methyl-D-aspartate (NMDA) receptors, voltage-gated calcium channels, APP, and the prion protein to mention few [85, 149, 162, 163]; and e) mechanisms of development that could account for defective cell positioning observed in Menkes gray matter [164].

3.2.2 Clinical and Pathological Characteristics of Menkes Disease

Menkes disease manifests itself between two to twelve months after birth with hypotonia, failure to thrive, focal and generalized seizures, impaired cognitive development, and brain atrophy at the expense of the gray and white matter. Hypotonia at birth evolves into spastic paresis. Systemic features associated with the disease include the characteristic hypopigmented “kinky hair”, which at the microscopic level reveal pili torti (twisted hairs), monilethrix (beaded hairs) and thickening or weak nodes that cause hair fragility (trichorrhexis nodosa). In

addition, affected Menkes infants exhibit sagging facial appearance, micrognathia and arched palate, laxity of the skin (cutis laxa) and joints, reduced bone density, bladder diverticula, aneurysms, vascular tortuousity, and bluish irises. This constellation of clinical features permits a high confidence of Menkes diagnosis when associated with serum copper deficiency and X-linked recessive transmission [149, 154, 156, 157, 165-168].

3.2.3 Menkes Disease Neuropathology

Menkes is characterized by widespread atrophy of the gray and white matter. At the light microscopic level there is focal degeneration that extends to all layers of the cerebral cortex. Neuronal cell loss is most pronounced in the cerebral cortex but affects hippocampus, striatum, hypothalamus and thalamus to a variable degree. In the cerebral cortex neuronal cell loss is commonly associated with astrocytosis [154, 169-172].

The cerebellum also shows astrocytosis, although this is more variable compared to the cerebrum. The cerebellum also exhibits marked atrophy in Menkes patients, a feature that is also observed in copper deficient animals giving rise to enzootic ataxia [173]. The Menkes cerebellum also shows scattered loss of Purkinje cells and pronounced reductions in neuronal numbers in the molecular and granular layers. The most prominent Purkinje cell phenotypes are defective cell positioning or heterotopia and abnormal cell architecture (Figure 8) [154, 169-171]. The

heterotopia is characterized by irregular alignment of Purkinje cells and displacement within the molecular and granular layers of the cerebellar cortex [154, 169-172, 174]. In addition, Purkinje cell dendrites are markedly swollen with an aberrant pattern of dendritic arborization (Figure 8) [170, 174, 175]. Sprouts directly extending from the Purkinje cell body, some of which resemble spines, appear less frequently with the age of the individual [170]. In addition to architectural phenotypes, the cell bodies of Purkinje cells contain clusters of mitochondria, which possess dense granules in their matrix and altered cristae [176-179]. This mitochondrial ultrastructure is important as it provides a morphological correlate to mitochondrial enzymatic defects, which has been postulated to underlie the neurodegeneration. However, despite their prominent mitochondrial ultrastructural pathology, the overall neurodegeneration of Purkinje cells is mild [179, 180]. This argues to factors in addition to mitochondria in the pathogenesis of Menkes disease neurodegeneration.

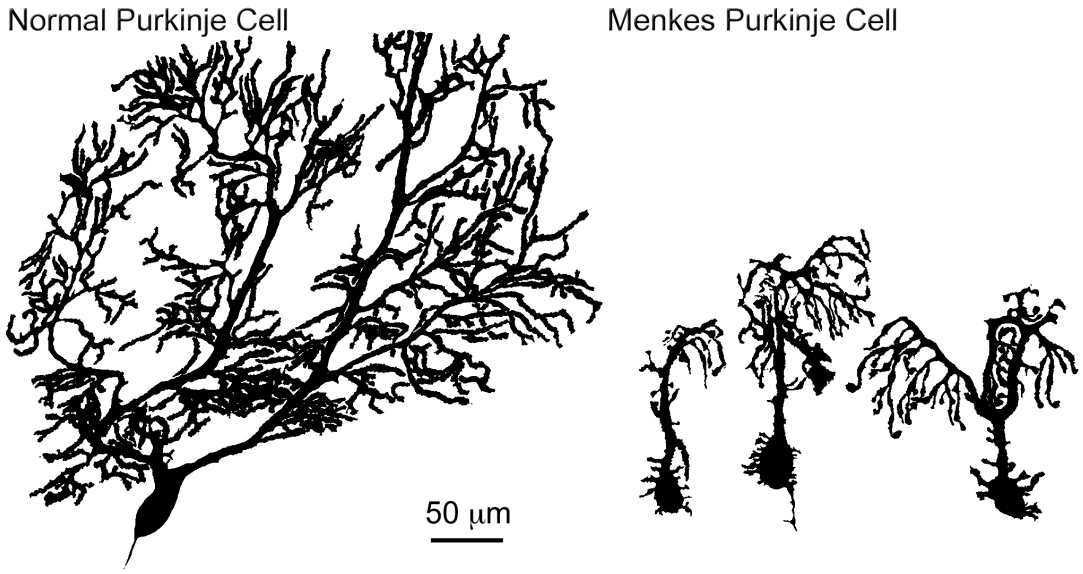


Figure 8. Branching abnormalities of cerebellar cortex Purkinje cells. Human tissue was processed with Golgi stain. Panels depict camera lucida drawings of Purkinje cells. Note the dendritic systems of Purkinje cells in Menkes' disease are atrophic and lack organized tertiary branches. Modified from Purpura et al. 1976 [174].

The emphasis on the neuropathology of Menkes disease has focused on the neurodegeneration associated with this disorder. However, it is important to emphasize that Menkes brains in humans or mice possess Purkinje cell heterotopy, abnormal neuroblast migration, and altered neuronal arborization [143, 170, 174, 181] (Figure 8). These defects precede neuronal cell death [143, 166, 181]. The cellular basis of these structural abnormalities observed in Purkinje cells point to defective developmental mechanisms such as cell migration, polarity, and lamination of cortical layers in the cerebellum and possibly in the cortex [164].

3.2.4 Cell Biology of Menkes Disease

Menkes disease is the product of either absent or impaired ATP7A copper pump activity and/or improper subcellular localization [149, 182-184]. The consequence of such defects at the cellular level is an impaired intraluminal Golgi or cytoplasmic copper homeostasis. At low extracellular copper concentrations, wild type ATP7A resides in the trans-Golgi network (TGN) where it pumps copper into the lumen of the trans-Golgi network as a cofactor for copper-dependent apoenzymes [185]. The most studied of these copper-dependent enzymes are tyrosinase, peptidylglycine α-amidating monooxygenase (PAM), dopamine beta hydroxylase (DBH), and lysyl oxidase (LOX), all of which are synthesized within the secretory pathway and loaded

with catalytic copper by ATP7A at the trans Golgi network [149, 186]. Under increased extracellular copper concentration, the resulting increase in cytosolic copper induces ATP7A translocation from the trans-Golgi network to post-Golgi vesicles and the plasma membrane [185-187]. ATP7A facilitates copper extrusion from cells upon fusion of copper-laden vesicles with the plasma membrane and/or by direct pumping of this metal across the plasma membrane. Finally, restoration of the extracellular copper levels to low micromolar levels induces the retrieval of ATP7A from the plasma membrane back to the Golgi apparatus via endosomes [185-189]. This requirement for ATP7A in copper extrusion plays a critical role in supplying the body with copper via transport across the intestinal enterocytes, thus accounting for the severe systemic copper deficiency in Menkes patients. In the CNS, this copper deficiency is exacerbated due to the requirement for ATP7A in copper transport across the choroid plexus and/or blood brain barrier. Thus, the dual functions of ATP7A in copper transport into the secretory pathway and copper efflux provide a reasonable foundation to think that defects in these and other copper-dependent enzymes account, in part, for diverse aspects of the Menkes phenotype [185-189].

The machinery required for anterograde trafficking of ATP7A from the Golgi is not fully understood. However, the retrieval of ATP7A via endosomes requires cytosolic trafficking complexes which include; clathrin, rab22, the clathrin adaptor complex AP-1, retromer, the WASH complex and possibly the BLOC-1 complex [190-193]. In particular, the WASH complex is part of a mechanism that also requires a complex of

proteins that include COMMD1, CCDC22, CCDC93, and C16orf62 (the CCC complex) for endosome to Golgi retrieval of ATP7A [194]. Among these CCC complex components, evidence supports a role for COMMD1 controlling ATP7A levels in cultured cells and COMMD1 mutations cause autosomal recessive copper toxicosis in Bedlington terrier dogs [195-197]. Mutations in AP-1 σ1A subunit cause the MEDNIK syndrome in humans [198-200], a distinctive syndrome with abnormalities in copper homeostasis that can be attributed, in part, to aberrations in AP-1-dependent steps in ATP7A trafficking. In contrast, copper content is normal in the brain of BLOC-1 deficient mice but at the expense of diverse molecular adaptations in copper homeostatic pathways (unpublished). Although the mechanisms by which these trafficking protein complexes are coordinated for the retrieval of ATP7A to the Golgi has not been elucidated, their connection with ATP7A trafficking offers new ways to understand mechanisms of Menkes neurodegeneration and neurodevelopmental abnormalities. For example, mutations that affect the retromer complex have been shown to cause certain cases of Parkinson's disease, a neurodegenerative disorder [191, 201, 202]. Moreover, mutations in AP-1 and the BLOC-1 complex are thought to contribute to neurodevelopmental disorders, such intellectual disability and schizophrenia [199, 203, 204]. Thus, because the proteins and mechanisms that control ATP7A trafficking appear to overlap with those of Parkinson's and other neurodevelopmental disorders, by extension these disorders could be caused in part by abnormal copper homeostasis.

3.2.5 The Oligoenzymatic Pathogenic Hypothesis of Menkes Disease

Is the Menkes neuropathology due to nutritional copper depletion or an intrinsic lack of ATP7A in neurons? Menkes disease neuropathology is recapitulated by conditional deletion of ATP7A in the gut [205]. This powerful evidence argues that copper depletion in the brain leads to Menkes neuropathology. Menkes neurological manifestations have been ascribed to five enzymes expressed in brain that require copper for their function. Presently, these enzymes include mitochondrial cytochrome oxidase C, and four enzymes that acquire copper in the Golgi apparatus: PAM, DBH, LOX, and tyrosinase [149, 165, 167]. We refer to this hypothesis as the 'oligoenzymatic hypothesis'.

The oligoenzymatic hypothesis was born in 1988 and its appeal was immediate due to its explanatory power for some of the disease features [157]. Defective tyrosinase activity explains the hypopigmentation observed in Menkes patients but not the neurological phenotypes (Table 1). Similarly, hypoactivity of LOX family members provides an explanation for the vascular defects, cutis laxa, and diverticula in Menkes patients [149, 185, 186]. LOX initiates the crosslinking of collagens and elastin catalyzing oxidative modification of lysine residues present collagens and elastin. Some of the systemic Menkes phenotypes are recapitulated by mouse Lox null alleles. However, none of these Lox mutant mice are known to present with neuroanatomical alteration (Table 1) [206-208]. In contrast, LOX propeptide excess regulates Purkinje cell development, thus a role for LOX in Purkinje dysfunction in the Menkes brain cannot be excluded [209].

PAM, DBH, and mitochondrial cytochrome oxidase C are frequently invoked to explain the neurological manifestation in Menkes [165, 167, 185, 186]. PAM and DBH participate in the synthesis of neurotransmitter and neuropeptides [87, 210], yet there is little evidence that they alone account for neurodegeneration and neurodevelopmental abnormalities in Menkes patients or mouse models. Mice homozygous for null alleles of PAM and DBH enzymes neither recapitulate the neurodevelopmental nor the neurodegenerative phenotypes observed in Menkes though the mice die in utero [211, 212] (Table 1). Consistent with these observations, embryonic lethality caused by DBH deficiency in mice can be rescued by systemic administration of L-threo-dihydroxyphenylserine, and while this treatment corrects brain neurochemical abnormalities in Menkes mice, it does not correct neurodegeneration [213]. PAM haploinsufficiency impairs long-term potentiation without neurodegeneration or abnormalities in cortical development [214]. PAM haploinsuficiency selectively alters copper content in the amygdala [215, 216]. This suggests that PAM could contribute to Menkes disease in an anatomically restricted manner.

The participation of mitochondrial mechanisms in Menkes disease neurodegeneration is appealing as Menkes patients and Atp7a mutant mouse models have ultrastructural alterations in mitochondria [176-179]. These observations have been linked to impaired function of the mitochondrial cytochrome oxidase C complex (complex IV). Cytochrome oxidase C is a 13-subunit copper-dependent complex necessary for oxidative phosphorylation [217].

Decreased content of cytochrome oxidase C subunits have been frequently reported in human cells in culture and mouse tissues affected by Menkes [218-229]. This is in contrast with our quantitative mass spectrometry studies performed in cultured Menkes fibroblasts. We have found that there is no appreciable difference in the levels of nearly 80 mitochondrial proteins including complex IV subunits in the Menkes mitochondrial proteome (unpublished data). The most consistent finding concerning complex IV is a reduction in the cytochrome oxidase C enzymatic activity [218-220, 223, 227-229]. Despite this decreased activity, the metabolism of high-energy phosphate compounds, such as ATP, does not seem to be affected in mouse cells lacking Atp7a [227, 228]. Thus, it is possible that redox alterations rather than triphosphate nucleotide depletion may account for part of the neurological phenotypes. Do mutations in complex IV phenocopy aspects of Menkes disease? Genetic defects affecting the assembly or function of the cytochrome oxidase C complex subunits generate diverse disorders. These disorders span from isolated myopathy to severe multisystem disease such as infantile cardioencephalomyopathy and Leigh syndrome. This spectrum of genetic disorders encompasses defects in mitochondria-rich tissues such as heart and skeletal muscle, severe neurodegeneration, and lactic acidosis (OMIM: 256000, 220110, 604377) [230, 231]. While mitochondrial disorders (e.g. Leigh's or Alper's) result in similar neurological defects to Menkes disease, and underscore the likely contribution of mitochondrial dysfunction to some aspects of Menkes neurodegeneration phenotypes, the constellation of neurological Menkes manifestations points to the contribution of non-mitochondrial defects in the neuropathology of this disorder.

These observations challenge the oligoenzymatic pathogenic hypothesis, which has remained unchanged for nearly 25 years [149, 165, 167].

3.2.6 Proposed Revisions to the Oligoenzymatic Hypothesis.

The oligoenzymatic hypothesis seeks to link ATP7A copper-sensitive targets to disease manifestations. However, the oligoenzymatic hypothesis alone may not adequately explain neurodegeneration and neurodevelopmental phenotypes due to the paucity of copper-sensitive targets that it considers. We propose that simply considering ontological categories to which these few enzymes belong can enhance the oligoenzymatic hypothesis. Cytochrome C oxidase, PAM, SOD3, DBH, LOX, and tyrosinase are part of the GO term GO:0005507, which defines gene products capable of copper ion binding (http://amigo.geneontology.org/amigo/landing). This ontological category encompasses 56 gene products in humans and mice (Table 1). This category includes many proteins documented to require copper for their activity and accordingly, their hypoactivity could directly contribute to disease pathogenesis. Analysis of these 56 copper binding proteins using the mouse genomic informatics server (http://www.informatics.jax.org/) shows that fundamental Menkes disease phenotypes can be identified using a bottom-up approach. For example, these 56 proteins predict as phenotypes hypopigmentation (MP0005408), abnormal blood vessel (MP0001614), abnormal skin tensile (MP0005275), and neurodegeneration (MP0002229) (Table 2). Among the neurodegeneration group MP0002229, there are several interesting gene products to consider in the pathogenesis of neurodegeneration and neurodevelopmental

phenotypes: PRNP which encodes the prion protein; SOD1; and SNCA, encoding alpha synuclein (Table 2). Searching the OMIM database with the 56 copper binding proteins offers additional insight (Table 3, http://www.ncbi.nlm.nih.gov/omim/). This search identifies Parkinson's disease and other dementias with significant association and commonalities to Menkes disease. However, data mining misses some suggestive molecules. Take for example Apoa4, Gpc1, Rnf7, and Sco2. The evidence supporting copper binding by some of these molecules is either incipient or indirect, yet when mutated in mice they produce diverse neurological manifestations (Table 1) [232-234].

The bioinformatics analyses presented here rest on the assumption of a copper requirement for all downstream targets of ATP7A. In addition to these copper sensitive targets, there may be ATP7A downstream effectors that are copper independent that contribute to disease. For example, proteins whose stability requires ATP7A polypeptides instead of their pump activity or molecules indirectly connected to ATP7A whose expression/activity is dependent on a copper-sensitive intermediary. Unbiased studies of the proteomes and transcriptomes of diverse Menkes mutations will provide insight into the existence of these copper independent effectors.

3.2.7 Conclusions

Menkes neurological and neurodevelopmental manifestations have been attributed to defects in a select group of enzymes that require copper. However, knowledge gained from genetic experiments affecting these enzymes indicates that in isolation

they are insufficient to account for the neurological manifestations in Menkes. We propose a revised version of the oligoenzymatic hypothesis that includes all molecules in the copper-binding ontological category and molecules that may be sensitive to ATP7A content rather than cellular copper content. This revised hypothesis suggests intriguing connections between Menkes and Parkinson's disease (SCNA), risk factors for Alzheimer's (APOE4), and neurodevelopmental disorders. We postulate that regional differences in the neuropathology of Menkes, as those observed between the cerebral and cerebellar cortex, emerge from varying degrees of impairment in molecular networks downstream of ATP7A, including cell migration, polarity, and survival.

Section 4: Introduction Summary, Hypothesis, and Contribution of this Dissertation to the Field

There is extensive documentation that copper plays a role in the pathogenesis of neurodegenerative and neurodevelopmental disorders, yet pathways and mechanisms that connect a genetic defect in a copper homeostatic gene, such as ATP7A, with the neurological phenotypes are mostly unknown. My dissertation fills this knowledge gap by defining the interactome of the copper transporter ATP7A and genetically testing the requirement of interactome components in cellular and neuronal tissue copper homeostasis.

4.1 Outstanding questions in ATP7A and copper biology

1) What mechanisms underlie the contribution of copper to neurodegenerative and neurodevelopmental disorders?

As explored in Section 2, copper exposure correlates with the development of neurodegenerative disease. Moreover, altered copper homeostasis is observed in diseases such as Parkinson's and Alzheimer's. Copper has been linked to these diseases through the oxidative damage pathogenesis hypothesis of neurodegeneration. However, there is no conclusive evidence to suggest a mechanism for the role of copper in developing neurodegeneration. One goal of this dissertation is to comprehensively define the molecular interactions of the copper transporter ATP7A, mutations in which lead to neurodegeneration. I predict that ATP7A molecular interactors will define novel pathways affecting neuronal development and viability.

2) Does copper influence other neurodegenerative diseases?

My dissertation will present evidence that the ATP7A interactome enriches gene products implicated in neurodevelopmental and neurodegenerative disorders. Newly identified interaction partners of ATP7A include VAC14, a regulator of endosomal lipid composition, and the COG complex, a multimeric Golgi tether, mutations in both of which are associated with neurodegeneration and neurodevelopmental disorders, respectively [235-252]. These findings suggest that aberrations in copper homeostasis may be common to diverse neurological diseases previously unsuspected to be affected by copper homeostasis mechanisms.

3) How is global copper homeostasis regulated?

The role of copper has long been explored in a cellular compartment-specific fashion, but there is increasing evidence for multi-compartment regulation of copper homeostasis. This idea is implicit in the emerging literature, but there is little experimental evidence to support this conception of copper homeostasis. In my dissertation, I discuss how my work and published findings support the multi-compartment regulation of copper homeostasis. I will present evidence that two molecules that localize to the Golgi complex and interact, ATP7A and the COG complex, affect the function of copper-dependent mechanisms at the plasma membrane and the mitochondria.

4) What proteins are required for the regulation of ATP7A localization and trafficking?

ATP7A translocates to the cell surface after copper exposure to then recycle from the plasma membrane to the Golgi apparatus via endosomes [188]. This recycling was uncovered two decades ago, yet we have a limited knowledge of the cytosolic molecular complexes required for its trafficking. I predict that complexes upstream of ATP7A trafficking are part of the ATP7A molecular interactors.

4.2 Hypothesis

I hypothesized that genetic defects in molecules belonging to the ATP7A interactome are required for the subcellular localization of copper transporters and neuronal tissue viability.

4.3 Contribution of this dissertation to the field

My dissertation provides answers to the four outstanding questions in ATP7A and copper biology listed. I describe and annotate the ATP7A interactome, finding an enrichment of proteins associated with neurodevelopmental and neurodegenerative disorders. I demonstrate that components of the ATP7A interactome are necessary for neuronal tissue integrity and present evidence of a COG-dependent mechanism connecting the plasma membrane, the Golgi complex and the mitochondria. These findings support a model of global cellular copper homeostasis and contribute to our understanding of the role of copper in the development of neuropathologies.

Table 1. Copper Binding Gene Products Define by Gene Ontology. NRF is the acronym for no reported phenotype.

Mouse Gene	MGI Entry Number	MGI Phenotypes	OMIM Entry Number	OMIM Phenotypes
Aoc1	MGI:1923757	NRF		
Adnp	MGI:1338758	Developmental defects. Failure of the cranial neural tube to close. Embryonic death between E8.5 and E9		
Ahcy	MGI:87968	NRF	613752	failure to thrive, mental and motor retardation, facial dysmorphism with abnormal hair and teeth, and myocardiopathy
Ang	MGI:88022	NRF	611895	ALS9 Parkinsonism
Aoc2	MGI:2668431	NRF		
Aoc3	MGI:1306797	Homozygous null display decreased lymphocyte migration and homing in response to inflammation		
Apoa4	MGI:88051	Homozygous null have neurodegeneration, lower HDL cholesterol levels but normal lipid absorption, growth, and feeding behavior.		
Atox1	MGI:1333855	Homozygote null mutation have impaired intracellular copper trafficking and exhibit high postnatal mortality, retarded growth, hypoactivity, loose skin, hypopigmentation, and seizures		

Atp7a	MGI:99400		309400	
Atp7b	MGI:103297	Copper accumulation in various organs, including brain. Liver cirrhosis that resembles Wilson disease in humans and the 'toxic milk' phenotype in mice	277900	Wilson disease
Commd1	MGI:109474	Embryonic lethal with growth retardation, failure to turn, increased apoptosis in brain mesenchyme and defects in extraembryonic tissue development		
Cox11	MGI:1917052	NRF		
Cox17	MGI:1333806	Homozygous null are growth retarded and die between E8.5 and E10, with severe reductions in cytochrome c oxidase activity at E6.5		
Cp	MGI:88476	Accumulation of iron in the liver, spleen, cerebellum, and brainstem, mild iron deficiency anemia, and impaired motor coordination associated with loss of brainstem dopaminergic neurons.	604290	dementia and diabetes mellitus, chorea, and ataxia
Cr1l	MGI:88513	die by E16.5 with abnormal C3 deposition		
Cutc	MGI:1913638	NRF		
Dct	MGI:102563	Pigmentation defect	191275	
Dbh	MGI:94864	Embryonic lethal probably	223360	orthostatic hypotension,

		due to cardiovascular failure		ptosis, nasal stuffiness, and a neonatal history of delayed eye opening, skeletal muscle hypotonia
F5	MGI:88382	50% of homozygous null allele die at E9-E10 with defects in yolk-sac vasculature and somite formation; the remaining half develop to term but die of massive hemorrhage within hours of birth.		
F8	MGI:88383	Prolonged, exsanguinating bleeding following tail-clipping	306700	hemophilia A
Gpc1	MGI:1194891	Reduced brain size with mild cerebellar patterning defects. Otherwise viable and fertile		
Heph	MGI:1332240	Small and pale at birth, exhibit a hypochromic anemia which tends to disappear with age. Mutants have impaired iron transport in the placenta and in the gut.		
Hephl1	MGI:2685355	NRF		
Il1a	MGI:96542	Development of Th2 helper cell responses and some antibody responses are compromised		
Lox	MGI:96817	Altered arterial wall structure, aortic aneurysms, cardiovascular dysfunction, diaphragmatic hernia, and perinatal death.		

		Abnormal development of the respiratory system, and elastic and collagen fiber abnormalities in the lung and skin are also observed		
Loxl1	MGI:106096	Elastic fiber homeostasis is disrupted, loose skin, abnormal lung morphology, intestinal defects, and post partum uterine prolapse		
Loxl2	MGI:2137913	NRF		
Loxl3	MGI:1337004	NRF		
Loxl4	MGI:1914823	NRF		
Mett11d1	MGI:1098577	NRF		
Mettl17	MGI:1098577	NRF		
Moxd1	MGI:1921582	NRF		
Moxd2	MGI:2388042	NRF		
mt-Co2	MGI:102503	NRF	540000	mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes, is a genetically heterogeneous mitochondrial disorder with a variable clinical phenotype. The disorder is accompanied by features of central nervous system involvement, including seizures, hemiparesis, hemianopsia, cortical blindness, and episodic vomiting

Mt1	MGI:97171	Abnormal zinc absorption and abnormal circadian rhythm response to melatonin. Mice homozygous for null alleles of Mt1 and Mt2 exhibit increased sensitivity to xenobiotics and injury with decreased wound healing and abnormal mineral aborption.		
Mt3	MGI:97173	Zinc deficiency in several brain regions, abnormal astrocyte morphology, increased susceptibility to kainic acid induced seizures, and altered zinc accumulation and neuronal death in certain brain areas following seizure-induced or acute brain injury		
P2rx2	MGI:2665170	Viable and show no gross pathology. Mice show abnormal ventilatory and electrophysiological responses to hypoxia	608224	autosomal dominant deafness-41
P2rx4	MGI:1338859	Hypertension, abnormal artery morphology, abnormal nitric oxide homeostasis, and impaired flow induced vascular remodeling and vasodilation		
P2rx7	MGI:1339957	Fertile and viable with no obvious phenotypic abnormality. Cellular responses of macrophages to extracellular ATP are frequently normal however. In addition, long bones are thinner than		

		normal in adult mice		
Pam	MGI:97475	Embryonic lethality during fetal growth and development, edema, abnormal yolk sac vasculature, thin arterial walls, and abnormal bronchial epithelial morphology		
Prnd	MGI:1346999	Homozygous null mice display male infertility		
Prnp	MGI:97769	Homozygous mutants also show impaired locomotor coordination and reduced mitochondria numbers with unusual morphology	123400 137440	memory loss, dementia, ataxia, and pathologic deposition of amyloid-like plaques in the brain
Rnf7			600072	insomnia with or without a diurnal dreaming state, hallucinations, delirium, and dysautonomia preceding motor and cognitive deterioration
Rnpep	MGI:2384902	NRF		
S100a13	MGI:109581	NRF		
S100a5	MGI:1338915	NRF		
Sco1	MGI:106362	NRF		
Sco2	MGI:3818630	Null allele exhibit embryonic lethality. Mice heterozygous for a knock-out allele and a knock-in allele exhibit muscle weakness and reduced exercise endurance.	220110	Leigh syndrome. heterogeneous, ranging from isolated myopathy to severe multisystem disease, with onset from infancy to adulthood
Slc11a2	MGI:1345279	Microcytic, hypochromic anemia associated with impaired intestinal iron	206100	hypochromic microcytic anemia with iron overload-1

		absorption and erythroblast iron uptake. Mutants have reduced viability and fertility.		
Snai3				
Snca	MGI:1277151	Disruptions in this gene display resistance to the effects of MPTP on dopamine levels. Mice expressing a knock-in allele exhibit impaired coordination, long stride length, abnormal response to reserpine and reduced brain dopamine levels.	127750 168601	Parkinson
Sod1	MGI:98351	Homozygous mutants exhibit increased motor neuron loss after axonal injury and enhanced susceptibility to ischemic reperfusion injury. Homozygous females have irregular and small litters, and for some alleles exhibit immature ovarian follicles with few corpora lutea.	105400	ALS1
Sod3	MGI:103181	Mice homozygous for a knock-out allele exhibit increased sensitivity to hyperoxia, increased LPS-stimulated spleen production of TNF, and enhanced severity of collagen-induced arthritis.		
Trp53	MGI:98834	Null homozygotes show high, early-onset tumor incidence; some have persistent hyaloid vasculature and cataracts. Truncated or	191170	Mutiple neoplasms

		temperature-sensitive alleles cause early aging phenotypes.		
Tyr	MGI:98880	Albinism or hypopigmentation. Albinism is associated with reduced number of optic nerve fibers and mutants can have impaired vision	606933	Occulo cutaneous albinism

Table 2. Enrichment Analysis of Mouse Phenotypes Associated to Genetic Defects in the Ontological Category Copper Binding.

Node Name	Corrected P-value Benjamini-Hochberg	Total Genes in Gene Set	Total Genes Intersected	Intersecting Genes
MP0005636_ABNORMAL_MINERAL_HOMEOSTASIS_	1.96E-10	195	10	MT3;ATP7B;MT1;CP;SLC11A2;HEPH;ATOX1;PRNP;ATP7A;SCO2
MP0003632_ABNORMAL_NERVOUS_SYSTEM_	2.01E-05	111	5	ATOX1;CP;PRNP;SNCA;ATP7B
MP0003631_NERVOUS_SYSTEM_PHENOTYPE_	7.06E-05	241	6	DBH;CP;ATOX1;PRNP;SNCA;ATP7B
MP0005408_HYPOPIGMENTATION_	1.30E-04	31	3	ATOX1;ATP7B;TYR
MP0003718_MATERNAL_EFFECT_	4.64E-04	49	3	ATOX1;SLC11A2;ATP7B
MP0002295_ABNORMAL_PULMONARY_CIRCULATION_	6.39E-04	55	3	LOX;ATP7A;ATOX1
MP0001186_PIGMENTATION_PHENOTYPE_	9.35E-04	258	5	ATOX1;ATP7B;TYR;ATP7A;TRP53
MP0005171_ABSENT_COAT_PIGMENTATION_	9.82E-04	14	2	ATP7A;TYR
MP0003186_ABNORMAL_REDOX_ACTIVITY_	8.53E-04	61	3	SOD1;ATP7A;MT1
MP0004147_INCREASED_PORPHYRIN_LEVEL_	1.11E-03	15	2	HEPH;SLC11A2
MP0008438_ABNORMAL_CUTANEOUS_COLLAGEN_	1.39E-03	17	2	LOX;ATP7A

MP0000609_ABNORMAL_LIVER_PHYSIOLOGY_	1.71E-03	442	6	ATP7B;ATP7A;ATOX1;SCO2;HEPH;SLC11A2
MP0001614_ABNORMAL_BLOOD_VESSEL_	1.58E-03	779	8	LOX;TRP53;CP;PAM;P2RX4;ATP7A;IL1A;APOA4
MP0002128_ABNORMAL_BLOOD_CIRCULATION_	1.87E-03	450	6	F5;LOX;TRP53;ATP7A;MT1;ATOX1
MP0005275_ABNORMAL_SKIN_TENSILE_	2.41E-03	23	2	LOX;ATP7A
MP0002229_NEURODEGENERATION_	2.32E-03	318	5	PRNP;ATP7B;CP;SOD1;SNCA
MP0008770_DECREASED_SURVIVOR_RATE_	2.66E-03	196	4	P2RX2;F5;TRP53;TYR
MP0003279_ANEURYSM_	3.24E-03	27	2	ATP7A;LOX
MP0003329_AMYLOID_BETA_DEPOSITS_	3.24E-03	27	2	ATP7B;PRNP
MP0005395_OTHER_PHENOTYPE_	4.46E-03	111	3	ATOX1;SLC11A2;ATP7B

Table 3. Enrichment Analysis of Human Phenotypes Associated to Genetic Defects in the Ontological Category Copper Binding.

Node Name	Corrected P-value Benjamini-Hochberg	Total Genes in Gene Set	Total Genes Intersected	Intersecting Genes
PARKINSON_DISEASE	5.68E-02	100	2	DBH;SNCA
LATERAL_SCLEROSIS	5.39E-02	97	2	ANG;SOD1
CUTIS_LAXA	4.26E-02	85	2	ATP7A;LOX
SKIN/HAIR/EYE_PIGMENTATION	3.04E-01	90	1	TYR
DEMENTIA	3.04E-01	90	1	SNCA
THROMBOPHILIA	3.01E-01	89	1	F5
ALBINISM	2.96E-01	87	1	TYR
WAARDENBURG_SYNDROME	2.93E-01	86	1	TYR
ATAXIA	2.15E-01	60	1	CP

CHAPTER 2

THE INTERACTOME OF THE COPPER TRANSPORTER ATP7A BELONGS TO A NETWORK OF NEURODEVELOPMENTAL AND NEURODEGENERATION FACTORS

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eLife 2017; 10.7554/eLife.24722. Published March 29, 2017.

ABSTRACT

Genetic and environmental factors, such as metals, interact to determine neurological traits. We reasoned that interactomes of molecules handling metals in neurons should include novel metal homeostasis pathways. We focused on copper and its transporter ATP7A because ATP7A null mutations cause neurodegeneration. We performed ATP7A immunoaffinity chromatography and identified 541 proteins co-isolating with ATP7A. The ATP7A interactome concentrated gene products implicated in neurodegeneration and neurodevelopmental disorders, including subunits of the Golgi-localized conserved oligomeric Golgi (COG) complex. COG null cells possess altered content and subcellular localization of ATP7A and CTR1 (SLC31A1), the transporter required for copper uptake, as well as decreased total cellular copper, and impaired copper-dependent metabolic responses. Changes in the expression of ATP7A and COG subunits in Drosophila neurons altered synapse development in larvae and copper-induced mortality of adult flies. We conclude that the ATP7A interactome encompasses a novel COG-dependent mechanism to specify neuronal development and survival.

INTRODUCTION

Copper, iron, and manganese act as micronutrients, yet in excess behave as environmental neurotoxicants [253-256]. These metals are handled by diverse membrane transporters and dedicated chaperones that deliver metals to cellular compartments to act as cofactors in essential enzymatic reactions while also maintaining cellular levels of these metals within a narrow range [257]. Mutations in these metal membrane transport and chaperoning mechanisms cause neurodegenerative disorders in both children and adults [149, 256]. In addition, metal exposure modulates the severity of neurological disease by yet unknown metal-sensitive mechanisms [126]. Thus, genetic and environmental factors, such as metals, converge to impinge on neurodegeneration and neurodevelopmental phenotypes.

Cellular copper content is regulated by two chief transporters, ATP7A and CTR1 (SLC31A1)[149, 256]. Cellular copper homeostasis is maintained by virtue of ATP7A and CTR1 subcellular localization and metal transport topology. ATP7A resides at the Golgi apparatus where it sequesters copper topologically into the Golgi lumen and away from the cytoplasm. In contrast, CTR1 localizes to the plasma membrane where it transports copper into the cytoplasm from the extracellular milieu [149, 185, 186]. The subcellular localization of ATP7A and CTR1 is modulated by copper-dependent vesicle traffic. After an extracellular copper challenge, ATP7A translocates from the Golgi complex to the plasma membrane where it extrudes copper out of cells while CTR1 undergoes copper-dependent endocytosis, thus

down-regulating CTR1-dependent copper transport across the plasma membrane [149, 185, 186]. Of these two transporters, ATP7A is associated with neurological phenotypes in vertebrates [98, 148, 149, 151, 154, 258], justifying it as our choice to test the hypothesis that interactomes of molecules specialized in the handling of metals encompass novel metal homeostasis mechanisms capable of modulating the expression of neurological traits.

ATP7A loss-of-function genetic defects cause three neurological diseases, including Menkes disease (OMIM 309400), occipital horn syndrome (OMIM 304150), and X-linked distal spinal muscular atrophy type 3 (OMIM 300489). Menkes causing mutations systemically deplete organisms and cells of copper due to defective gut copper uptake, which in turn reduces the activity of cuproenzymes involved in neurotransmitter, neuropeptide, melanin, mitochondrial respiration, and extracellular matrix synthesis [98, 148, 149, 151, 154, 186, 258]. Menkes disease is dominated by early childhood neurodegeneration whose mechanisms remain poorly understood [96], thus making ATP7A an ideal candidate to identify metal homeostasis mechanisms capable of modulating neuropathology phenotypes.

Here we defined the ATP7A interactome which was enriched in products implicated in neurodegeneration and neurodevelopmental disorders, including the conserved oligomeric Golgi (COG) complex. COG is a Golgi vesicle tether necessary for intra Golgi vesicular traffic and the retention of Golgi-localized enzymes at the Golgi complex, such as glycosyltransferases [237, 244, 259-261]. COG genetic defects

cause disruption of the Golgi complex function characterized by the loss of Golgi-resident enzymes and alterations in the glycosylation of membrane proteins traversing the exocytic route. These COG mutations cause a systemic disorder that includes cerebral atrophy, developmental delay, hypotonia, ataxia and epilepsy [237, 244, 259-261]. A requirement for the COG complex in cellular copper homeostasis and ATP7A-dependent metal buffering mechanisms was previously unrecognized and it has became the focus of our attention.

RESULTS

The ATP7A Interactome

In order to identify ATP7A-dependent and copper-sensitive mechanisms, we developed an unbiased approach to comprehensively define the ATP7A interactome in neuroblastoma cells (Fig. 1). We used human SH-SY5Y neuroblastoma cells to perform immunoaffinity chromatography isolation of cross-linked ATP7A complexes. ATP7A was isolated with a monospecific monoclonal antibody that robustly recognizes a band in SH-SY5Y cells whose immunoreactivity is abolished in ATP7A-null cells (Fig. 1A, lanes 1-4 and Fig. 1B). Like other cells, SH-SY5Y ATP7A surface content increased after copper addition as determined by surface biotinylation followed by streptavidin precipitation and immunoblot against ATP7A. Transferrin receptor, a copper-insensitive membrane protein, did not increase at the surface upon copper addition (Fig 1, compare lanes 1’ and 4’). These results validate our choice of cells and antibody to define the ATP7A interactome.

Intact cells were first cross-linked with dithiobis(succinimidyl propionate) to identify ATP7A protein interaction partners with weak or transient interactions (DSP, Fig. 1C). DSP is a short arm 12 Å cross-linker, which is cell permeant and cleavable by reducing agents [262-264]. Cross-linking increases the coverage of interactome components co-purifying with membrane proteins of complex topology, such as ATP7A [265, 266]. We developed a multipronged approach to maximize ATP7A-specific interactions. First, non-selective binding to magnetic bead-ATP7A antibody complexes was determined by the addition of an excess of the antigenic ATP7A peptide recognized by the monoclonal antibody (Fig. 1C1-2 and 2B). Second, the ATP7A antigenic peptide was used to selectively elute cross-liked ATP7A interacting proteins from magnetic beads instead of Laemmli sample buffer (Fig. 1C1-4). Third, we mock isolated ATP7A from ATP7A-null human fibroblasts (ATP7A-/-), along with the same cells rescued by expression of recombinant ATP7A (Fig. 1C3-4, ATP7AR/R) [267]. Proteins isolated from ATP7A-null cells were eliminated from all experimental datasets (Fig. 1C3). Fourth, we performed label free quantitative mass spectrometry and thresholded positive protein identification as a ratio >2 between samples immunoisolated in the absence and presence of the ATP7A antigenic peptide (Fig. 1C and 2A) [268]. Finally, we isolated cross-linked ATP7A complexes from SH-SY5Y neuroblastoma cells preincubated in the presence of either CuCl2 or the copper chelator bathocuproine disulphonate (Fig. 1C1-2, BCS). Figure 1D depicts immunoaffinity chromatography experiments with cell extracts from SH-SY5Y cells pre-treated in the absence (Fig. 1D, BCS) or presence of copper (Fig. 1D). Non-specific interactors bound to ATP7A antibody-decorated magnetic

beads are depicted in lanes 3 and 3' where incubations were performed with an excess of the ATP7A antigenic peptide (Fig. 1D). The presence of ATP7A and one previously described interactor, dopamine beta hydroxylase (DBH), was confirmed by immunoblot of isolated cross-linked complexes (Fig. 1D) [269]. Protein complexes copurifying with ATP7A isolated from BCS or copper-treated cells were not evidently different (Fig. 1D, compare lanes 2 and 2' and traces, and Fig 2C). Therefore, we reasoned that proteins common to BCS and copper conditions would represent stable and/or strong interactors carried by ATP7A irrespective of its subcellular location (Fig. 2C).

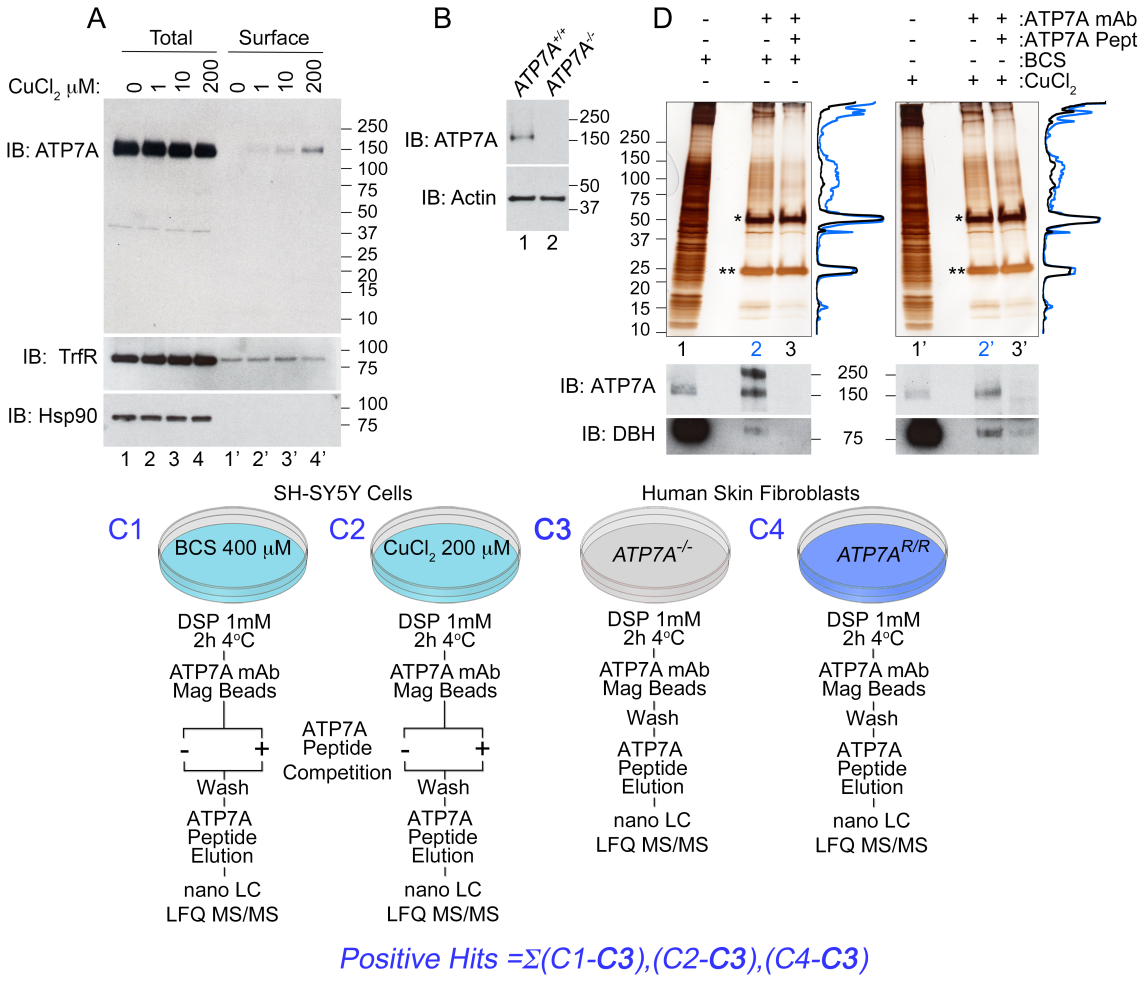

Figure 1 Isolation of ATP7A interactome.

(A) In SH-SY5Y neuroblastoma cells, the addition of increasing amounts of copper leads to an increase of ATP7A at the cell surface as measured by surface biotinylation followed by streptavidin pull-down (lanes 1'-4'), while the total levels of ATP7A remain unchanged (lanes 1-4). Transferrin receptor shows consistent surface expression regardless of copper addition (lanes 1'-4'). The cytosolic chaperone Hsp90 was used to assess the selectivity of streptavidin pulldowns (B) The monoclonal ATP7A antibody used in these studies recognizes a single band by immunoblot. This band is missing in ATP7A null human Menkes fibroblasts (lane 2). (C1-C4) Experimental designs to isolate ATP7A interactomes. ATP7A immunoaffinity chromatography was performed in two cell types, SH-SY5Y cells (C1-2) and human skin fibroblasts (C3-4). In the former, left, SH-SY5Y cells were incubated with either 400 μM BCS (C1), a copper chelator, or 200 μM CuCl2 for 2 hours (C2). Cells were crosslinked with DSP, cell extracts were immunoprecipitated with the monoclonal ATP7A antibody either in the absence or presence of 22 μM ATP7A antigenic peptide. The same peptide was used to elute samples, which were then analyzed by label free quantitative mass spectrometry or silver stain (D). C3-4, ATP7A immunoaffinity chromatography was performed in ATP7A-null human skin fibroblasts (C3) as well as the same cells recombinantly expressing ATP7A (C4). The experiment was performed as in SH-SY5Y cells, with the exception of the ATP7A antigenic peptide being omitted for outcompetition. (D) Silver stain from ATP7A immunoprecipitation depicted in (C) except that immunocomplexes were eluted with Laemmli sample buffer. Immunoprecipitations were performed for BCS-treated (lanes 1-3) and CuCl2 treated (lanes 1'-3') SH-SY5Y cell extracts. Asterisks indicate immunoglobulin G chains, and densitometry profiles show differential protein enrichment in samples immunoprecipitated with (lanes 3 and 3', black traces) and without (lanes 2 and 2', blue traces) the antigenic ATP7A peptide. Below are immunoblots performed in parallel revealing positive identification of ATP7A and known interacting partner dopamine beta hydroxylase (DBH).

Quantitative mass spectrometry identified five hundred and forty one positive hits co-isolating with ATP7A, as defined in Figure 1C and Supplementary Tables 1-3. These five hundred and forty one proteins are the result of a curation with a dataset obtained under the same conditions but using cellular homogenates from ATP7A null human cells (Fig. 1C3, see Supp Tables 1-3). We confirmed the quality of this curation step by comparing the five hundred and forty one positive hits to a published control dataset of three hundred and thirty five proteins that spuriously

bind magnetic beads (Fig. 2C, CRAPome) [270]. The overlap between our five hundred and forty one ATP7A interactors and the CRAPome was just thirty one proteins, a 5.7% overlap (Fig. 2C, CRAPome, Supp Table 3). One hundred and thirty four proteins co-isolated with ATP7A regardless of whether cells were incubated with BCS or copper (Fig. 2A and C, see Supp. Tables 1-3). Mass spectrometry identified ATP7A peptides corresponding primarily to the antigenic primary sequence at the C-terminal domain of ATP7A (Fig. 2B). The interactome includes known ATP7A interactors such as dopamine beta hydroxylase [269]; subunits of the WASH complex, FAM21 and WASH1 [194, 271]; subunits of the retromer complex, VPS26 and 35 [191, 194]; and components of clathrin-coated vesicles such as CLTB [190, 193, 199, 272]. In order to prioritize candidate proteins for functional studies, we analyzed the ATP7A interactome with gene ontology algorithms (Fig. 3, Suppl. Tables 3A-B). Gene ontological categories were enriched in Golgi-related terms such as Golgi transport complex (GO:0017119, $p< 5E-6$), which contained six of the eight subunits of the Golgi localized COG tethering complex. Three of these six COG subunits were identified in the BSC and copper-challenged ATP7A interactomes (Fig. 2C, COG2, 4 and 7). Additionally, bioinformatics identified neuronal ontological terms including growth cone, neuron projection, and dendrite (GO:004300, 0030426, 003042 respectively; all $p< 10E-4$, Fig 3A, Supp. Table 3A-B). Similar bioinformatic results were obtained irrespective of the algorithm used, DAVID (Fig. 3B, see Supp. Table 3A) or ENRICHR (Fig. 3C, see Supp. Table 3B) [273-275]. The ATP7A interactome is overrepresented in biological processes related to membrane trafficking and vesicular transport, Golgi-related transport in particular. Proteins

contained in these ontological terms were among the products identified in both BSC and copper-challenged ATP7A interactomes, such as the COG complex subunits (COG2, 4 and 7, Fig. 2C). In fact, the COG complex was among the most prominently enriched complexes present in the ATP7A interactome (CORUM database, Fig. 3D, combined score >7, see Supp. Table 3B) [276]. These gene ontology tools point to the Golgi-dependent and neuronal trafficking mechanisms as key components of the ATP7A interactome.

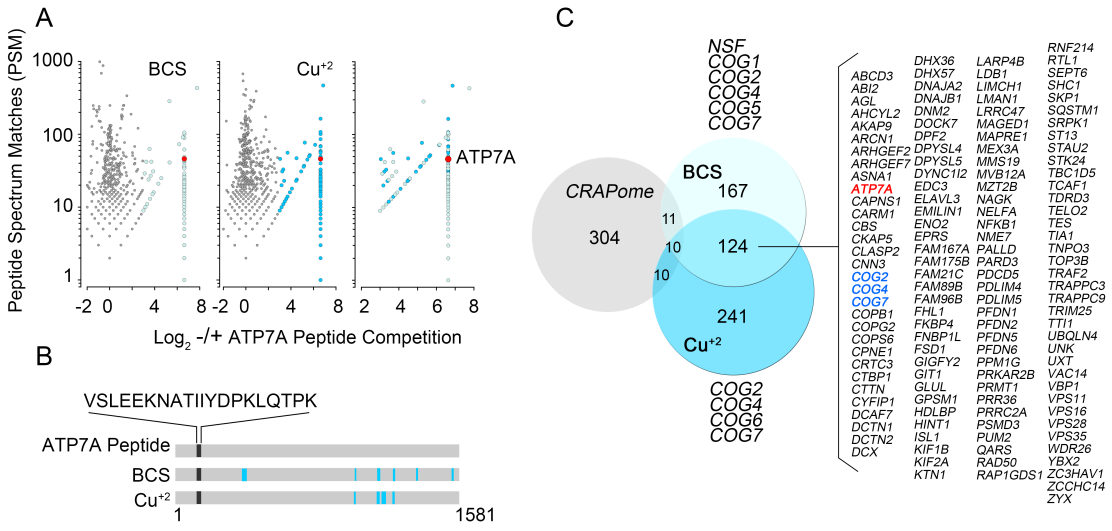

Figure 2 Identification of ATP7A interactome components by mass spectrometry.

(A) Peptide spectrum matches (PSM) from quantitative mass spectrometry of proteins co-isolating with ATP7A are plotted for cells incubated with copper chelator BCS (left), CuCl2 (middle), and peptides identified in both samples (right). Blue dots represent peptides that were enriched 2-fold over negative control samples incubated with an antigenic ATP7A peptide. (B) ATP7A peptides identified by mass spectrometry. Peptides corresponded to the antigenic peptide sequence shown above the black line as well as other ATP7A peptides identified via mass spectrometry (blue lines). (C) Five hundred and forty one proteins co-isolated with ATP7A, one hundred thirty three of which were present regardless of cellular copper status are listed. Three COG subunits were present in both BCS and copper-treated samples (blue text), the other three subunits were found either in BSC or copper-treated samples. Curation with a dataset from the CRAPome reveal minimal overlap.

We next asked what traits would associate with loss-of-function mutations in components of the ATP7A interactome using mouse phenotypic databases [275]. Analysis of gene sets describing mouse phenotypes demonstrated that gene products contained in the ATP7A interactome were significantly enriched in categories describing neuronal pathology such as abnormal brain-neuronal morphology and neurodegeneration (Fig. 3E, combined score >9.5, Supp. Table 3B). To further our understanding of phenotypes associated with members of the ATP7A interactome, we utilized the GDA bioinformatic tool that derives disease-gene links from human databases OMIM and Genopedia [277]. This analysis revealed a significant association with neurodegenerative nervous system diseases (Fig. 4A, Supp. Table 3C) and psychiatric disorders (Fig. 4B, Supp. Table 3C). The ATP7A interactome enriched 62 neurodegeneration associated or causative genes (MeSH, Medical Subject Heading C10.574, $p<0.0001$), which include COG2, VPS26, VPS35, DBH, and NSF. Moreover, 181 gene products contained in the ATP7A interactome were associated with mental disorders (Medical Subject Heading F03, $p<0.0001$, Supp. Table 3C). Within the mental disorder category the MeSH term neurodevelomental disorders stood out (Medical Subject Heading F03.625, $p<0.0001$, Supp. Table 3C) represented with 34 gene products. These analyses indicate that the ATP7A interactome enriches gene products previously implicated in neurodegenerative and neurodevelopmental disorders, suggesting that these associations could participate in the neuropathogenesis of ATP7A genetic deficiencies.

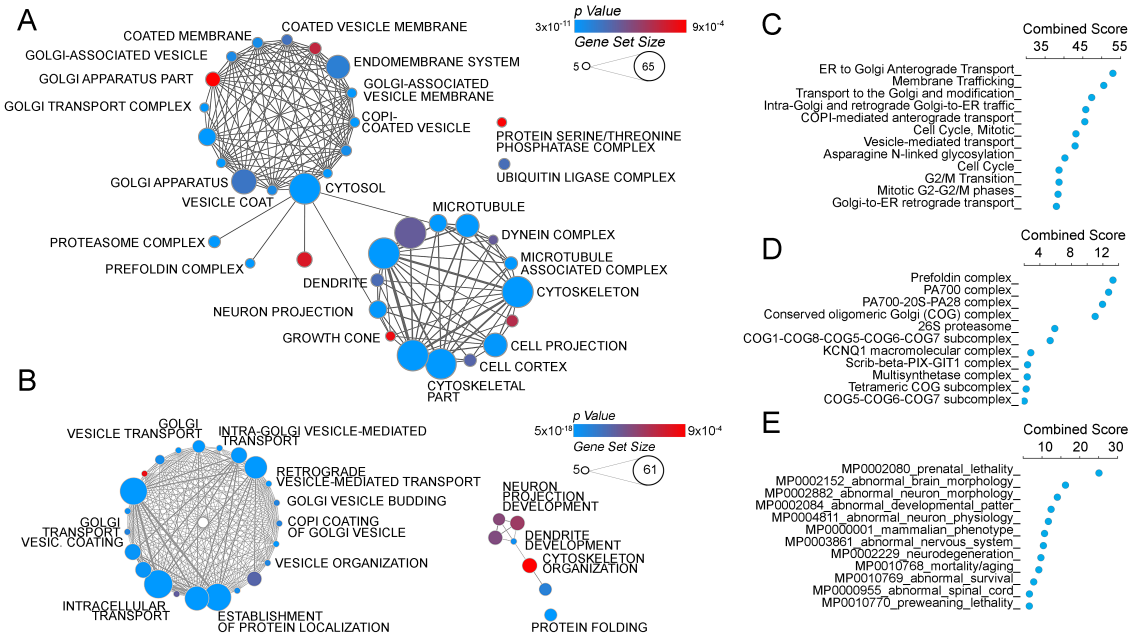

Figure 3 The ATP7A interactome enriches gene products implicated in Golgi function and neuropathologies.

(A-B) The gene ontology algorithm DAVID was used to analyze the ATP7A interactome using the GO Terms Cellular Component (A) and Biological Process (B). Size of the circles increases with gene set size, and p-values are represented by colors ranging from blue (p = 3x10-11) to red (p = 9x10-4). Lines connecting circles depict ontology categories with shared gene products. (C-F) The ATP7A interactome was also evaluated using ENRICHR algorithm, to characterize dataset enrichment in GO Term Biological Process (C), protein complexes from the CORUM database (D), and phenotypes in mice (E). Significance is represented as a combined score (z-score x -log(p-value)).

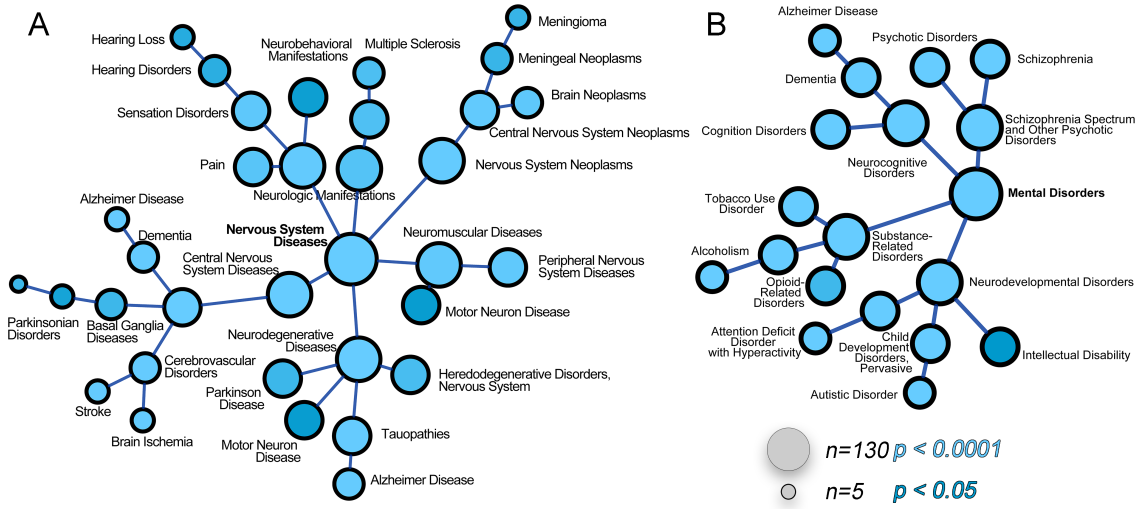

Figure 4 The ATP7A interactome enriches gene products associated with nervous system diseases and mental disorders.

(A-B) The ATP7A interactome was analyzed using the GDA bioinformatics tool, which derives disease-gene links from human databases OMIM and Genopedia Nervous system diseases (A) and mental disorders (B) were both significantly enriched in the dataset ($p < 0.0001$). Circle size represents number of gene product from the ATP7A interactome in the disease category. Shades of blue depict p values.

We prioritized for confirmation ATP7A interactome candidates due to their association with neurodegenerative and neurodevelopmental disorders. We immunoprecipitated ATP7A from cell extracts of cross-linked SH-SY5Y neuroblastoma cells and immunoblotted for proteins of interest. We focused on proteins that were either co-enriched with ATP7A to a similar extent as the bait (fold of enrichment log2 >6), were present in the BCS and copper treated ATP7A interactomes (Fig. 2C), were associated with or causative of neurodegeneration (VAC14, strumpellin, NFKB1, and GIGYF2/PARK11) as well as mental disorders (NSF, DOCK7, GIGYF2, and COG complex subunits, Fig. 3), and/or were present in compartments where ATP7A traffics to and from. The WASH complex subunit strumpellin and clathrin heavy chain (CHC) served as controls for previously described ATP7A interacting proteins [190, 193, 194, 199, 271, 272]. The ATP7A monoclonal antibody co-immunoprecipitated strumpellin and clathrin heavy chain, and other proteins belonging to the ATP7A interactome listed above (Fig. 5A, lane 2). The addition of ATP7A immunogenic peptide prevented the coprecipitation of ATP7A with these selected interactome components (Fig. 5A, lane 3). To further establish specificity of our immunoprecipitation method, we immunoblotted for abundant cytosolic proteins that were identified by mass spectrometry either in the

negative control samples or that fell below the threshold of enrichment established for significance. These proteins include actin, several peroxiredoxins, thioredoxin, and the ubiquitin hydrolase UCHL1 (Fig. 5B). None of these proteins co-isolated with ATP7A even in the presence of a crosslinker (Fig. 5B, lane 2). Neither specific interactors of ATP7A (Fig. 5A) nor proteins used as controls (Fig. 5B) changed their association with ATP7A after pretreating cells with copper chelator (BSC-treated cells, Fig. 1C) or copper (data not shown). These results validate the ATP7A interactome and confirm that this interactome enriches neurodevelopmental and neurodegenerative gene products.

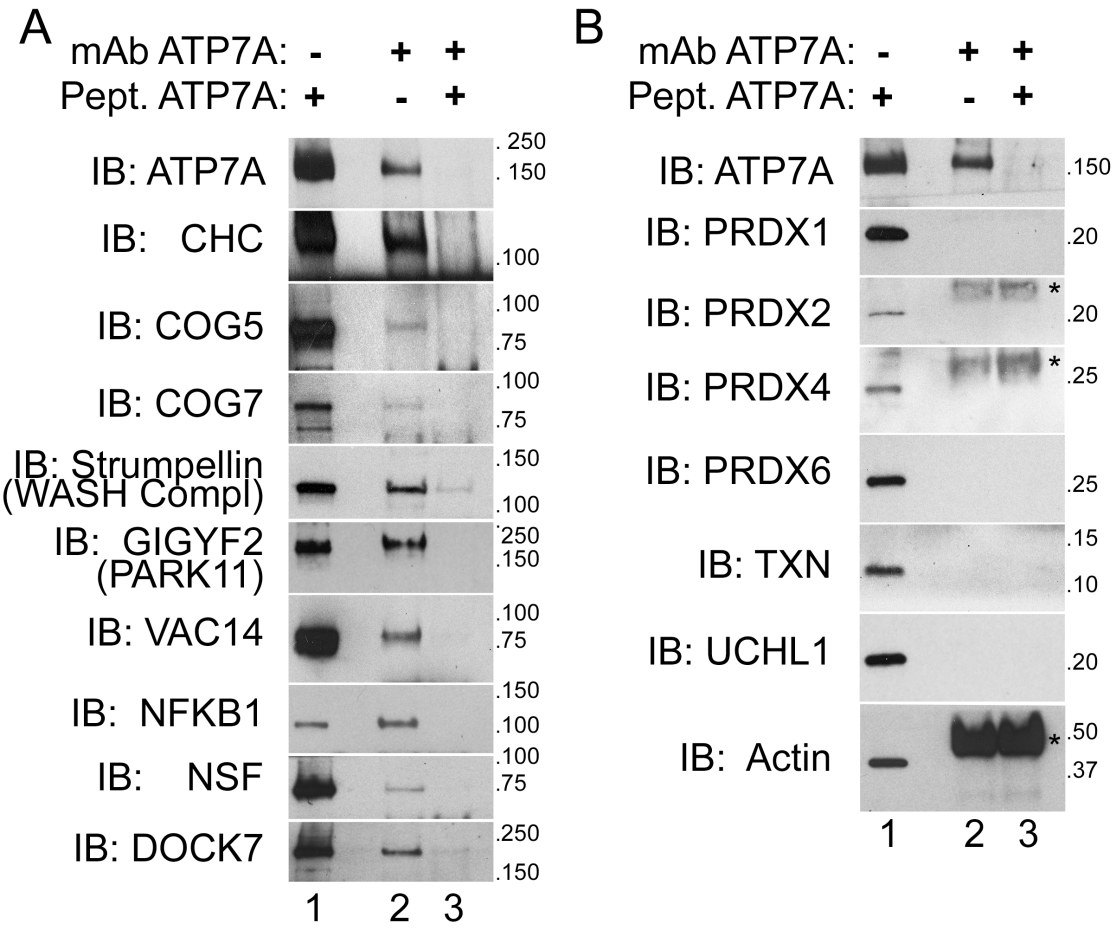

Figure 5 ATP7A co-immunoprecipitates with proteins implicated in neurodegeneration and neurodevelopmental disorders.

(A-B) ATP7A was immunoprecipitated from DSP-crosslinked SH-SY5Y neuroblastoma cell lysates. Whole cell extracts (lanes 1) and immunoprecipitated samples (lanes 2 and 3) were resolved by SDS-PAGE and analyzed by immunoblot. Lane 3 contains samples in which an antigenic ATP7A peptide was added during immunoprecipitation as a negative control. (A) ATP7A and previously characterized interactors, clathrin heavy chain (CHC) and strumpellin, were selectively identified, along with newly identified interactome components that were highly enriched by mass spectrometry and associated with neuropathologies. Note the presence of two COG complex subunits. (B) Abundant cytosolic proteins that fell below the significant fold of enrichment by mass spectrometry or were identified in the negative control samples failed to co-purify with ATP7A. None of these proteins coprecipitate with ATP7A demonstrating the specificity of the interactions depicted in A. Asterisks denote mouse IgG chains.

The COG Complex is Necessary for Copper Transporter Stability and Cell Surface Expression.

We selected the COG complex to test the hypothesis that genetic defects in components of the ATP7A interactome impair copper homeostasis by metal transporters for three reasons. First, we find that ATP7A co-purifies with six of the eight COG complex subunits to a similar extent (Fig. 2A and C; 5A). Secondly, our bioinformatics analysis prioritizes this complex among components of the ATP7A interactome (Fig. 3A-E). Finally, mutations in the human COG subunits result in neurological defects some overlapping with Menkes disease (Figs. 3E and 4).[239-243, 245-251, 278].

We used HEK293 cells rendered null for COG subunits 1 ($COG1^{\Delta/\Delta}$) or 8 ($COG8^{\Delta/\Delta}$) using CRISPR-Cas9 genome editing [279, 280]. These cells reproduce cellular phenotypes associated with COG complex deficiencies, such as defective Golgi-

dependent glycosylation of membrane proteins and degradation of Golgi-localized proteins in lysosomal compartments [279-281]. Both COG null cell lines exhibited decreased expression of a known COG-dependent and Golgi-localized membrane protein, GPP130 (Fig. 6A). In addition, the GPP130 protein was found to migrate faster during SDS-PAGE, an indication of defective GPP130 glycosylation in COG null cells (Fig. 6A) [282-284]. Similarly, ATP7A expression was reduced by ~25-50% (Fig. 6A-B), and ATP7A SDS-PAGE migration was increased in COG-null cell lines (Fig. 6A).

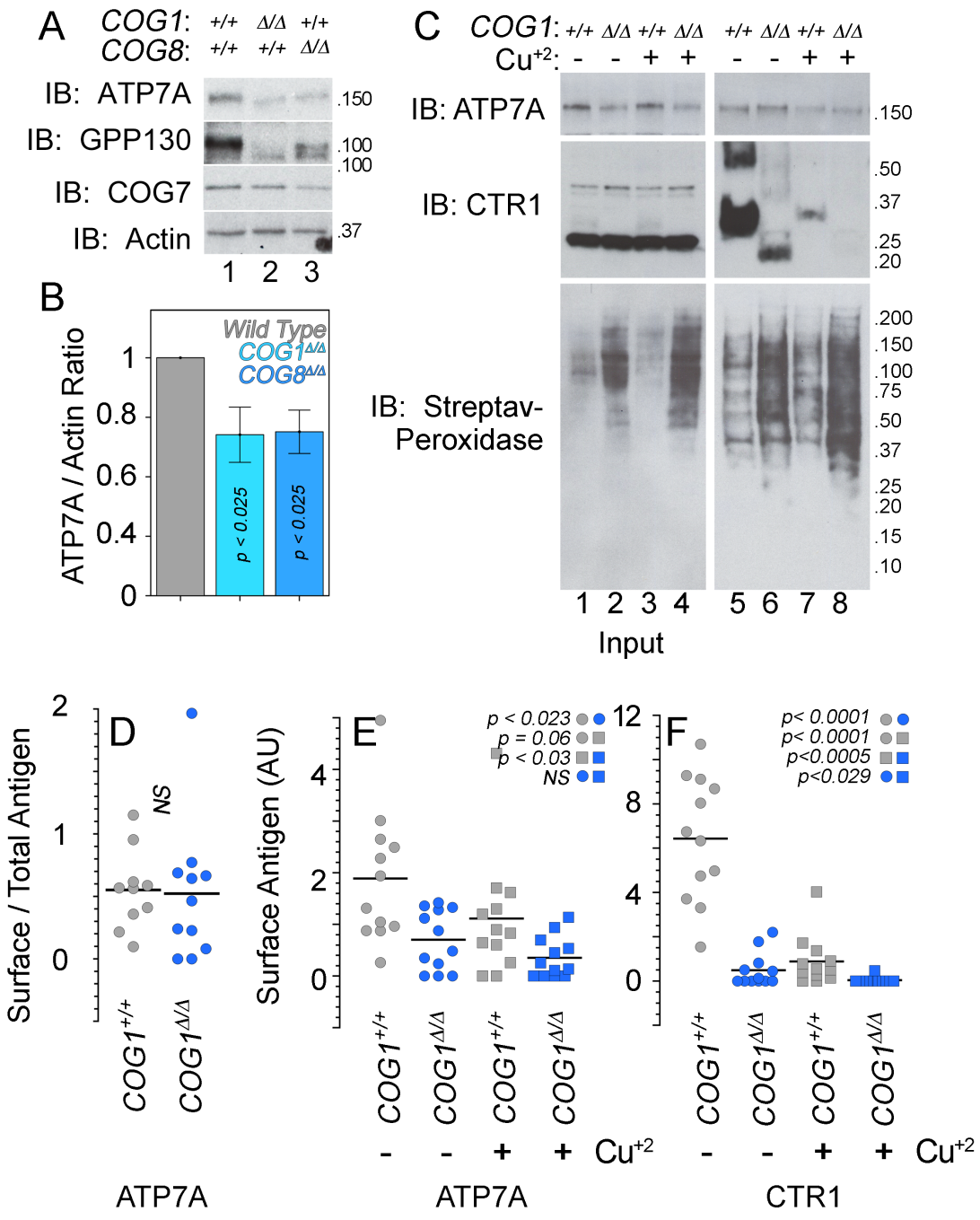

Figure 6 ATP7A stability and surface expression requires the COG complex.

(A and B) Immunoblots for ATP7A, known COG-dependent protein GPP130, COG 7 and actin were performed in wild type HEK293 cells (6A, lane 1) and cells null for COG subunit 1 (6A, lane 2, $COG1^{\Delta/\Delta}$) or COG subunit 8 (6A, lane 3, $COG8^{\Delta/\Delta}$). (C) Surface biotinylation of the same cell types was performed with (6C, even lanes) and without (6C, odd lanes) the addition of 200μM CuCl2 for two hours. Total protein extracts (6C, lanes 1-4) and surface biotinylated proteins precipitated by streptavidin beads (6C, lanes 5-8) were probed for ATP7A and CTR1, along with streptavidin-

peroxidase to compare biotinylation efficiency. (D-F) ATP7A and CTR1 quantitations to measure the ratio of surface to total ATP7A (D), total surface ATP7A with and without copper (E), and total surface CTR1 with and without copper (F). D to F surface signals were corrected by the efficiency of biotinylation that was 1.84 ± 0.7 higher in COG1$^{\Delta/\Delta}$ cells (average ± SEM). Surface levels of ATP7A and CTR1 for each experimental condition were compared using non parametric Kriskal Wallis test followed by pairwise Mann-Whitney U test comparisons, n=7.

We determined whether COG deficiency also affected the surface expression of ATP7A by surface biotinylation. Wild type, COG1$^{\Delta/\Delta}$, and COG8$^{\Delta/\Delta}$ HEK293 cells were incubated at 37°C for two hours in the absence and presence of 200 μM CuCl2, followed by surface biotinylation at 4°C. Biotinylated proteins were precipitated with streptavidin beads and analyzed by immunoblot with antibodies against the copper transporters ATP7A and CTR1 (Fig. 6C-F). The efficiency of cell surface biotinylation was at nearly two-fold higher in COG-null cells as compared to wild type cells (Fig. 6C compare odd and even lanes), thus we normalized all transporter surface expression levels as a ratio of the surface content to the biotinylation efficiency. Normalized surface levels of ATP7A were decreased in COG1$^{\Delta/\Delta}$ HEK293 cells as compared to controls (Fig. 6C, compare lanes 5-6, Fig. 6D). Copper addition to wild type HEK293 cells modestly decreased the normalized surface levels of ATP7A. In contrast, normalized ATP7A surface levels in COG1$^{\Delta/\Delta}$ HEK293 cells did not change after copper (Fig. 6C, compare lanes 5 and 7 and 6 and 8, Fig. 6E). The mobilization of ATP7A from the surface in the presence of excess copper in HEK293 cells is in contrast with the ATP7A response in SH-SY5Y neuroblastoma cells (Fig. 1A). These findings demonstrate that the total and cell surface levels of ATP7A are decreased in cells with genetic defects in the COG complex.

Our findings suggested that either the ATP7A trafficking to and from the plasma membrane in the presence of copper differ between wild type HEK293 and SH-SY5Y cells or, alternatively, HEK293 cells are poorly responsive to a copper challenge. We tested the latter hypothesis by asking if the normalized surface content of plasma membrane copper transporter CTR1 was sensitive to copper addition. CTR1 is the main plasma membrane transporter required for copper influx into cells [285, 286]. We used the well-known copper-induced endocytosis of CTR1 as a tool to assess if HEK293 cells respond to copper addition (Fig. 6C) [287, 288]. The CTR1 antibody recognized monomeric and oligomeric species when CTR1 was enriched in cell surface biotinylated proteins, thus we could not assess CTR1 total cellular expression (Fig. 6C compare inputs 1-2 to lanes 5-8). However, we found pronounced down-regulation of normalized cell surface CTR1 in COG1$^{\Delta/\Delta}$ HEK293 cells in basal conditions (Fig. 6C compare lanes 5 and 6, and Fig. 6F). Addition of copper to wild type and COG1$^{\Delta/\Delta}$ HEK293 cells further exacerbated the depletion of CTR1 at the surface (Fig. 6C compare lanes 5 and 7, lanes 6 and 8, and Fig. 6F), demonstrating that wild type and COG1$^{\Delta/\Delta}$ HEK293 cells are responsive to copper challenge. These results demonstrate that COG complex genetic defects decrease the surface expression of two copper transporters, ATP7A and CTR1, thus suggesting complex copper metabolism phenotypes in COG deficiencies.

COG complex genetic defects decrease cellular copper and modify the expression of ATP7A and other copper-sensitive transcripts.

We addressed whether COG genetic defects impair cellular copper uptake by directly measuring metal content in cells. In addition we determined the secondary effects of copper imbalances by quantifying transcripts whose expression is sensitive to metals and metal pathway dysfunction. We determined total copper and zinc content in wild type, COG1$^{\Delta/\Delta}$, and COG8$^{\Delta/\Delta}$ HEK293 cells with inductively-coupled plasma mass spectrometry. Copper was readily detectable in wild type HEK293 cells. However, copper content in COG-null HEK293 cells was below detection limit (1 ng/sample, Fig. 7A). COG-null cellular copper phenotype was selective since zinc levels remained similar to wild type (Fig. 7B). Addition of the copper-selective chelator BCS brought down copper content in wild type cells while addition of copper chloride or the copper ionophore disulfiram increased cellular copper levels in all three genotypes (Fig. 7A). However, copper chloride uptake was impaired in COG null cells as compared to wild type HEK293 cells even when COG-null cells were exposed to 25 μM copper (Fig. 7A). Only the addition of the copper ionophore disulfiram increased the copper content at or above wild type levels in COG null HEK293 cells (Fig. 7A). These results demonstrate that the decreased expression of ATP7A and CTR1 observed in COG mutant cells results in selective copper deficiency.

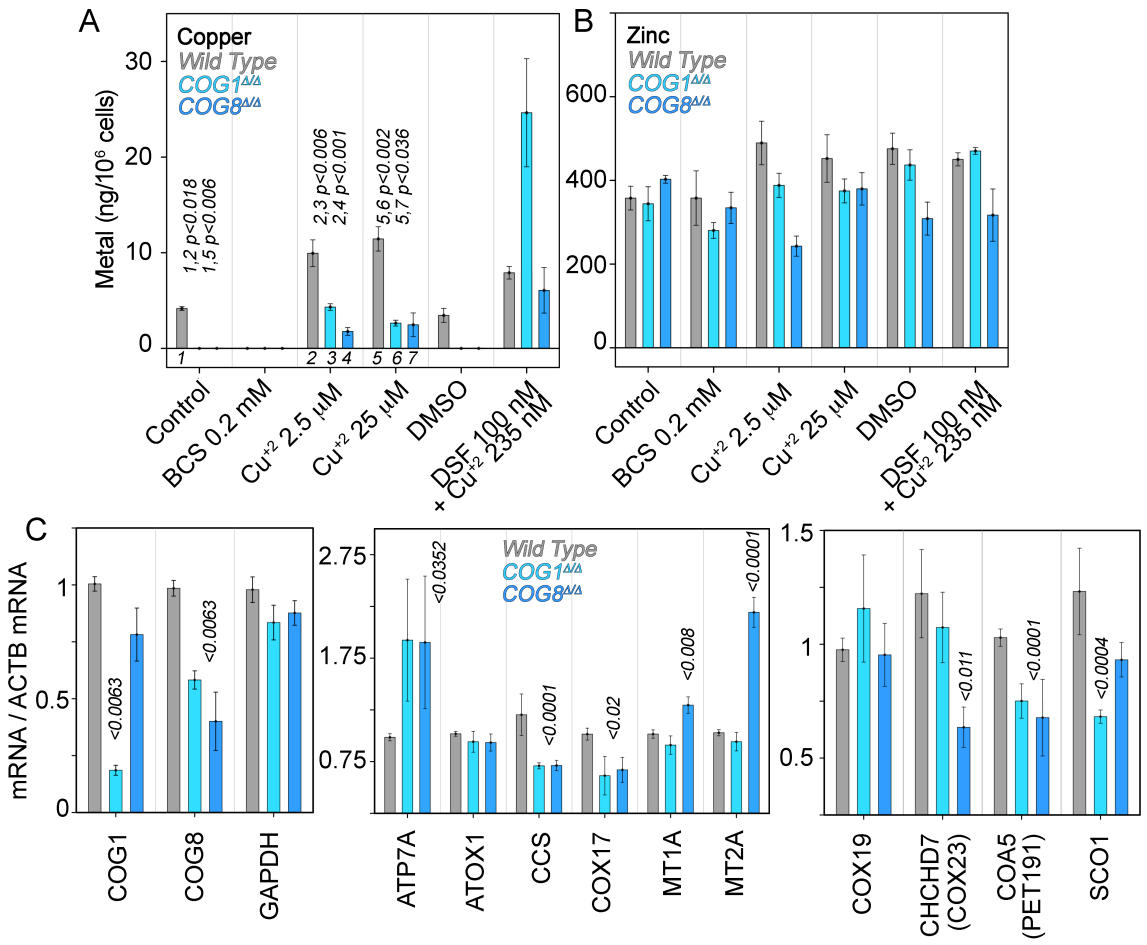

Figure 7 Copper content and expression of copper-sensitive transcripts is altered in COG deficient cells.

Copper (A), zinc (B), and transcript (C) levels from wild type (grey bars), COG1$^{\Delta/\Delta}$ (light blue bars), and COG8$^{\Delta/\Delta}$ (dark blue bars) HEK293 cells were measured either by inductively coupled plasma mass spectrometry (A-B) or quantitative real-time PCR (C). Transcripts were normalized to beta-actin mRNA. Inductively coupled plasma mass spectrometry was performed in two independent biological replicates where each determination was in triplicate. Five independent biological replicates were performed for each determination in triplicate for quantitative real-time PCR. For metal determinations, significant p-values were determined by ANOVA followed by Dunnett test. P values associated to transcript changes were determined by non-parametric Kriskal Wallis test followed by pairwise Mann-Whitney U test comparisons; all unlabeled comparisons are not significant in (C).

Imbalances in cellular copper metabolism modify the expression of transcripts encoding copper transporters and metallochaperones that carry this metal to distinct molecules and subcellular compartments [257]. We previously used copper-sensitive gene expression to document copper metabolism imbalances in mutations of an ATP7A complex interactor, the BLOC-1 complex [269]. We predicted that transcripts of ATP7A and/or metallochaperones should be altered in COG deficient cells if these cells have copper dyshomeostasis. We focused on the metallochaperones ATOX1, which delivers copper to ATP7A [18, 58, 289, 290]; CCS, which carries copper to the mitochondrially localized SOD1 and that itself is imported into mitochondria [291, 292]; COX17, which is required for copper delivery to the mitochondrial cytochrome c oxidase [293]; and two isoforms of metallothioneins, both cysteine rich proteins that bind metals in the cytoplasm [294].

We used a qRT-PCR assay capable of detecting changes in the expression of COG1 and COG8 transcripts in COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ HEK293 cells (Fig. 7C). We normalized all transcript determinations against beta-actin mRNA. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase message showed no differences among cell genotypes when normalized to actin mRNA (Fig. 7C, GAPDH). In stark contrast with the ATP7A protein expression levels, both COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ deficiency doubled ATP7A transcript levels as compared with wild type cells. These changes in ATP7A mRNA contrasted with the expression of the ATP7A metallochaperone, ATOX1, which remained unchanged (Fig. 7C). Transcripts encoding

metallothioneins IA and IIB were increased only in COG8$^{\Delta/\Delta}$ (MT1A and MT2A, Fig. 7C). However, mRNA levels of two metallochaperones that traffic copper to mitochondria, CCS and COX17, were significantly down-regulated in both COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ cells (Fig. 7C). CCS mRNA was reduced to 58% in both COG deficient cells whereas COX17 mRNA was decreased to 64% of the control values. These findings indicate that the expression of mitochondrial copper homeostasis factors is perturbed in COG deficient HEK293 cells. We further tested this hypothesis by measuring mRNA levels of mitochondrially localized co-factors that work in concert with COX17 to load copper into cytochrome c oxidase (COX19, COX23, PET191, and SCO1, Fig. 7C) [293]. Of these factors PET191/COA5 mRNA was significantly decreased to ~70% in both COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ HEK293 cells (Fig. 7C). In contrast, SCO1 and COX23 were selectively down-regulated in COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ cells, respectively (Fig. 7C). Thus, COG complex deficient cells have altered expression of at least four transcripts implicated in the delivery of copper to mitochondrial enzymes. These results suggest that COG complex deficiency cellular copper depletion leads to copper-dependent cellular and mitochondrial metabolism.

COG-Dependent ATP7A and CTR1 Defects Impairs Copper Homeostasis

We assessed the effects of increasing extracellular copper on cellular and mitochondrial metabolism with tetrazolium salts. Tetrazolium is reduced into formazan by NAD(P)H-dependent oxidoreductases and dehydrogenases localized to cytoplasm and mitochondria [295]. We measured 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide reduction (MTT) in HEK293 cells either wild

type, COG1$^{\Delta/\Delta}$, COG8$^{\Delta/\Delta}$, or carrying combined defects in COG1 and COG8 (Fig. 8, COG1,8$^{\Delta/\Delta}$). Cells were exposed to increasing extracellular copper concentrations for 72 hours and MTT reduction was measured. Wild type cells exposed to low copper, 3-20 μM, increased MTT reduction as compared to wild type cells incubated with media alone (Fig. 8A, grey symbols). In contrast, COG1$^{\Delta/\Delta}$, COG8$^{\Delta/\Delta}$, and COG1,8$^{\Delta/\Delta}$ HEK293 cells exposed to low copper failed to increase MTT metabolization (Fig. 8A, blue symbols and Fig. 7A). Irrespective of the cell genotype, all cells experienced a decrease of MTT metabolization at copper concentrations above 50 μM, likely due to copper toxicity (Fig. 8A). These COG- and copper-dependent phenotypes do not reflect general cellular sensitivity to metal-based toxicants as assessed with cisplatin, a cytotoxic agent whose import into HEK293 cells does not require CTR1 copper transporter activity (Fig. 8B) [296]. These results demonstrate that COG deficient cells fail to respond to extracellular copper as predicted from the defects in ATP7A and CTR1 surface transport mechanisms.

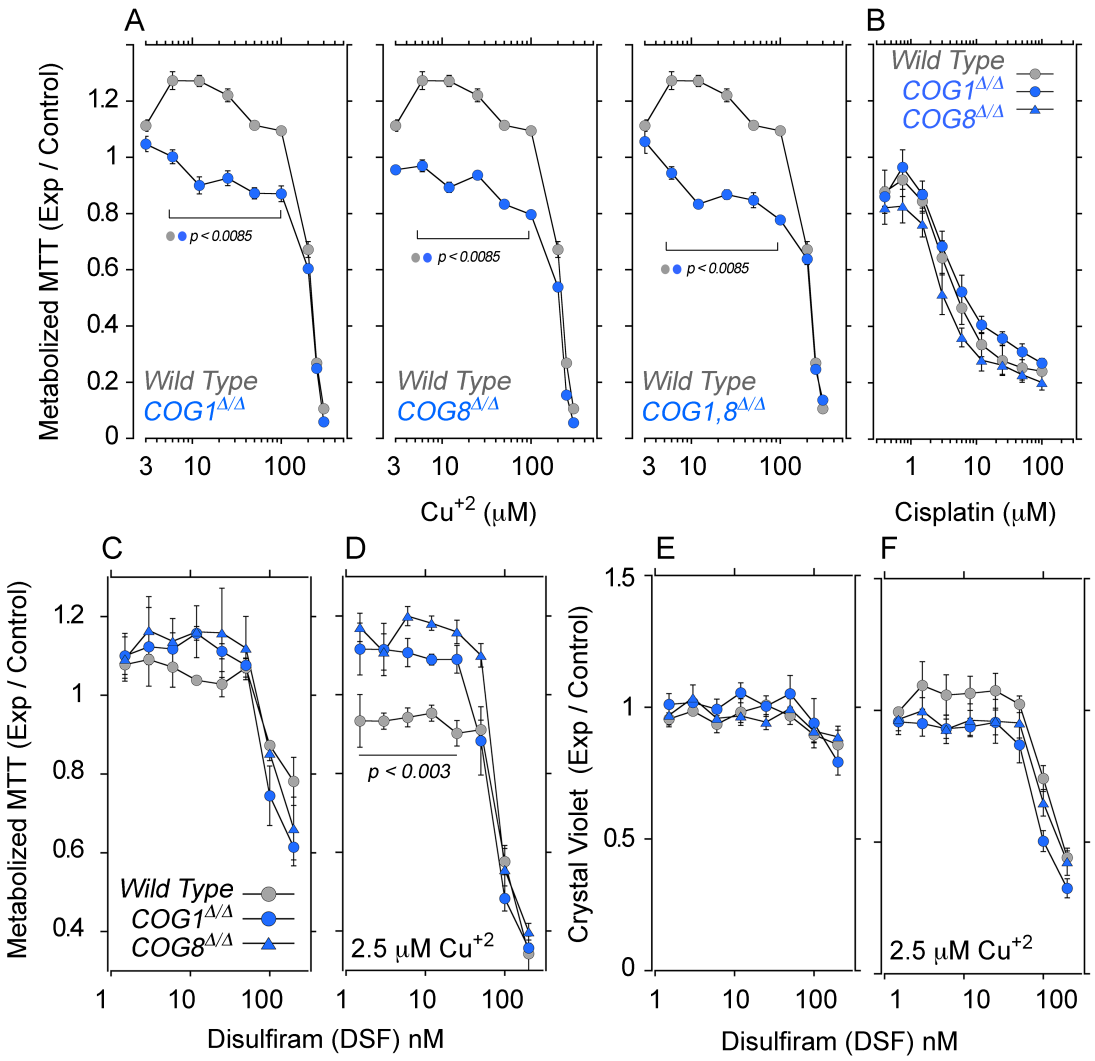

Figure 8 COG null cells possess copper metabolism defects.

(A) Wild type, COG1$^{\Delta/\Delta}$, COG8$^{\Delta/\Delta}$ and COG1,8$^{\Delta/\Delta}$ HEK293 cells were incubated with CuCl2 ranging from 3-300μM for 72 hours. Each condition was carried out in quadruplicate, and the activity of NAD(P)H-dependent oxidoreductases was measured by the reduction of MTT. Each dot represents the average absorbance at 595nm ± SEM, normalized to a baseline reading (n=5). One Way ANOVA followed by Bonferroni's All Pairs Comparisons (B) Wild type and COG null cells were incubated with 0.4-100μM cisplatin for 72 hours and MTT reduction was measured as above (n=2). (C-D) The copper ionophore disulfiram (DSF) was added to wild type, COG1Δ/Δ , and COG8Δ/Δ HEK293 cells for 24 hours either in concentrations ranging from 1.5-200nM either in the absence (C) or presence (D) of 2.5μM CuCl2; each condition was carried out in quadruplicate. Reduction of MTT by NAD(P)H-dependent oxidoreductases was measured and normalized to a baseline reading with no drug added. Each dot represents the average of five independent biological replicates ± SEM. Non-parametric Kriskal Wallis test followed by pairwise Mann-Whitney U test comparisons. (E-F) Crystal violet staining was performed in parallel to MTT analysis to measure changes in cell number.

We hypothesized that if decreased cellular copper and normalized surface levels of ATP7A and CTR1 in COG deficient HEK293 cells (Fig. 6) prevent MTT metabolization in cytoplasmic or mitochondrial compartments, then direct copper delivery across the plasma membrane via a copper ionophore should revert MTT phenotypes (Fig. 9 and 7A). Disulfiram is a cell permeant copper chelation agent that inhibits copper dependent enzymes in diverse compartments including mitochondria [86, 297-299]. However, disulfiram complexed with copper increases metal cellular levels [300, 301] (Fig. 7A), and rescues respiration phenotypes in CTR1 null cells [302]. We incubated wild type and COG deficient HEK293 cells with disulfiram in the absence (Fig. 9A and C) or presence of copper (Fig. 9B and D). Cells were incubated for 24 hours to minimize the effect of modifications in cell numbers on MTT activity readings. We controlled for cell numbers with a crystal violet colorimetric assay [303] (Fig. 9C-D). Addition of increasing disulfiram to wild type and COG-null HEK293 cells did not affect MTT activity at low concentrations, yet disulfiram above 25 nM decreased MTT activity (Fig. 9A). None of these disulfiram concentrations significantly affected cell numbers (Fig. 9C) indicating that MTT metabolization was impaired by the copper chelation activity of disulfiram at high doses. Next, we added increasing disulfiram concentrations to wild type and COG null HEK293 cells in the presence of 2.5 μM of copper (Fig. 9B and D). Disulfiram concentrations above 25nM in the presence of added copper decreased MTT activity and cell numbers irrespective of the cell genotype (Fig. 9B and D). Thus, we focused on disulfiram and

copper conditions that did not compromise cell numbers (Fig. 9D). Loading cells with copper using low concentrations of disulfiram (< 25nM) significantly increased MTT metabolization in COG1$^{\Delta/\Delta}$ and COG8$^{\Delta/\Delta}$ HEK293 cells as compared to wild type controls (Fig. 9B, compare gray and blue symbols). These results show that delivering copper with a copper ionophore to bypass copper transporter plasma membrane defects increases the metabolization of MTT in COG null cells.

Genetic Interactions between ATP7A and COG complex subunits in *Drosophila melanogaster*.

COG null HEK293 cells have copper-dependent cellular and metabolic phenotypes that can be rescued with a copper-loaded ionophore, disulfiram. We focused on ATP7A overexpression because it cell-autonomously decreases cellular levels of copper due to ATP7A mistargeting to the cell surface [304, 305]. Thus, we hypothesized that phenotypes induced by genetically increasing ATP7A expression in neurons should be modulated by loss-of-function mutations in the COG complex, which decreases ATP7A expression (Fig. 6A-B).

We first tested whether Drosophila ATP7A and COG complex subunits genetically interact to specify synapse morphology in the developing neuromuscular junction of the third instar larva (Fig. 9). We overexpressed ATP7A in Drosophila neurons using the pan-neuronal elav GAL4 c155 driver (C155) [306]. Overexpression of ATP7A reduced the cumulative synapse branch length; thus, inducing a collapse of the

synapse as measured as an increased synaptic bouton density (Fig. 9A image C155>UAS-ATP7A, Fig. 9B-C, column 4. Compare 4 to control columns 1 and 2). In contrast, animals carrying one copy of the null allele cog1e02840 increase cumulative synapse branch length while maintaining wild type synaptic bouton density (Fig. 9A-C, column 3). As predicted by our hypothesis, overexpression of ATP7A in cog1e02840 flies restored synaptic bouton density to wild type levels (Fig. 9A and B, compare columns 4 and 5). These results demonstrate that a component of the ATP7A interactome, the COG complex, genetically interact with ATP7A to specify a neurodevelopmental synapse phenotype.

Second, we examined whether ATP7A and COG complex subunits genetically interact to specify neurodegeneration in the Drosophila adult nervous system (Fig. 10). We controlled the expression of ATP7A in adult dopaminergic neurons, a group of cells frequently used to model Parkinson's disease in Drosophila [307-311]. We drove the expression of UAS-ATP7A selectively in dopaminergic and serotoninergic neurons with the dopa decarboxylase (Ddc)-GAL4 driver [307]. We reasoned that overexpression of ATP7A, which decreases cellular levels of copper[304, 305], should reduce the toxicity to copper diet exposure. We previously observed a high sensitivity to copper in the diet of wild type animals [269]. Copper feeding progressively increased mortality in wild type male (Fig. 10A) and female adults (Fig. 10B) over a period of three days. Overexpression of ATP7A in adult dopaminergic neurons was sufficient to significantly protect males and female adult animals from the toxic effect of copper feed at 48 hours (Fig. 10A-B, (Ddc>UAS-

ATP7A)). Similarly, mutation of the COG complex subunit cog1 protected animals from copper diet induced death (Fig. 10A-B, (Ddc x cog1e02840)). In contrast, the mortality phenotype observed in animals overexpressing ATP7A was restored to the levels of wild type lethality by adding in trans a genetic defect in cog1 (Fig. 10A-B, (UAS-ATP7A; Ddc x cog1e02840)). Importantly, mortality was negligible when copper was omitted from the diet fed to animals of any genotype (Fig. 10C-D). Our experiments demonstrate that the COG complex and ATP7A genetically interact in adult dopaminergic neurons to specify copper-dependent mortality.

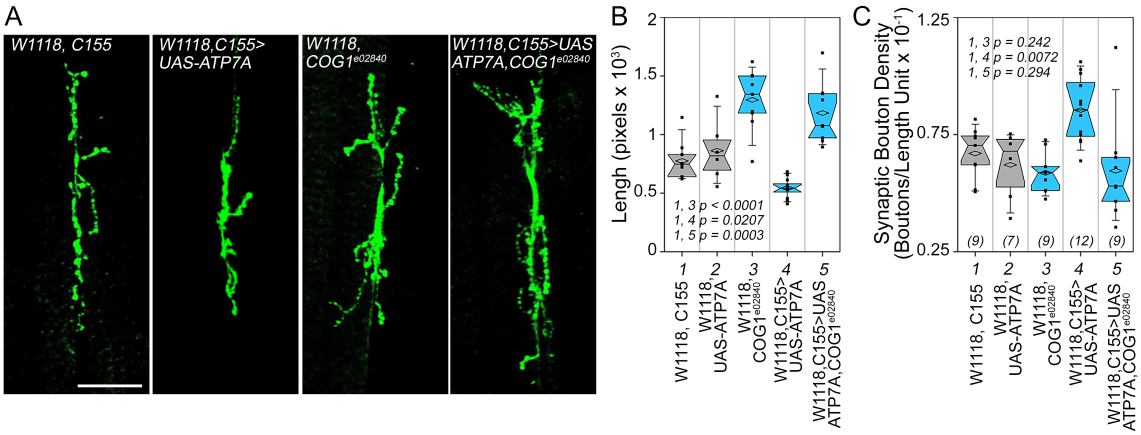

Figure 9. Drosophila ATP7A and COG1 genetically interact to specify *Drosophila melanogaster* synapse development.

Third instar larvae neuromuscular junction synapses were stained with anti HRP antibodies (A) imaged and their morphology assessed using as parameters branch length (B) and bouton density (C). Scoring was done blind to the animal genotype. Control animals (C155 outcross, column 1; or UAS-ATP7A outcross, column 2), animals carrying one copy of the null allele cog1e02840 (cog1 e02840 outcrossed, column 3), flies overexpressing ATP7A in neuronal cells (c155>UAS-ATP7A; column 4), and animals overexpressing ATP7A and mutant for cog1 (C155> UAS-ATP7A x cog1e02840, column 5) were analyzed. Numbers in parentheses and italics in C depict the number of animals. Statistical comparisons were performed with One Way ANOVA followed by Fisher's Least Significant Difference Comparison. Box plots depict percentiles 5th and 95th. Box line represents sample median and diamonds sample mean and notches mark the half-width.

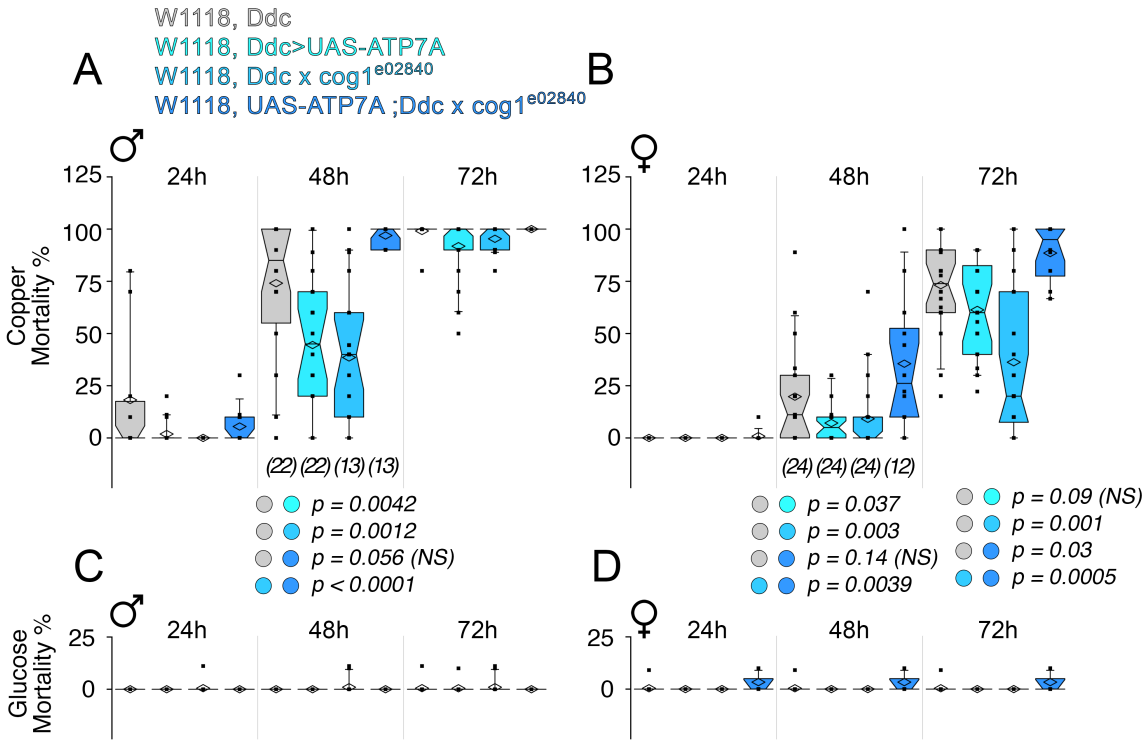

Figure 10. Drosophila ATP7A and COG1 genetically interact in dopaminergic neurons to specify copper-induced *Drosophila melanogaster* viability.

Control animals (Ddc outcross), animals carrying one copy of the null allele cog1e02840 (cog1 e02840 outcrossed), flies overexpressing ATP7A in dopaminergic neuronal cells (Ddc>UAS-ATP7A), and animals overexpressing ATP7A and mutant for cog1 (UAS-ATP7A; Ddc x cog1e02840) were fed a glucose diet (C-D) or a glucose diet supplemented with 1mM CuCl2 for three consecutive days. Numbers in parentheses and italics depict the number of independent experiments each one performed with at least 10 animals per genotype. Statistical comparisons were performed with Non-parametric Kriskal Wallis test followed by pairwise Mann-Whitney U test comparisons. Box plots depict percentiles 5th and 95th. Box line represents sample median and diamonds sample mean and notches mark the half-width.

DISCUSSION

We isolated and defined the ATP7A interactome to identify novel metal homeostasis mechanisms capable of modulating the expression of neurological traits. We selected candidate gene products from the ATP7A interactome based on three criteria; they strongly coenriched with ATP7A, were present in Golgi and post-Golgi compartments where ATP7A is present at steady state or traffics through after a

copper challenge, and phenocopied some of the chief neurological phenotypes in Menkes disease. One such candidate that met all of these criteria, the COG complex, is associated with severe neuropathologies when mutated in humans and as we demonstrate have a severe depletion of cellular copper [239-243, 245-251, 278]. Here, we demonstrate that the COG complex is a hub where two copper transporters, ATP7A and CTR1, converge to regulate cellular copper content, homeostasis, synapse development and copper-induced neurodegeneration.

We compared the ATP7A interactome from cells treated in the presence of either excess copper or the copper chelator BCS (Figure 1). We reasoned that copper-dependent translocation of ATP7A from Golgi to post-Golgi compartments would reveal putative compartment-specific ATP7A interactors. However, we found that the most enriched proteins were associated with ATP7A regardless of whether cells were challenged with excess copper or not. It may be possible that we did not identify proteins that selectively associate with ATP7A in a copper-dependent manner because of our purification strategy. ATP7A peptides identified by mass spectrometry from the interactome were predominately sequences enriched at the C-terminal domain of ATP7A, which could either be a reflection of the tertiary structure of the protein (Figure 2) or an indication that copper-induced protein associations with ATP7A mask the N-terminal domain from recognition by the antibody used as a bait. We favor the latter hypothesis because the antibody used to isolate the interactome is directed to the N-terminal domain, the same domain where the copper binding sites reside in ATP7A and a domain necessary for ATP7A

interaction with ATOX1, the copper chaperone that shuttles copper to ATP7A [18, 58, 289, 290]. Consistent with this hypothesis, we did not detect ATOX1 in the ATP7A interactome although it is a robustly documented ATP7A interactor. However, our ATP7A antibody bait allowed the identification of both known and novel interacting proteins. ATP7A possesses 664 predicted and 13 experimentally tested interactors according to a comprehensive computational interactome of the human genome [312]. Our experimentally generated ATP7A interactome includes 541 proteins, of which only 21 proteins are in common with a computational ATP7A interactome. Therefore, the majority of the ATP7A interactome components identified here consisted of uncharacterized relationships. We identified several known trafficking proteins and complexes present at the plasma membrane, endosome, or Golgi complex that are known to regulate the trafficking of ATP7A, including the WASH complex and components of clathrin coated vesicles, as well as novel factors such as VAC14 and the COG complex (Figure 2). We independently confirmed that these trafficking components co-isolated with ATP7A (Figure 5). Strikingly, these trafficking proteins and complexes also associate with diverse neuropathologies, suggesting that they may share common mechanisms with the neuropathology observed in Menkes disease (Figures 3-4) [235, 237, 313].

One prominent finding was the strong co-enrichment of COG complex subunits with ATP7A. We predicted that if the COG complex were responsible for trafficking of ATP7A, deletion of the complex would alter cellular copper content, subcellular localization and levels of copper transporters, the expression of copper-sensitive

molecules, and cellular susceptibility to copper challenges. We found that in COG$^{\Delta/\Delta}$ cells, the total cellular and normalized surface levels of ATP7A are decreased (Figure 6), a molecular defect leading to selective copper depletion in COG null cells (Fig. 7A). While the non-normalized surface levels of ATP7A measured by immunoblot were comparable in wild type and COG $^{\Delta/\Delta}$ cells at steady state, we normalized by the biotinylation efficiency, which was greater in the COG null cells. We attribute this increased biotinylation efficiency to a reduction in the surface glycocalyx negative charge in COG null cells, which would increase surface biotinylation efficiency with the anionic sulfo-NHS-biotin reagent [314]. When observing normalized surface levels of ATP7A after a copper challenge, we were surprised to find that, unlike in previously observed cell types including Caco-2, CHO cells, and human fibroblasts, ATP7A did not increase at the surface of wild type HEK cells [189, 315]. We were unable to assess if this was due to an accelerated rate of endocytosis of ATP7A after copper addition, or a small pool of ATP7A at the Golgi unable to sustain increased ATP7A surface expression. However, we were able to document copper dependent membrane traffic of CTR1 in the same cells [285-288], thus excluding unresponsiveness to copper in HEK293 cells. We also assessed the total and normalized surface levels of CTR1, a copper importer that is known to internalize in the presence of excess copper. CTR1 normalized surface levels were dramatically decreased in COG $^{\Delta/\Delta}$ cells in resting conditions, and this deficiency was exacerbated by the addition of copper, leaving no measurable CTR1 protein on the surface. This led us to hypothesize that COG deletion is perturbing not only copper

regulation by ATP7A, but copper homeostasis in other cellular compartments that require the entry of copper from the plasma membrane via CTR1.

We decided to use copper-sensitive mitochondrial function to determine whether the effects of COG deletion mutations affecting ATP7A and CTR1 surface expression extended beyond ATP7A-dependent Golgi trafficked enzymes, such as DBH, LOX and PAM [149, 186]. Cytochrome c oxidase, though not directly downstream of ATP7A, requires copper as a cofactor, and thus mitochondrial function is impaired by decreased activity of cytochrome c oxidase when copper delivery to mitochondria is impaired [49, 316]. Our hypothesis that copper homeostasis is impaired in $COG^{\Delta/\Delta}$ cells is supported by reports describing that RNAi or mutation of COG subunits alters copper content in mammalian cells and yeast, respectively [302, 317] and by our data that levels of copper and copper sensitive transcripts encoding proteins present in or transferring copper to different subcellular compartments are altered in COG null cells (Figure 7). Copper regulatory genes are known to undergo coordinated regulation in response to changing copper levels, and we found that multiple copper sensitive transcripts are decreased in $COG^{\Delta/\Delta}$ cells [318]. Of particular interest are two metallochaperones that deliver copper to mitochondria, CCS and COX17, whose message levels are reduced in COG null cells [257, 293]. We found that mitochondrial function is impaired in COG null cells due to defects in copper cellular homeostasis. These data suggest the existence of a mechanistic link between a Golgi-dependent metal buffering and mitochondrial-dependent copper homeostasis. Several lines of evidence further support a model of a

multicompartment regulation of copper homeostasis. Genetic evidence indicates that the expression of CTR1 is modulated by the mitochondrial metallochaperone SCO1 [53], down-regulation of mitochondrial components including complex I subunits (NDUFB4, NDUFA1) and the mitochondrial transporter SLC25A41 lead to global cellular changes in copper [317], and the elimination of ATP7A rescues mitochondrial metallochaperone mutant phenotypes [52, 317].

Along with the neurodegeneration characteristic of Menkes disease, disruptions in copper homeostasis have been implicated in several other prevalent neurodegenerative diseases, including Parkinson's and Alzheimer's disease [319, 320]. There is evidence that therapeutic modulation of copper levels may be effective in alleviating symptoms of or delaying the onset of Parkinson's and Alzheimer's disease, yet there are gaps in our understanding of the regulation of copper homeostasis, particularly in the brain [126, 321, 322]. Polymorphisms in genes encoding the COG complex subunits COG4, 6, and 8 associate with neurodegenerative and neurobehavioral phenotypes in humans [323-326]. Our bioinformatic studies also indicate that the COG2 subunit is one of 42 gene products present in the ATP7A interactome associated with tauopathies and Alzheimer's disease; one of fourteen genes products associated with Parkinson's disease; and one of the 49 gene products in the ATP7A interactome associated with neurocognitive disorders, such as dementia (Fig. 3). Deletions of COG complex subunits lead to type II congenital disorders of glycosylation (CDG II) and are strongly associated with cerebral atrophy, developmental delay, hypotonia, ataxia

and epilepsy [239-243, 245-251, 278]. Since COG mutations drastically decreased the cellular copper levels (Fig.7A Since COG mutations drastically decreased the cellular copper levels (Fig.7A), it is possible that COG neurological phenotypes reflect in part decreased neuronal copper much like is the case in Menkes disease. Mutations in cog1 prevent toxicity of dietary copper in Drosophila (Fig. 10) supporting this concept of decrease copper content in COG deficient neurons. Similarities between Menkes and congenital disorders of glycosylation are suggested by neurological phenotypic overlap between these disorders. Common phenotypes include developmental delay, seizures, hypotonia and cerebral atrophy [98, 148, 149, 151, 154, 258]. We genetically tested whether phenotypes caused by ATP7A dosage increase could be modulated by genetic defects in COG using the Drosophila developing neuromuscular synapse and adult dopaminergic neuron (Figs. 9-10). Phenotypes due to overexpression of ATP7A in developing synapses and adult neurons could be reverted to wild type by adding a mutation in cog1. Inspired by the effects of COG deficiency in human ATP7A (Fig. 6A-B), we interpret this Drosophila rescue results as a reduction in the levels of overexpressed ATP7A in fly neurons due to a ATP7A down-regulation effect of the cog1 mutant allele. This interpretation assumes cell autonomous effects of the cog1 mutation on neuronal overexpressed ATP7A and it does not consider possible non-cell autonomous contributions of the cog1 genomic defect. Our findings suggest that part of the neurological phenotypes in COG genetic defects may be due to impaired copper content and metabolism as we report here.

While we initially focused on the role of the COG complex in ATP7A trafficking, it is clear that copper related phenotypes downstream of COG extend beyond cuproenzymes that traverse the Golgi complex. Our strategy to identify an ATP7A interactome has revealed novel ATP7A interacting partners and may be a fruitful way to further explore networks related to other copper regulatory proteins. Our finding that the COG complex, a Golgi localized tether is implicated in mitochondrial copper homeostasis indicates that essential mineral homeostasis, such as copper, results from coordinated multicompartment metabolite sensing and response mechanisms.

MATERIAL AND METHODS

Cell culture

SH-SY5Y (ATCC) and HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml penicillin and streptomycin (Hyclone) at 37°C in 10% CO_2. HEK293T cells deficient for COG subunits were generated as described in [279]. Two lines of Menkes deficient fibroblasts were used: one in conjunction with a rescue line expressing recombinant ATP7A (described in [267]) and the other in conjunction with a familial control (Coriell GM01981 and GM01983). The former were cultured in DMEM supplemented with 10% FBS and 100 µg/ml penicillin and streptomycin at 37°C in 5% CO_2, while the latter were cultured in minimum essential media (MEM) (Thermo Fisher 11095080) supplemented with 15% FBS and 100 µg/ml penicillin and streptomycin at 37°C in 5% CO_2. Antibodies and primers can be found in supplementary table 1.

Immunoprecipitation of ATP7A

To assess interactions of ATP7A in the presence and absence of copper, we performed cross-linking in intact cells with dithiobis(succinimidylpropionate) (DSP) followed by immunoprecipitation as previously described but with the following modifications [269]. Briefly, SH-SY5Y neuroblastoma cells or $ATP7A^{-/-}$ and $ATP7A^{R/R}$ human fibroblasts were washed with ice cold PBS with $MgCl_2$ and $CaCl_2$ (PBS/Mg/Ca). 200 µM Copper chloride or 400 µM BCS diluted in PBS/Mg/Ca buffer was then added to the respective plates and they were placed back in the 37°C

incubator for 2 hours. The plates were then placed on ice, rinsed twice with PBS/Mg/Ca, and incubated with 10 mM DSP (Thermo Scientific 22585), diluted in PBS for 2 h on ice. Tris, pH 7.4, was added to the cells for 15 min to quench the DSP reaction. The cells were then rinsed twice with PBS and lysed in buffer A (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM $MgCl_2$, pH 7.4) with 0.5% Triton X-100 and Complete anti-protease (catalog #11245200, Roche), followed by incubation for 30 min on ice. Cells were scraped from the dish, and cell homogenates were centrifuged at 16,100 × g for 10 min. The clarified supernatant was recovered, and at least 500 µg of protein extract was applied to 30 µl Dynal magnetic beads (catalog #110.31, Invitrogen) coated with 5 µl ATP7A antibody (NeuroMab Cat No. 75-142), and incubated for 2 h at 4°C. In some cases, immunoprecipitations were done in the presence of the antigenic ATP7A peptide as a control. The peptide (sequence VSLEEKNATIIYDPKLQTPK, custom made by Biosynthesis) was prepared in 10 mM MOPS and used at 22 µM The beads were then washed 4–6 times with buffer A with 0.1% Triton X-100. Proteins were eluted from the beads with sample buffer. Samples were resolved by SDS-PAGE and contents analyzed by immunoblot or silver stain. In the case of the large-scale proteomic analysis, proteins eluted from the beads were combined and concentrated by TCA precipitation.

Mass spectrometry

Sample Digestion: The IP beads were spun down and residual buffer was removed. Digestion buffer (200 ul of 50 mM NH4HCO3) was added and the bead

solution was then treated with 1 mM dithiothreitol (DTT) at 25°C for 30 minutes, followed by 5 mM iodoacetimide (IAA) at 25°C for 30 minutes in the dark. Proteins were digested with 1ug of lysyl endopeptidase (Wako) at room temperature for 2 hours and further digested overnight with 1:50 (w/w) trypsin (Promega) at room temperature. Resulting peptides were desalted with a Sep-Pak C18 column (Waters) and dried under vacuum.

LC-MS/MS analysis: The dried peptides were resuspended in 10 uL of loading buffer (0.1% formic acid, 0.03% trifluoroacetic acid, 1% acetonitrile). Peptide mixtures (2 uL) were separated on a self-packed C18 (1.9 um Dr. Maisch, Germany) fused silica column (25 cm x 75 uM internal diameter (ID); New Objective, Woburn, MA) by a Dionex Ultimate 3000 RSLCNano and monitored on a Fusion mass spectrometer (ThermoFisher Scientific , San Jose, CA). Elution was performed over a 120 minute gradient at a rate of 350nl/min with buffer B ranging from 3% to 80% (buffer A: 0.1% formic acid in water, buffer B: 0.1 % formic in acetonitrile). The mass spectrometer cycle was programmed to collect at the top speed for 3 second cycles. The MS scans (400-1600 m/z range, 200,000 AGC, 50 ms maximum ion time) were collected at a resolution of 120,000 at m/z 200 in profile mode and the HCD MS/MS spectra (0.7 m/z isolation width, 30% collision energy, 10,000 AGC target, 35 ms maximum ion time) were detected in the ion trap. Dynamic exclusion was set to exclude previous sequenced precursor ions for 20 seconds within a 10 ppm window. Precursor ions with +1, and +8 or higher charge states were excluded from sequencing.

Database search: Spectra were searched using Proteome Discoverer 2.0 against human Uniprot database (90,300 target sequences). Searching parameters included fully tryptic restriction and a parent ion mass tolerance (± 20 ppm). Methionine oxidation (+15.99492 Da), asaparagine and glutamine deamidation (+0.98402 Da) and protein N-terminal acetylation (+42.03670) were variable modifications (up to 3 allowed per peptide); cysteine was assigned a fixed carbamidomethyl modification (+57.021465 Da). Percolator was used to filter the peptide spectrum matches to a false discovery rate of 1%. Peptide spectral match (PSM) counts were used as the semi-quantitative measure.

Inductively-coupled plasma mass spectrometry

Cells were washed three times in cold phosphate based saline and detached by gentle squirting. Cell pellets were heated to 55°C for 3 hours with concentrated nitric acid then diluted to 10 mL with buffer. Copper and zinc were analyzed using inductively-coupled plasma mass spectrometry. Samples were quantified with an 8-point calibration using indium as an internal standard. The limits of detection were 1 ng/sample for copper and 0.1 ng/sample for zinc.

Bioinformatic analysis

Gene list to disease associations were performed with GDA algorithm (http://gda.cs.tufts.edu[277]. We performed gene ontology analysis with ENRICH (http://amp.pharm.mssm.edu/Enrichr/) [275], and Database Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov) [273,

274]. Cytoscape with Enrichment Map plugin for visualizing DAVID outputs was used in order to depict integrations between GO terms associated with the ATP7A interactome as described [327, 328]. Charts were narrowed down in order to simplify Cytoscape representations by eliminating broad GO terms.

MTT Assay

Cells were collected and seeded in a 96-wells plate at a density of 10×10^3 cells/well in DMEM + 10% FBS +100 U/ml penicillin, 100 µg/ml streptomycin and incubated overnight. On day 2, cells were treated with serial dilutions of the appropriate drug (0.4-100 µM cisplatin, 3-300 µM copper chloride, or 1.5-200nM disulfiram). After incubation at 37°C for either 24 or 72 hours depending on the experiment, 20µl 5 mg/ml (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) (Life Technologies M6496) was added to each well, and the plates were incubated for an additional 3.5 h at 37°C. MTT was aspirated, 150 µl DMSO was added and cells were agitated on an orbital shaker for 15 min. The absorbance was read at 595 nm using a microplate reader. Each condition was carried out in quadruplicate, and MTT absorbance was expressed as percentage absorbance of untreated cells.

Crystal Violet Staining

Cells were plated and treated with the appropriate drug as described for MTT experiments above. After incubation with drug, cells were washed once with PBS-Ca-Mg. Cells were then fixed for five minutes in 65% MeOH, followed by a fifteen minute fixation with 100% MeOH. The methanol was removed and wells allowed to

dry completely, after which 100µl 0.1% w/v crystal violet in H_2O was added to each well for five minutes. Plate was washed 3-5 times with H_2O, and crystal violet was solubilized with 2% sodium deoxycholate for 10 minutes. The absorbance was read at 595 nm using a microplate reader. Each condition was carried out in quadruplicate, and crystal violet absorbance was expressed as percentage absorbance of untreated cells [303].

Surface labeling and streptavidin pulldowns

Plates of ~75% confluent HEK cells were incubated for 2 hours at 37°C in the absence or presence of 200 µM copper chloride and then moved to an ice bath. Plates were then washed two times with ice-cold PBS-Ca-Mg (0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ in PBS). All solutions used were ice-cold when applied to cells, and cells were maintained on an ice bath throughout. A biotin labeling solution of 0.5mg/ml Sulfo-NHS-Biotin (Thermo Scientific 21217) in PBS-Ca-Mg was applied for 15 min, aspirated, and fresh labeling solution was applied for an additional 15 minutes. The labeling solution was then aspirated and cells were washed three times with 1mg/mL glycine in PBS-Ca-Mg followed by one wash with plain PBS. Cells were collected from plates and incubated for 30 min in buffer A with 0.5% Triton X-100, supplemented with Complete antiprotease. Lysates were spun at 16,100 × g for 15 min. The supernatant was recovered and diluted to 1 mg/ml. A small volume (50 µl of NeutrAvidin-coated agarose bead slurry (Thermo Scientific 29200) was prewashed two times in buffer A with 0.1% Triton X-100, and 500 µg of cell lysate was incubated with the beads for 2 h with end-over-end rotation at 4°C. Beads were

then washed five times in buffer A with 0.1% Triton X-100 for 5 min with end-over-end rotation at 4°C. Proteins were eluted from the beads by boiling in Laemmli sample buffer at 75°C for 5 min, and samples were analyzed by SDS–PAGE followed by immunoblot.

qRT-PCR

Quantiative rt-PCR was performed as described in [269, 329]. RNA from HEK293T cells was TRIzol-extracted (Invitrogen), and isolated RNA was reverse transcribed into cDNA using SuperScript III first strand synthesis (Invitrogen). PCR amplifications were performed on a LightCycler480 real time plate reader using LightCycler 480 SYBR Green reagents (Roche).

Drosophila Strains and Procedures

The following strains where obtained from the Bloomington Drosophila Stock Center, Bloomington, Illinois: w[1118].

w[1118]; PBac{w[+mC]=RB}CG4848[e02840]/TM6B, Tb[1]; w[1118]; w[1118]; P{w[+mC]=Ddc-GAL4.L}Lmpt[4.36] and C155 GAL4 animals were from Blominton (P{GawB}elavC155, Fly Base ID FBti0002575). w[1118]; UAS-ATP7-wt was a gift of Richard Burke, Monash University, Australia.

Flies were reared on standard Molasses Food (Genesee Scientific) at 25C in 12 hr:12hr light:dark cycle. Copper feeding toxicity experiments were performed as

described using 5% glucose as control feed or 5% glucose supplemented with 1 mM CuCl2[269].

Larval dissections, immunohistochemistry, and confocal microscopy were performed as described previously [268, 330, 331] . Wandering third-instar female larvae were dissected in normal HL3, fixed in 4% paraformaldehyde for 1 h, and stained with HRP–FITC conjugated antibody for 2 h at room temperature (1:500). An inverted 510 Zeiss LSM microscope was used for confocal imaging of synapses at muscle 6/7 on either the second or third segment. Bouton counts and branch length were calculated using FIJI [332] with experimenters blind to the genotype of the animal.

Statistical analysis

Experimental conditions were compared using Synergy Kaleida-Graph, version 4.1.3 (Reading, PA) or Aabel NG2 v5 x64 by Gigawiz as specified in each figure.

Acknowledgements

This work was supported by grants from the National Institutes of Health: NS088503 to VF, DK093386 to MP, GM083144 to VL, and the HERCULES grant (NIEHS: P30 ES019776). We are indebted to the Faundez lab members for their comments. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) were used in this study. SR-R was supported by a fellowship from the Marion T. Clark Research Fund & William Joe Frierson Research Fund. We would like to thank Maria Olga Gonzalez Gonzalez for support during the manuscript figure making.

Supplementary Table 1

Antibody	Catalog Number	Source	WB Dilution
Monoclonal Anti ATP7A	75-142	Neuromab UC Davis	1:500
Monoclonal Anti TrfR	13-6800	Zymed	1:2000
Monoclonal Anti Hsp90	610418	BD Bioscience	1:1000
Monoclonal Anti β Actin	A5451	Sigma	1:500
Polyclonal Anti DBH	AB1536	Millipore	1:500
Monoclonal Anti Clathrin heavy chain (23)	610499	BD Transduction Lab.	1:1000
Polyclonal Anti COG5	HPA020300	Sigma	1:1000
Polyclonal Anti COG7	N/A	Lupashin laboratory	1:1000
Polyclonal Anti Strumpellin C-14	sc87442	Santa Cruz	1:500
Polyclonal Anti GIGYF2	A303-731A	Bethyl	1:2000
Polyclonal Anti VAC14		Weissman laboratory	1:2000
Polyclonal Anti NF-kappaB1	A301-820A-T	Bethyl	1:1000
Polyclonal Anti NSF	D31C7	Cell Signaling	1:500
Polyclonal Anti DOCK7	1300-1	PTG	1:1000
Monoclonal Anti PRDX1 (3G5)	LF-MA0214	Biovendor	1:3000
Monoclonal Anti PRDX2 (1E8)	LF-MA0144	Biovendor	1:2000
Monoclonal Anti PRDX4	ab16943	Abcam	1:2000
Monoclonal Anti PRDX6	ab16947	Abcam	1:1000
Polyclonal Anti Thioredoxin 1	SC20146	Santa Cruz	1:1000
Monoclonal Anti UCHL1	N/A	Li Lab	1:28000
Polyclonal Anti GPP130	PRB-144C	Biolegend	1:1000
Polyclonal Anti SLC31a1 (CTR1)	MABS398	Millipore	1:1000

Gene	FWD	REV
COG1	AAGCCAGACTCCAGAATTGAG	ACCAGTCACCAATCCAAACAG
COG8	AAGCCAGACTCCAGAATTGAG	TTGGCAGGATAAAGGCGAG
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG
ATP7A	TCTTCCAGGATTGTCTGTTATGAA	ACCAGCCTCCGAAAAACTG
ATOX1	ACAGCCCACAGGATGGAC	GACTGCCAAGTCCCAGGTC
CCS	TCATCGAGGGAACTATTGACG	AGGGTTAAAGTGATTCCCACAG
COX17	AAGTGACTGCGGACGAATC	TTTGAGTCAACCAGACCCG
MT1A	TCCTGCAAATGCAAAGAGTG	GCACACTTGGCACAGCTC
MT2A	CTAGCCGCCTCTTCAGCTC	GCAGGTGCAGGAGTCACC
COX19	TGGAGAAACTGGGATTTGGAG	TGGGCCGTGTTCAGTGGTCT
CHCHD7 (COX23)	CGTGAGCCATTGACGTGTT	TTTGCATGAGTTCCTCGTGT
COA5 (PET191)	TTATGAGGACAAGCCGCAG	GCGTACTTCAAAGAGTTGCAG
SCO1	GGAATGAAGCACGTCAAGAAAG	TCCTTGTCAGTTTTACGCTCC

CHAPTER 3
DISCUSSION

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Overview of Findings and Significance

Genetic and environmental factors interact to influence the development of neurodegenerative diseases, and determination of environmental risk factors offers insight into the mechanisms underlying progressive neuronal death. Loss of copper homeostasis is not only a consequence of mutations in copper binding proteins but also a common feature of prominent neurodegenerative diseases including Alzheimer's and Parkinson's disease [93]. Both copper deficiency and excess in the brain can lead to neurodegeneration, but the mechanisms that underlie the contribution of copper to neurodegeneration are poorly understood. As described in Chapter 1 Section 3, the current understanding of the neurodegenerative phenotypes in Menkes disease, a disease of copper deficiency, is incomplete. Determination of the mechanisms underlying this monogenic disorder might offer insight into more genetically complex neurodegenerative disorders, particularly those modulated by environmental risk factors. The oligoenzymatic hypothesis of Menkes disease etiology has focused attention on a small number of ATP7A-dependent cuproenzymes [96]. We sought to expand the known interaction network of ATP7A to better understand ATP7A regulatory mechanisms and in turn uncover novel copper homeostasis mechanisms. In Chapter 2, I described the ATP7A interaction proteome, finding an enrichment of proteins associated with neuropathologies. Further investigation focused on the interaction between ATP7A and the conserved oligomeric Golgi (COG) complex, a multi-subunit Golgi tether that we hypothesized may be acting upstream of ATP7A. We found that deletion of COG subunits perturbs the protein expression and localization of not only ATP7A, but

also the plasma membrane copper transporter CTR1. We used inductively coupled plasma mass spectrometry to show that the copper content in COG null cells was significantly reduced and that the addition of a copper ionophore augmented cellular copper levels and bypassed the requirement for CTR1 at the plasma membrane. Using an MTT assay to measure the activity of mitochondrial reductases, we also found that mitochondrial function is compromised in COG null cells and that, like copper content, this deficiency could be alleviated by the addition of a copper ionophore. Finally, we overexpressed ATP7A in a *Drosophila melanogaster* system to induce copper deficiency. In this background, deletion of COG subunits significantly reduced the viability of the flies as compared to wildtype flies and those overexpressing ATP7A, demonstrating a genetic interaction between COG and ATP7A. Further, both deletion of COG subunits and overexpression of ATP7A produce neurodevelopmental phenotypes at the neuromuscular junction as well as copper-sensitivity in the adult animal. Expression of both mutations alleviates these effects. Thus, the COG complex is a novel interacting partner of ATP7A that regulates cellular copper homeostasis. While an extensive functional analysis was carried out to characterize the interaction between ATP7A and the COG complex, the ATP7A proteome yielded dozens of additional novel interactions that we plan to investigate further in the future.

Revisiting the Central Hypothesis

In Chapter 1, I hypothesized that molecules belonging to the ATP7A interactome are required for the subcellular localization of copper transporters and neuronal tissue

viability. In Chapter 2, I experimentally tested this hypothesis and found the following evidence in support:

1. The ATP7A interactome is enriched in gene products associated with neurodegenerative and neurodevelopmental disorders.
2. The COG complex, a multi subunit Golgi tethering complex, is a novel interaction partner of ATP7A.
3. The COG complex is required for the stability and surface expression of the copper transporters ATP7A and CTR1.
4. Cells containing deletions of COG subunits are copper deficient.
5. $COG^{-/-}$ cells modify the expression of copper-sensitive transcripts for proteins localized to multiple organelles.
6. Mitochondrial function is perturbed in $COG^{-/-}$ cells and can be rescued by the addition of a copper ionophore.
7. COG and ATP7A genetically interact in *Drosophila melanogaster*.

In Chapter 1 Section 4, I posed four open questions in the field pertaining to our understanding of ATP7A and copper biology. Below, I revisit those questions in light of the findings presented in Chapter 2.

1) What mechanisms underlie the contribution of copper to neurodegenerative and neurodevelopmental disorders?

Chapter 1 Section 2 described the associations between altered copper homeostasis and neurodegenerative disease, including Alzheimer's, Parkinson's, and prion

disease. In all three, copper is found sequestered in extracellular protein aggregates in the brain. The predominant hypothesis regarding a potential role for copper in the pathogenesis of these diseases is that excess unbound copper accumulates in the protein aggregates, leading to the production of reactive oxygen species, which then trigger neuronal damage and ultimately cell death [93]. However, neurodegeneration is also a hallmark of Menkes disease in which the brain is depleted of copper [170]. The specific removal of ATP7A from neurons demonstrated that it is not a loss of ATP7A from neurons that is predictive of neuronal phenotypes, but rather a loss of copper in the brain when ATP7A is absent from the blood brain barrier [147].

The results in Chapter 2 conducted in $COG^{-/-}$ cells provide some insight into the relationship between copper deficiency and decreased mitochondrial function. MTT and extracellular flux measurements performed in $COG^{-/-}$ cells revealed decreased mitochondrial function that could not be alleviated by the addition of copper alone. Inductively coupled plasma mass spectrometry measurements confirmed that these cells were copper deficient, and the addition of copper in conjunction with an ionophore was sufficient to restore mitochondrial function. One prominent theory to account for neurodegeneration is that reduced mitochondrial function leads to the production of reactive oxygen species and in turn contributes to neuronal cell death. Inhibition of COX function in particular has been shown to generate reactive oxygen species, induce caspase-3-like activity, and lead to DNA fragmentation [333]. Further work suggests that while the activity of individual electron transport chain

subunits must be inhibited by 50% or more before ATP synthesis is affected, the generation of reactive oxygen species can occur at lower degrees of inhibition [334]. Prior work has shown that COX activity is reduced ~50% in the brain of patients with Alzheimer's disease [335]. Mutations in the mitochondrial proteins PINK1 and Parkin lead to familial Parkinson's disease, strengthening the connection to mitochondrial dysfunction [134, 336]. Copper is essential for COX function, and decreased copper content or loss of proper copper trafficking to the mitochondria is one mechanism by which COX function could be perturbed [42].

As discussed in Chapter 1, the presence of extracellular excess copper in the protein aggregates associated with neurodegenerative diseases is well established [162, 322]. However, there is much less information regarding the intracellular copper content or the spatial localization of copper in these disorders. There is emerging evidence that suggest copper deficiency, despite the excess copper present in Aβ plaques of Alzheimer's patients and Lewy bodies of Parkinson's patients, plays a role in the etiology of these neurodegenerative disorders. For example, the induction of SOD1 deficiency in a mouse model of Alzheimer's disease accelerated both Aβ oligomerization and the development of cognitive phenotypes [337]. In this study, levels of SOD1 protein were manipulated, but copper depletion would also lead to SOD1 deficiency [34]. Another study found that intraneuronal copper, as measured by synchrotron radiation X-ray fluorescence microscopy (SRXFM), and Ctr1 levels, as measured by Western blot, were specifically decreased in degenerative brain tissue from Parkinson's patients as compared to controls [338].

Future work will shed light on whether irregular copper homeostasis is a hub of neurodegenerative disease and what upstream mechanisms disrupt appropriate homeostasis.

2) Does copper influence other neurodegenerative diseases?

Alzheimer's, Parkinson's, and Menkes disease are just a few of the copper related neurodegenerative disorders I have discussed in this dissertation. Could other neuropathologies not currently described as diseases influenced by copper homeostasis be identified based on the findings presented in this dissertation? In Chapter 2, I describe the ATP7A interactome and the finding that the interactome is enriched in gene products implicated in neurodevelopmental and neurodegenerative disorders. These novel interaction partners include VAC14, a regulator of endosomal lipid composition, and the COG complex, a multimeric Golgi tether, mutations in both of which are associated with neurodegeneration and neurodevelopmental disorders, respectively [235-252]. Neither of these has previously been linked to copper homeostasis.

While we originally hypothesized that the COG complex regulates the trafficking of ATP7A, we found through our characterization of this relationship that the COG complex is tied to copper homeostasis at not only the Golgi complex, but also the plasma membrane and the mitochondria. Prior work suggests that the COG complex plays a role in copper homeostasis by regulating the import of copper by CTR1. In $COG^{-/-}$ cells, CTR1 appears to be proteolytically cleaved, as suggested by the

presence of a low molecular weight band observed in COG$^{-/-}$ cells but not in controls. The surface levels of CTR1 are also significantly reduced in COG$^{-/-}$ cells, and this depletion is exacerbated by the addition of excess copper to these cells. The reduction of CTR1 at the plasma membrane correlates with reduced copper content in these cells. The addition of copper in conjunction with an ionophore that bypasses the requirement for CTR1 import alleviates the copper deficiency in COG$^{-/-}$ HEK cells, suggesting that aberrant CTR1 function is upstream of copper deficiency in these cells. Interestingly, the addition of copper and the ionophore DSF increases the copper content in COG$^{-/-}$ cells substantially more than in control cells. There are two experimental observations that could account for this marked increase. The first is that ATP7A levels are decreased in the COG$^{-/-}$ cells, and there appears to be decreased mobilization to the plasma membrane under acute conditions of copper addition. Thus, loss of ATP7A activity could prevent the COG$^{-/-}$ cells from eliminating excess copper over the course of 24 hour exposure. Second, levels of copper-sensitive transcripts were altered in COG$^{-/-}$ cells, including transcripts for metallothionein-1 and -2, both of which were elevated in COG8 deletion cells (though not in COG1 knockouts). This alteration of transcript levels suggests that multiple regulatory mechanisms, including metallothioneins that are able to buffer excess copper, may be perturbed in COG$^{-/-}$ cells. COG loss of function mutations in patients lead to systemic phenotypes that vary depending on which subunit is mutated, but neuronal phenotypes are the most consistent [252]. The current understanding of COG function is limited, and existing work pertains to its role in the regulation of glycosylation enzymes (Figure 1) [314]. Our results suggest that

the COG complex possesses regulatory roles outside of its known function, and that disruption of copper homeostasis may contribute to the neuronal phenotypes associated with COG-related congenital disorders of glycosylation.

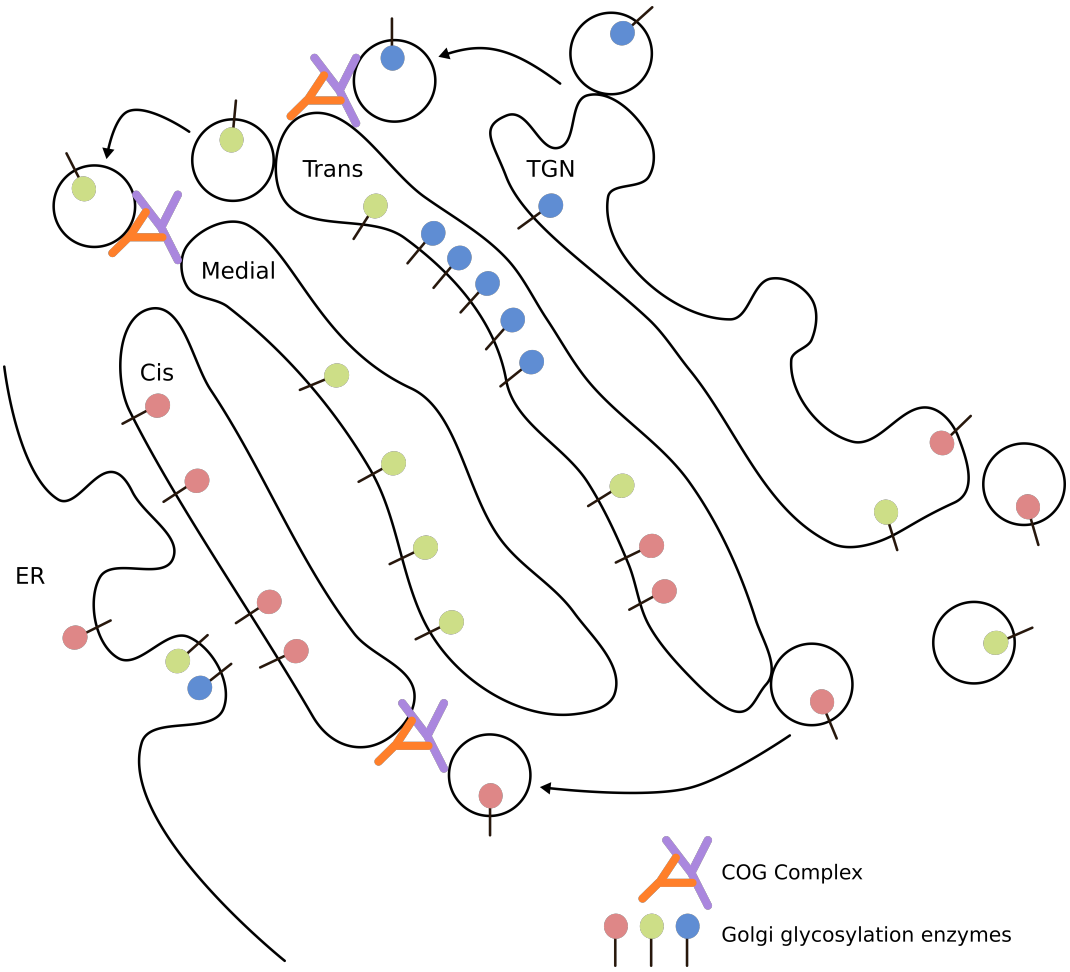

Figure 1 The COG complex maintains the integrity of the Golgi complex.
As the cisternae of the Golgi mature, the COG complex (orange and purple) is required for the retrograde trafficking of Golgi-resident enzymes. The prevailing model describes the COG complex as a multi-subunit tethering complex that facilitates binding of vesicles and the target membrane. Deletion of COG subunits leads to mislocalization of Golgi enzymes, perturbations in glycosyation, and fragmentation of the Golgi complex. Modified from Foulquier 2009.

Several other genes associated with neuropathologies previously not linked with copper homeostasis appear in the ATP7A interactome, including VAC14, a lipid

regulatory protein, and N-Ethylmaleimide-sensitive factor (NSF), a protein that resolves SNARE complexes. Mutations in VAC14 have been observed in pediatric-onset neurodegeneration, while mutations in NSF have been tied to a form of epilepsy [235, 236, 339]. Table 1 presents the set of genes associated with neurodegeneration and neurodevelopmental phenotypes that were identified in the ATP7A interactome. This includes known interaction partners, such as DBH, along with novel interactions. While the ATP7A interactome yielded hundreds of novel interaction partners that we hope will further our understanding of ATP7A biology, these genes associated with neuropathologies offer a promising starting point as it may be revealed that copper plays a pathogenic role in the progression of associated neuropathologies as well.

3) How is global copper homeostasis regulated?

Much of the research aimed at understanding copper homeostasis has focused on discrete cellular compartments. Mitochondrial copper homeostasis has been considered as distinct from Golgi copper homeostasis, perhaps due in part to the existence of specialized copper chaperones. However, recent work in the field, including results presented in Chapter 2, point to the presence of global copper homeostasis mechanisms in which signals are relayed between multiple cellular compartments to respond to changing copper levels. Section 1.2.2 details a role for the COX assembly proteins, SCO1 and SCO2, in mediating copper homeostasis via COX19 and ATP7A. The study described in this section demonstrated that the redox state of the copper-binding cysteines of SCO1, along with the abundance of

oxidoreductase SCO1, correlate with copper status in the cell [52]. Mutation of SCO1 or SCO2 results in copper deficiency that can be rescued by reducing ATP7A expression, suggesting that copper efflux via ATP7A is tied to the redox state of SCO1. Given that SCO1, SCO2 and ATP7A are all membrane proteins, it was hypothesized that a protein intermediary existed to relay this copper-sensing signal from the mitochondria to the Golgi complex. Soluble COX assembly factors including COX17, COX19, COX23, and PET191 were strong candidates as they all contain copper-binding motifs and partition between the intermembrane space and the cytosol. Further characterization revealed that only COX19 partitions between the inner mitochondrial space and cytosol in a copper-dependent manner, and knockdown of COX19 in *SCO* fibroblasts significantly increased cellular copper levels. The communication of regulatory signals between these compartments is an exciting finding as it is one of the first reports positing a possible mechanism of copper sensing and response that involves multiple cellular compartments.

The same research group also described a possible role for SCO1 in the regulation of CTR1 activity, again connecting copper homeostasis mechanisms to multiple cellular compartments [53]. A mouse with a conditional SCO1 knockout in the liver was generated and exhibited copper deficiency and reduced COX function. In the liver of these mice, CTR1 levels were also reduced and the remaining protein was not properly localized to the plasma membrane. Inhibition of the proteasome significantly increased CTR1 levels, suggesting a mitochondrial signaling pathway regulating CTR1 localization and, consequently, copper uptake. The internalization

and degradation of CTR1 in response to elevated copper levels is an established regulatory mechanism [287]. The aberrant degradation of CTR1 in *SCO1* cells is indicative of a novel CTR1 regulatory mechanism that is not directly dependent on copper concentration.

Expanding on the body of evidence describing multi-compartment copper regulatory mechanisms, I present evidence in Chapter 2 that the Golgi-localized COG complex is required for maintaining cellular copper homeostasis, and that deletion of the COG complex results in mitochondrial function deficits that can be rescued by copper addition. My results suggest that the cellular levels and trafficking of at least two copper transports, ATP7A and CTR1, are affected by loss of COG subunits. Given the known function and localization of the COG complex, we hypothesized the complex could be required for the trafficking and/or the glycosylation of ATP7A. Levels of ATP7A are reduced by an average of 50% in HEK cells bearing deletion of COG subunits. In addition to the total reduction in ATP7A protein levels, the migration of the protein on a Western blot was increased in a COG deletion background. Previously characterized COG-dependent proteins, termed GEARS, also exhibit this increase in migration that correlates with glycosylation defects [283]. This phenotype is a known consequence of COG subunit deletions, and it is possible that glycosylation of ATP7A is dependent on the COG complex as well [340]. While glycosylation defects may contribute to the instability and degradation of ATP7A, the surface biotinylation experiments presented in Chapter 2 suggest that deletion

of the COG complex is also impairing the anterograde trafficking of ATP7A, an idea I will explore further in the following section.

Likewise, surface biotinylation experiments also demonstrate that CTR1 function and localization are perturbed in cells bearing COG deletions. The surface levels of CTR1 are significantly reduced in COG null cells, and this depletion is exacerbated by the addition of copper. Further, the antibody to CTR1 recognizes an additional low molecular weight band in $COG^{-/-}$ cells that is not present in control cells. This observation is consistent with previous findings that demonstrate loss of O-linked CTR1 glycosylation produces a proteolytic cleavage product [341]. Inductively coupled plasma mass spectrometry experiments revealed that $COG^{-/-}$ cells are significantly copper depleted and that this depletion can be rescued by the addition of the copper ionophore DSF. This finding suggests that decreased copper import via CTR1 is the primary driver of copper depletion in $COG^{-/-}$ cells. These cells also exhibited decreased mitochondrial function as measured by oxygen consumption rate, and this deficit can be rescued by the addition of copper in conjunction with an ionophore.

Taken as a whole, the results in Chapter 2 demonstrate a role for a Golgi-resident complex in regulating cellular and mitochondrial copper homeostasis. Given the previous observation that COX19 acts as an intermediary between mitochondrial copper sensing and a response from ATP7A at the Golgi complex, we aim to investigate COX19 protein levels and function in $COG^{-/-}$ cells [52].

4) What proteins are required for the regulation of ATP7A localization and trafficking?

One aim of defining the interactome of ATP7A was to uncover candidates that regulate the localization and trafficking of ATP7A. Relatively few such proteins are known, and a mechanistic understanding of their role is unspecified. There are also open questions in ATP7A regulation that are unaddressed by the known regulatory proteins, including what signals the translocation of ATP7A to the plasma membrane upon elevation of copper content. It is unknown whether this signal is directly dependent on copper status, or if it is relayed by an intermediary protein.

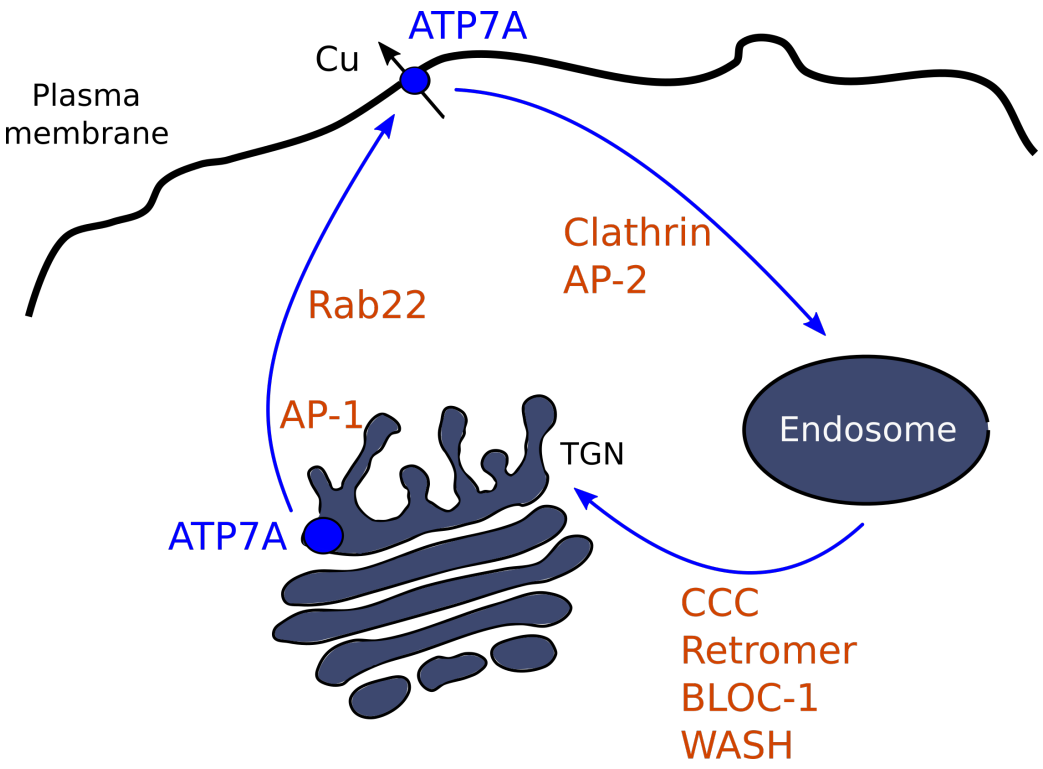

Figure 2 Trafficking of ATP7A.

Under steady state conditions, ATP7A resides at the Golgi complex where it provides copper to holoenzymes requiring the metal as a cofactor. When intracellular copper is elevated, ATP7A translocates to the plasma membrane; Rab22 and AP-1 are required for this anterograde movement. ATP7A contains a dileucine motif required for its internalization from the plasma membrane under the regulation of clathrin and the AP-2 adaptor. Finally, delivery from the endosome to the Golgi complex requires multiple trafficking complexes, including the CCC, retromer, BLOC-1, and WASH complexes.

The known set of proteins required for ATP7A trafficking and localization are depicted in Figure 2. Trafficking of ATP7A, as it is currently understood, can be broken down into three steps: (1) anterograde movement from the Golgi complex to the plasma membrane, (2) retrograde movement from the plasma membrane to the endosome, and (3) return to the Golgi complex from the endosome. An assessment of well-characterized trafficking complexes revealed that Rab22 is required for anterograde trafficking of ATP7A [193]. Expression of a constitutively active Rab22a mutant prevents targeting of ATP7A to the plasma membrane and retains ATP7A at the TGN, a non-canonical role for Rab22 activity. Rab22 is the only known protein required for anterograde trafficking of ATP7A, and the mechanism of copper-sensitive ATP7A trafficking is still unknown. In the same screen for trafficking proteins, it was revealed that AP-1 plays a role in retaining ATP7A at the TGN, and later work determined a physical interaction between AP-1 and ATP7A occurs via the C-terminal domain of ATP7A [193, 272].

While relatively few proteins are known to regulate anterograde trafficking of ATP7A, many more trafficking proteins have been identified for the internalization of ATP7A from the plasma membrane and return to the TGN. Internalization occurs

via clathrin-mediated endocytosis and requires a carboxy-terminal dileucine motif along with the adaptor AP-2 [189, 193]. Upon delivery to the endosome, there is evidence that several trafficking complexes, including the BLOC-1, retromer and WASH, and CCC complexes, mediate transport of ATP7A to the TGN, and this step in particular may be cell type specific [191, 194, 342]. While an association between COMMD1 deficiency and irregular copper homeostasis was established, it was not until COMMD1 was identified as part of a complex also containing CCDC22 and CCDC93, hence named the CCC complex, that its role in copper homeostasis was uncovered [343]. The CCC complex localizes to endosomes and is required for the trafficking of ATP7A from endosomes to the TGN [194]. Further, the CCC complex interacts with components of the WASH complex, which also plays a role in retrograde ATP7A trafficking from endosomes [194]. Work from our lab supports this model as WASH has been demonstrated to regulate the trafficking of endosomal BLOC-1 cargoes [271]. While the mechanistic details are not clear, there is evidence that retromer in conjunction with sorting nexin 27 is required for ATP7A stability. Loss of either component leads to lysosomal degradation of ATP7A [191]. Work from our lab also determined a requirement for the BLOC-1 complex in copper homeostasis via ATP7A [269]. Subunits of the BLOC-1 complex genetically and biochemically interact with ATP7A, and BLOC-1 deficient cells exhibit increased susceptibility to copper challenge. Given the endosomal localization of BLOC-1 and the known trafficking route of ATP7A, we hypothesize that BLOC-1 regulates the retrograde trafficking of ATP7A. This hypothesis is supported by work in

melanocytes, in which BLOC-1 is required for delivery of ATP7A to melanosomes where it provides copper to the cuproenzyme tyrosinase [342].

Given the localization of the COG complex at the Golgi and its function in the retrograde-trafficking of Golgi-resident enzymes, one possibility is that it is required for one or more steps in ATP7A trafficking. We tested the requirement for COG in anterograde trafficking of ATP7A by subjecting $COG^{-/-}$ cells to a copper challenge and assessing the mobilization of ATP7A to the surface by biotinylation. The biotinylation efficiency in $COG^{-/-}$ HEK cells was substantially greater than in control cells, confounding quantification of ATP7A surface levels. However, normalizing the biotinylation efficiency reveals that ATP7A surface content as compared to total levels is reduced in $COG^{-/-}$ cells. Experiments conducted in *Drosophila* also support an interaction between ATP7A and the COG complex. Flies overexpressing ATP7A in combination with COG deletion exhibit increased mortality as compared to either genetic manipulation alone. Further work to assess how COG deletion affects the localization of ATP7A will strengthen our understanding of the interaction between the two. Determination of ATP7A localization by microscopy is limited by antibody specificity, particularly in HEK cells where ATP7A levels are low, but development of tools to assess ATP7A by immunofluorescence could provide great insight into the role of the COG complex in ATP7A trafficking.

Summary

My dissertation provides an interactome for the copper transporter ATP7A along with a characterization of the interaction between ATP7A and the COG complex, a novel interaction partner. The ATP7A interactome enriches genes associated with neurodegenerative and neurodevelopmental disorders, and these newly identified interactions offer pathways to explore pathological mechanisms. Aberrant copper homeostasis is a feature not only of mutations in copper binding proteins, but also in common neurodegenerative diseases such as Parkinson's and Alzheimer's disease, and the genes identified in the interactome may be implicated in both copper homeostasis and the pathology of neurodegeneration.

I showed that the COG complex, known for its role in retrograde Golgi trafficking, regulates copper homeostasis at the Golgi complex, plasma membrane, and mitochondria. This finding strengthens the evidence for a framework in which copper homeostasis is regulated at the level of the cell rather than the organelle. The copper deficiency observed in $COG^{-/-}$ cells correlates with defects in mitochondrial function that can be rescued by the provision of copper. This connection between mitochondrial function and copper deficiency is one that can be explored as a neurodegenerative mechanism, particularly in light of recent work that points to copper deficiency in the brain as sufficient to induce neuronal phenotypes. The findings in this dissertation lay the groundwork for future work to account for the role of copper homeostasis in neuropathologies.

Table 1 ATP7A Interactome: Genes Associated with Neuropathologies

The ATP7A interactome is enriched in genes associated with neurodegenerative and neurodevelopmental disorders. Table 1 lists these genes (Column 1) along with the OMIM entry for each gene (Column 2) and the Entrez Identifier (Column 3); entries in Columns 2 and 3 are hyperlinked to their source. Columns 4-6 list the neuropathologies associated with each gene, the Unified Medical Language System identifier, and the PMID for the primary literature establishing the disease association. The PMID entries are hyperlinked to the PubMed source.

Gene	OMIM	Entrez	Disease Association	UMLS	PMID
ABAT	137150	18			
			Seizures	C0036572	1407778
			Autistic Disorder	C0004352	15830322
ABCA7	605414	10347			
			Alzheimer's Disease	C0002395	21460840
			Schizophrenia	C0036341	19721717
ADH5	103710	128			
			Schizophrenia	C0036341	19165527
AKAP9	604001	10142			
			Alzheimer's Disease	C0002395	25172201
			Schizophrenia	C0036341	25943950
ANXA7	186360	310			
			Epilepsy	C0014544	21432772
BCAS2	605783	10286			
			Autistic Disorder	C0004352	24189344
CASP3	600636	836			
			Status Epilepticus	C0038220	18571097

			Diabetic Neuropathies	C0011882	19555701
			Alzheimer's Disease	C0002395	18818379
CBS	613381	875			
			Cystathionine beta-Synthase Deficiency	C0751202	22267502
			Homocystinuria	C0019880	11230183
CDK5	123831	1020			
			Peripheral Neuropathy	C0031117	81423835
			Brain Ischemia	C0007786	14502288
			Alzheimer's Disease	C0002395	18480410
CHMP2A	610893	27243			
			Intellectual Disability		25356899
CTNNA2	114025	1496			
			Bipolar Disorder	C0005586	19416921
			Attention deficit hyperactivity disorder	C1263846	18839057
CUL7	609577	9820			
			Autistic Disorder	C0004352	25961944
CYFIP1	606322	23191			
			Prader-Willi Syndrome	C0032897	16982806
			Autistic Disorder	C0004352	20029941
			Schizophrenia	C0036341	24996170
DBH	609312	1621			
			Dopamine Beta Hydroxylase Deficiency	C0342687	11857564

			Paranoia	C1456784	10673769
			Parkinson Disease, Late Onset	C3160718	27177268
			Peripheral Neuropathy	C0031117	20864405
DCLK2	613166	166614			
			Epilepsy	C0014544	19342486
DCTN1	601143	1639			
			Neuropathy, Distal Hereditary Motor, Type Viib	C1843315	12627231
			Amyotrophic Lateral Sclerosis	C0002736	18305234
			Parkinsonian Disorders	C0242422	24676999
DCX	300121	1641			
			X-Linked Lissencephaly	C1848199	9489700
			Subcortical Band Heterotopia	C1848201	10807542
DOCK4	607679	9732			
			Austistic Disorder	C0004352	19401682
			Schizophrenia	C0036341	23720743
DYRK1A	600855	1859			
			Down Syndrome	C0013080	18509201
			Alzheimer's Disease	C0002395	18005339
ELAVL4	606852	1996			
			Learning Disorders	C0023186	23545166
			Sciatic Neuropathy	C0149940	12957493
			Status Epilepticus	C0038220	17577668
			Parkinson Disease	C0030567	18587682

EPHX2	132811	2053			
			Cerebrovascular Accident	C0038454	19940276
			Cerebral Infarction	C0007785	16306811
FAM120C	300741	54954			
			Autistic Disorder	C0004352	25258334
			Mental Regardation, X-Linked	C1136249	18498374
FARP1	602654	10160			
			Alzheimer's Disease	C0002395	21116278
			Brain Diseases	C0006111	20171287
GIT1	608434	28964			
			Attention deficit hyperactivity disorder	C1263846	21499268
			Huntington Disease	C0020179	15383276
HIP1	601767	3092			
			Huntington Disease	C0020179	16847693
IRS2	600797	8660			
			Epilepsy, Temporal Lobe	C0014556	25458098
MAP1B	157129	4131			
			Seizures	C0036572	7790894
			Spinal Muscular Atrophy	C0026847	1881920
NSF	601633	4905			
			Parkinson Disease	C0030567	21812969
			Epilepsy, Temporal Lobe	C0014556	11226670
			Schizophrenia	C0036341	18077426

PARK7	602533	11315			
			Autosomal Recessive Early Onset Parkinson Disease	C1853445	15254937
			Parkinson Disease	C0030567	23792957
PDE4B	600127	5142			
			Schizophrenia	C0036341	16293762
			Bipolar Disorder	C0005586	19350560
			Autistic Disorder	C0004352	18090323
PPP3CB	114106	5532			
			Schizophrenia	C0036341	21531385
PRKRA	603424	8575			
			Dystonia	C0013421	25142429
			Parkinsonian Disorders	C0242422	18243799
PSMC1	602706	5700			
			Neurodegenerative Diseases	C0524851	18701681
			Ataxia Telangiectasia	C0004135	19147735
RBFOX1	605104	54715			
			Autistic Disorder	C0004352	24613350
			Parkinson Disease	C0030567	21623373
SKP1	601434	6500			
			Machado-Joseph Disease	C0024408	23171848
			Parkinson Disease	C0030567	23988235
SQSTM1	601530	8878			
			Amyotrophic Lateral Sclerosis	C0002736	22972638

			Alzheimer's Disease	C0002395	19481695
STXBP1	602926	6812			
			Epileptic Encephalopathy, Early Infantile, 4	C2677326	18469812
			Epilepsy	C0014544	22596016
			Mental Retardation	C0025362	19557857
VGF	602186	7425			
			Amyotrophic Lateral Sclerosis	C0002736	18432310
			Austistic Disorder	C0004352	19598235
VPS26A/B	610027	112936			
	605506	9559			
			Parkinson Disease	C0030567	25475142
VPS35	601501	55737			
			Parkinson Disease 17	C3280133	22517097
			Parkinson Disease	C0030567	24980502
			Schizophrenia	C0036341	21822266

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