The Involvement of Copper in Leigh Syndrome

By

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Abstract

Leigh Syndrome is a neurodegenerative disease that presents with bilateral lesions in the brain. This condition is characterized by progressive loss of mental and movement abilities in which these symptoms start as early as three months old and usually results in death within two to three years. One major cause of Leigh syndrome is a mutation in the SURF1 gene. The loss of SURF1 causes a defect in cytochrome *c* oxidase (COX) assembly. Cytochrome *c* oxidase is required for the electron transport chain in cellular respiration. COX is the terminal electron accepting enzyme that requires more than 25 factors to assemble the subunits of this enzyme, including components to coordinate the early stages of mitochondrial translation and cofactor insertion such as MITRAC. For function COX requires several metals including copper. Copper is an essential element that is required for the proper functioning of mammalian cells. It is required for iron uptake, oxidative stress prevention, and the electron transport chain. Copper is especially important in mitochondria for COX, SOD, and storage in the matrix. Although copper is essential, maintaining a suitable balance of copper is important to prevent toxicity in the cell that causes dysfunctions and diseases to occur. There are many different measures in place to keep copper balanced, the details of how, what, who, and how many players involved are not completely understood. Much is known on the pathway of copper getting into the cell, into mitochondria to be distributed to enzymes, and through the mitochondrial carrier family

protein (SLC25A3) into the matrix, but it is unknown how copper is exported out of the matrix. In this dissertation I investigated a potential role for copper in Leigh Syndrome and potential role for SURF1 in regulating copper availability in mitochondria. Understanding of this pathway could lead to much needed therapies to combat this disease and prevent infant death.

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List of Abbreviations

COX	Cytochrome *c* Oxidase
IMS	Inner membrane space
IM	Inner membrane
OM	Outer Membrane
SOD	Superoxide Dismutase
ETC	Electron Transport Chain
ABC	ATP-Binding Cassette
MCF	Mitochondrial Carrier Family
MPC	Mitochondrial Pyruvate Carrier
MITRAC	Mitochondria translation regulation assembly intermediate of cytochrome c oxidase complex

Chapter 1: Literature Review

Medlock AE, Hixon JC, Bhuiyan T, Cobine PA. Prime Real Estate: Metals, Cofactors and MICOS.

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1. Introduction

Mitochondria are a hub of metabolism and signaling [1]. At the heart of many of these activities are metal cofactors such as heme, iron sulfur clusters and other metalloenzymes [2]. Therefore, coordinating the flux of metals and controlling cofactor assembly should be closely linked with overall mitochondrial health. While the mitochondrial outer membrane (OM) is porous thus allowing for gated diffusion of metabolites, the inner membrane (IM) is folded into cristae and is impermeable to most small molecules and especially metals, Figure 1.1. Yet the internal matrix compartment requires metals for processing proteases as well as enzymes of the tricarboxcylic acid cycle. The IM houses many metalloenzymes and contains the highest ratio of proteins to lipids of all eukaryotic membranes [3]. The assembly and insertion of the cofactors into the complexes of electron transport chain requires assembly proteins localized in the intermembrane space (IMS) and the matrix [4]. Some cofactors are produced (heme and Fe-S) or stored in the matrix (copper) and they must be redistributed to the IMS for assembly into the target enzymes [5]. Therefore, coordinating multiple proteins in multiple compartments with the availability of the metals/cofactors is a task that requires strategies to position the proteins involved at the right time in the right place.

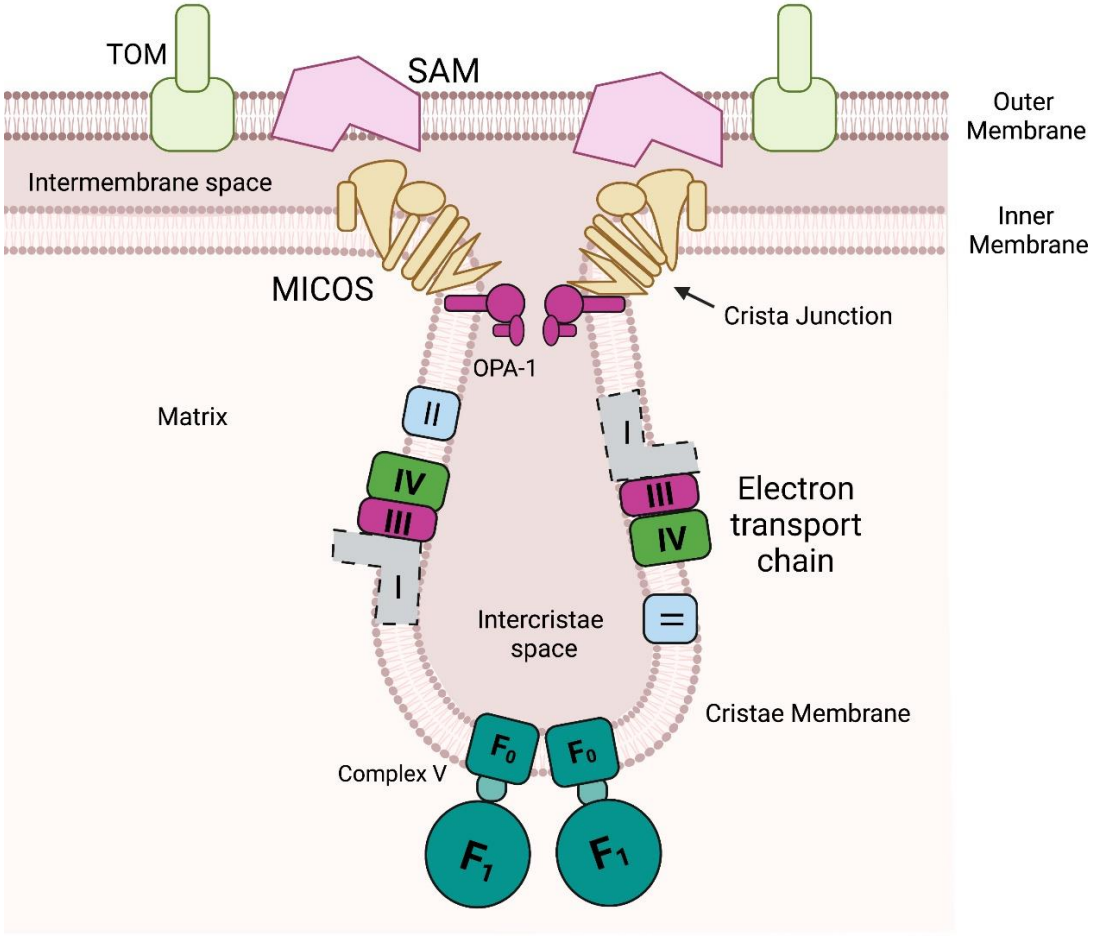

Figure 1.1: **Mitochondrial Cristae Structure**

Mitochondria have multiple unique compartments. Outer membrane is a porous membrane that allows exchange of metabolites and small molecules with the cytosol but restricts the entry of proteins to the intermembrane space. The inner membrane is a highly protein loaded membrane that folds into invaginations known as cristae. The cristae house the assembled components of the oxidative phosphorylation (Complex I, II, III and IV and ATP synthase (F_0F_1 ATPase, Complex V). In mammals this includes a membrane spanning NADH oxidoreductase (Complex I) (shown with dotted lines due differences between yeast and mammals), in yeast this enzyme is soluble in the matrix. The mitochondrial contact site and cristae organizing system (MICOS) and Opa-1 cooperate to form cristae junctions that encloses the intercristae space. MICOS also provides a structural point of contact between the inner and outer membrane via an interaction with the sorting and assembly machinery (SAM).

The processes involved in mitochondrial ultrastructure, energetics, and metal homeostasis are conserved from yeast to mammals. While the nomenclature and naming of the proteins has been partially unified, examples of differently named homologs still exist. In this review we discuss a mixture of model systems that have been used to further our understanding of mitochondrial physiology. We have noted when evidence is from yeast or mammals in the text and have used all caps for protein names from mammals (including mouse and human) (e.g., SLC25A3) and have used sentence case (e.g., Pic2) for yeast proteins.

Multiple pathways evolved to facilitate the correct localization of the mitochondrial proteome [6]. Importantly, all the import pathways require proteins to be unfolded and therefore they must receive the cofactors required for activity in the organelle. Import and insertion is tightly regulated by protein complexes that allow transit of unfolded polypeptides to the correct location without causing disruption of the membrane potential as depolarization of mitochondria is a well described inducer of cell death. Using a combination of cytosolic and mitochondrial proteins, newly translated proteins enter the mitochondrion via translocase of the outer membrane (TOM). Then multiple pathways exist for the delivery of proteins to their final localization [6]. Two different TIM complexes, either Tim22-or Tim23-containing complexes, sort and facilitate the insertion of IM and matrix proteins, while the β-barrel sorting and assembly machinery (SAM) is required for OM protein insertion [6]. Cysteine-containing IMS localized proteins have a dedicated pathway called the mitochondrial intermembrane space import and assembly (MIA) machinery. Mia40 is the major protein that participates in import via transient disulfide bond formation with the incoming targets and then, in the final stages of the import process, facilitates the formation of disulfide bonds within the IMS protein

[7]. In addition, the proteins known as the small TIMs assist in directing OM and IM proteins to the distinct complexes that facilitate insertion. The concerted action of all these complexes allows for the insertion and activity of the ~1000 nuclear encoded proteins that function in mitochondria.

Mitochondrial genomes content can vary in different organisms but generally it encodes ribosomal RNAs, tRNAs and select subunits of the OXPHOS machinery. The mRNAs encode the proteins that are core elements of the OXPHOS complexes (3 subunits of complex IV, 3 subunits of complex V, 1 subunit complex III in yeast; 3 subunits of complex IV, 2 subunits of complex V, 1 subunit complex III, 7 subunits of complex I in humans) are transcribed, translated within the matrix by a dedicated machinery. Once transcribed and translated these proteins are inserted into the IM to form distinct complexes and assembly intermediates that form in a modular fashion. OXA1 is essential for the correct insertion and assembly of many of these proteins [8; 9]. Mutations in human OXA1 result in fatal encephalitis, hypotonia, and developmental delay due to assembly defects in complexes I, IV and V [9]. Both human and yeast Oxa1 have been shown to interact with the mitochondrial ribosome. In yeast, mtDNA encoded subunits of complex V are synthesized on the cristae membrane, while complex III and IV components were synthesized both at the inner boundary membrane and cristae membrane as defined by the localization of the specific translational activators [10]. Using fluorescent noncanonical amino acid tagging it was shown that in human cells the majority of mtDNA encoded proteins are synthesized at the cristae membrane [11]. The authors of this study note that while the technique cannot follow synthesis in real time it is unlikely that the assembly intermediates could pass through the cristae junctions [11]. Therefore, the cofactor containing subunits of

OXPHOS requires the coordination of these translational factors, insertases, the available cofactor at specific localizations to ensure correct assembly.

2. Mitochondrial morphology and organization

Aberrant mitochondrial morphology is associated with cellular dysfunction suggesting that mitochondrial architecture and function are intimately linked. The mitochondrial contact site and cristae organizing system (MICOS) complex consists of numerous proteins essential for formation of mitochondrial structure, Figure 1.2 [12; 13]. The MICOS complex establishes and maintains the inner membrane architecture, provides contacts between the IM and OM, and facilitates the closure of the cristae junction [12]. It is known that membrane potential, lipid and protein composition affect cristae formation and that the maintenance of cristae shape is linked to content of ATP synthase. The conserved dynamin related GTPase, OPA1 catalyzes membrane fusion and is found at crista junctions and regulates both the number of cristae and the release of cristae contents [14].

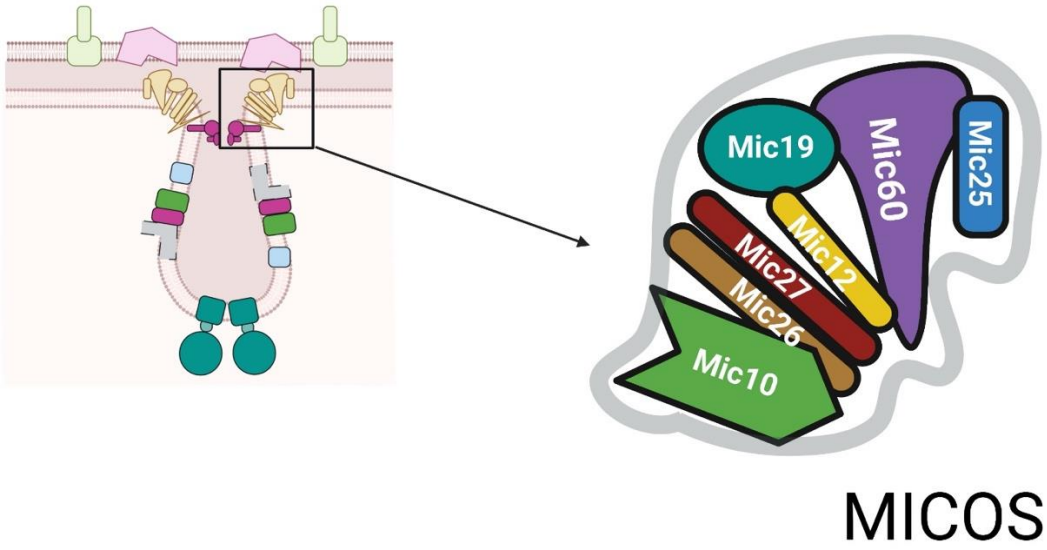

Figure 1.2: **MICOS complex**

The MICOS complex consists of multiple proteins named for their molecular weight. The numbers shown are based on mammalian system and a slightly different in yeast system.

Depending on the organism the number of core components identified to date varies [12]. The core components of MICOS are: Mic10, Mic12, Mic19, Mic25, Mic26, Mic27, and Mic60, Figure 2 [15]. In addition, to the role in closing the cristae junction, the complex is required for the formation of contact sites between the IM and OM to facilitate communication between these two mitochondrial compartments. MICOS proteins also interact with proteins such as Ugo1, a part of mitochondrial fusion machinery, and porin, the abundant OM channel for small metabolites [16]. Through its links with multiple proteins, the MICOS complex functions as an organizer of mitochondrial architecture and integration platform for processes that are centered on mitochondrial membranes.

A recurring theme in mitochondria is that large complexes interact and work together exchanging proteins into and out the complexes to regulate functions critical to cellular physiology. This MICOS network includes interactions of Mic60 with the protein translocases TOM, SAM, and the oxidoreductase Mia40. Mic60 stimulates the TOM-SAM-mediated import of β-barrel proteins into the outer membrane forming a complex with TOM and SAM. In yeast, Tim23 and Mic60 interact to facilitate precursor handover from the TOM complex to the Tim23 complex by bringing the OM and IM into close contact. While TIM22 and MICOS work synergistically in human mitochondria to drive the translocation of mitochondria carrier family (MCF/SLC25) proteins [17]. Mic60 also interacts with the MIA machinery to mediate the import of intermembrane space proteins that have containing characteristic cysteine motifs [18]. Since Mic60 transiently binds to both TOM and Mia40, Mic60 helps to position the receptor Mia40 to the intermembrane space side of the TOM complex. This spatial coupling function of Mic60 facilitates the formation of intermolecular disulfide bonds between Mia40 and the preprotein

near the TOM complex preventing the exposure of potentially redox-sensitive cysteine residues in the preproteins. Thus, Mia40 can rapidly bind to precursor proteins passing through the TOM channel and promote their efficient import [19]. Mia40 pathway plays critical roles in the import of multiple metalloprotein and metalloprotein related assembly proteins. Therefore, MICOS has a critical role in the translocation of most mitochondrial proteins. This review will focus on the roles MICOS complexes have on metal homeostasis and the roles metalloproteins and metals have on MICOS stability.

3. Transporters in mitochondria

Membranes provide the greatest barrier to metal distribution in cells [20]. Therefore, understanding metal homoeostasis starts with understanding the transporters that facilitate entry and exit of the metals/cofactors. Transporters have the potential to function as scaffolds for loading metals however limitations in our ability to monitor these interactions, or a lack of knowledge about the transporters in particular compartments has slowed our progress in defining such roles. Mitochondria have five major classes of transporters in the IM that include the mitochondrial carrier family (MCF/SLC25), ATP-binding cassette (ABC) transporters, mitochondrial pyruvate carrier (MPC/SLC54), mitochondrial cation/H^+ exchangers (LETM/SLC55), and sideroflexins (SLC56) [1; 21]. Here we will focus on transporters involved in metals in mitochondria with the MPC and LETM families discussed elsewhere [21].

3.1 Mitochondrial Carrier Family

Mitochondria contribute to intracellular signaling and regulatory networks in part by coordinating trafficking of metabolites into and out of mitochondria[1]. The mitochondrial carrier family (MCF/Slc25 family) form the largest group of transporters in the IM and are responsible for most of the metabolic flux to, and from, the organelle. In humans there are 53 members that are collectively responsible for the transport of numerous substrates including TCA cycle intermediates, nucleoside di- and triphosphates, amino acids, and metals [1]. Sixteen of the 53 MCFs have no known substrate and the established promiscuity of some of the characterized transporters raises the possibility that even those 37 with known substrates may have additional substrates. Notable members of this family involved in metals and metal cofactor assembly are SLC25A3/Pic2 which is a copper and phosphate transporter [22; 23], SLC25A37/MITOFERRIN1/Mrs3 and SLC25A28/MITOFERRIN2/Mrs4 which are involved in mitochondrial iron homeostasis and necessary for iron transport [24; 25; 26; 27; 28], as well as SLC25A38 which transports glycine for heme synthesis [29].

MCF transporters have a conserved fold consisting of three repeats of approximately 100-amino acids that contain two transmembrane helices connected by a loop with a short α-helix [30]. This structural insights into the family of transporters is based on the structure determined for ADP/ATP exchanger that was crystalized with inhibitors that stabilized the protein in either an open to the IMS state (c-state) or open to the matrix state (m-state) [31; 32]. The MCF fold is stabilized by salt bridges that form at the closed end of the substrate binding pocket depending on which state the protein adopts. The 1st,3rd, and 5th

transmembrane helices contain a conserved PX(D/E)XX(R/K) motif that forms salt bridge and hydrogen bonding contacts on the matrix side of the binding pocket. A complementary (Y/F)(D/E)XX(R/K) motif in 2^{nd},4^{th}, and 6^{th} helices is found on the IMS side [30]. The strength of these salt bridge interactions is an important predictor of the requirements and directionality of transport. Two examples exist of single amino-acids changes in MCF that give rise to changes in specificity. In yeast, Rim2 is a bifunctional pyrimidine and iron transporter but E248A mutant specifically disrupts mitochondrial iron transport activity while K299A mutant specifically abrogated pyrimidine nucleotide transport and exchange [33]. Similarly, a L175A mutation can make the bifunctional copper and phosphate transporter SLC25A3 specific for copper transport [34].

The metal transporting MCF SLC25A3 and SLC25A37 are predicted to be uniporters that import metals into the matrix. Both SLC25A3 and SLC25A37 have been successfully reconstituted into proteoliposomes to demonstrate transport activity [22; 28]. The yeast homologs, Pic2 and Mrs3 and mammalian SLC25A3 have also been expressed in *L. lactis.* Expression in *L. lactis* allows for monitoring import of metals into this organism. Both Pic2 and Mrs3 can transport copper in *L. lactis* although for Mrs3 to facilitate copper transport iron had to be chelated/limited presumably to prevent competition [35]. The plant variant of SLC25A37 was also shown to have the ability to transport iron and copper [36; 37]. But our understanding about the interplay between these transporters *in vivo* is limited.

In addition to a role in transporting metals the MCF family has the potential to form a scaffold for protein-protein interactions to enhance the assembly of the mitochondrial metalloproteome. The MCF proteins have been isolated in many distinct large complexes. The

large interactome of these proteins is exemplified by the ADP/ATP exchange proteins [38; 39; 40]. These proteins play critical role in delivery of the energy currency to the cell and have been isolated in large multiple protein complexes. These ADP/ATP exchanger protein complexes can also include other MCFs including the copper and phosphate carrier (SLC25A3), calcium dependent carriers (SLC25A13, SLC25A24, SLC25A25), the tricarboxylate carrier (SLC25A1), and the carnitine/acylcarnitine carrier (SLC25A20) [40]. How these interactions affect and/or regulate the specificity and/or transport activity is unknown due to the difficulties in assessing these biochemical characteristics *in vivo* or with purified components.

3.2 ABC-transporters

There are 4 mitochondrially-localized ABC-transporters (ABCB6, ABCB7, ABCB8 and ABCB10) which are part of a large superfamily of proteins that mediate nucleotide-dependent transport [1]. ABC-transporters have four core domains for function. Two transmembrane domains that provide specificity by forming a binding site, and two nucleotide binding domains to hydrolyze ATP to facilitate transport of the bound ligand. Conservation is found in the nucleotide binding domains with variability in the transmembrane domains to allow for a broad number of substrates [41]. The four mitochondrial ABC-transporters engage in moving Fe-S clusters and heme related metabolites into and out of mitochondria. ABCB6 is localized to the OM (and other cellular locations including the cell surface). ABCB6 has been proposed as porphyrin transporters [42] and has been shown to specify the Langereis blood group [43].

ABCB7, ABCB8 and ABCB10 are all localized to the IM and the exact molecule transported by each is still unclear. ABCB7 links to cofactor assembly are because it was

identified as a candidate causative gene for X-linked sideroblastic anemia with spinocerebellar ataxia and the yeast homolog *ATM1* is regulated by iron and participates in maintaining Fe-S cluster homeostasis [44; 45]. Similarly phenotypic data links ABCB8 to iron homeostasis as a mutation of *Abcb8* results in cardiomyopathy with increased mitochondrial iron accumulation with decreased activity of cytosolic Fe-S clusters proteins. Finally, the *Abcb10* knockout in mouse is embryonic lethal due to anemia. In support of a role of this transporter in iron homeostasis, *Abcb10* forms a complex with MITOFERRIN1 (SLC25A38) and ferrochelatase (FECH) that enhances heme synthesis, Figure 3 [46; 47; 48]. While the substrate for ABCB10 has not be definitively identified, multiple studies suggest different substrates related to heme synthesis and it is clear this protein plays an important role in iron metabolism. The characterization of a larger complex that includes multiple heme synthesis related proteins is a continuation of the theme observed for the MCF proteins, that complexes of transporters and targets can form a potential regulatory mechanism for metal cofactor assembly or metal homeostasis.

3.3 Other transporters

Other notable mitochondrial transporters involved with metals are sideroflexins (Slc56). One of the five sideroflexin proteins, sideroflexin-1, has been shown to have a role in serine transport in mitochondria, and both sideroflexin-1 and sideroflexin-3 have been linked to iron homeostasis and Fe-S cluster biogenesis [49]. Sideroflexins are five transmembrane domain proteins that contain a conserved mitochondrial tricarboxylate/iron carrier domain (PFAM: 03820). All members share the same topology with the amino-terminal inside and the carboxy-

terminal outside. Sideroflexins 1-4 have sites of post-translational modification including acetylation that regulate activity and stability.

Cellular zinc transporters have been reported to localize to different mitochondrial membrane in some cell types. Zinc transporters can be broadly categorized in two groups ZIP (Irt-like) and ZnT [50]. ZIP transporters function as in zinc import while ZnT class function as zinc exporters. ZIP family members contain 8 transmembrane domains, with the proposed metal-binding residues embedded in transmembrane helices 4 and 5 and a cytoplasmic (inside/IMS in mitochondria) region between transmembrane 3 and transmembrane 4 that is important for regulation including protein stability the exact roles of this domain are still under investigation [50]. It is also important to recognize that the ZIP family homologs have been shown to transport a variety of divalent ions including but not limited to iron, manganese, copper. Therefore, these transporters could have effects on other metals in mitochondria.

4. Metals in mitochondria

4.1 Copper

Copper is found within each of the mitochondrial compartments. In the IM, cytochrome c oxidase is the multi-subunit complex that catalyzes the final steps of the electron transport chain (ETC). It accounts for about 25% of mitochondrial copper and is the major cuproenzyme present in this organelle [51]. In addition, a small percentage of cellular Cu, Zn superoxide dismutase (Sod1) and its copper chaperone, Ccs1, is localized to the IMS. However, these proteins do not account for a significant proportion of the total mitochondrial Cu. The majority

(up to 70%) is found in the matrix and is bound to a biochemically characterized but structurally undefined complex known as the copper ligand (CuL)[5]. The copper in the matrix is transported by MCF SLC25A3/Pic2, that is capable of transporting Cu and CuL as a substrate[22; 23; 34]. The data in multiple experimental models shows that the copper in the matrix is redistributed to the IMS for the assembly of cytochrome c oxidase (COX) and superoxide dismutase (Sod1). The matrix distribution model that is based on phenotypic data from mutants of the copper importer and biochemical competition assays in yeast under copper stress. These phenotypes are reproduced in mammalian cell culture without the need for copper restriction [22]. However, the identity of the transporter responsible for copper export is unknown and therefore the regulation of this pathway is poorly understood. An alternative pathway that feeds copper directly from the cytosol into these enzymes has been suggested based on purification of copper complexes that are thought to be in the IMS, this alternative pathway could operate under conditions where copper is abundant [5; 52].

Copper availability in the IMS is critical for the assembly of COX [5]. However, copper levels are restricted and therefore chaperone proteins are required to enhance the assembly process. This evidence for the copper delivery pathway is a combination of *in vitro* observations and genetic suppression experiments. In the IMS, Cox17 presents copper to Sco1 and Cox11 for assembly of the copper sites in COX called Cu_A and Cu_B sites, respectively [5; 91]. Cox17 is a soluble protein that adopts a coiled coil-helix-coiled coil-helix fold stabilized by two disulfide bonds. The Cox17-Sco1 interaction is the most thoroughly studied copper transfer reaction that occurs in the IMS. Cox17 donates copper to the exposed CxxxC site on Sco1. Studies have shown that the reactions proceed from the Cu-loaded, partially oxidized conformer of human

COX17 to SCO1 via transient interactions [88; 89; 92]. This Cu-delivery pathway is further supported by the observation that *SCO1* overexpression can rescue the phenotype of a *COX17* mutant cell [93]. Sco1 subsequently insert the Cu into Cox2 to form the Cu_A site in COX. Multiple redox related steps are involved in the COX assembly process. Mammalian COA6 (and yeast Coa6) and SCO2 are two of the proteins with roles in regulating the reduction state of assembly proteins and the target COX2 [56; 57; 94] . Most recently an additional interplay between the Cu assembly process and heme has been identified with a role discovered for COA7, a heme binding protein, that transiently interacts with the copper metallochaperones SCO1 and SCO2 and catalyzes the reduction of disulfide bonds within these proteins [95]. To build the Cu_B site, cells use a different battery of assembly proteins. Cox11 is a membrane bound factor required for the insertion of the Cu in COX1 and is also a recipient of copper from Cox17 [96]. Like Sco1 pathway specific accessory factors are required to maintain the redox state of the proteins in the pathway. Cox19 is required for the reduction of two critical copper binding cysteines in Cox11 [97]. Copper transfer occurs via an interface than that is distinct from that used for interaction with Sco1 [91; 98]. The requirement for this multi-step interplay of protein-protein interactions in the IMS to facilitate copper loading reinforces the idea that limited copper availability is a major complication for the correct metalation of cuproenzymes. It is also important to note that mutation in SCO proteins can cause remodeling of cellular copper homeostasis. The mutant cells and mice show copper deficiencies due to inappropriate degradation of the high affinity importer CTR1 or excess activity of the copper exporter ATP7A [96; 99; 100; 101]. During the cellular copper deficiency mitochondrial copper is maintained suggesting a possible prioritization of the matrix copper under these conditions [102].

To date at least six gene deletions in yeast have been shown to limit matrix copper accumulation based on analysis of the purified mitochondria and mitochondrial targeted copper responsive reporters. The proteins implicated in copper import/maintenance are the MCF proteins: Pic2, Mrs3 and the assembly factors Coa1, Coa4, Coa6 and Shy1[53; 54; 55]. Coa6 is a Cx_9C containing protein of the IMS that is responsible for regulating the redox state of proteins involved in Cox2 assembly machinery [56; 57; 58]. Coa1, Coa4 and Shy1 are all linked to the translation and assembly of Cox1 [53]. The mammalian homologs of Shy1 (SURF1) and Coa1 (COA1/MITRAC15) are part of the mitochondrial translation regulation assembly intermediate of cytochrome c oxidase (MITRAC) complex [59]. This complex is another example of a large protein complexes that form in mitochondria to facilitate more efficient processing and/or substrate channeling. The assembly of the respiratory chain complexes proceeds in modular fashion and require complex-specific assembly factors to stabilize the intermediates for the complete maturation of complexes. Because maturation of the enzyme complexes is a sequential process during which new components and cofactors are added, the composition of the MITRAC complex changes during the process [59]. Since MITRAC is facilitating the translation and assembly of COX1 and this is the heme and copper containing subunit it is intriguing to proposed that this complex could be critical for regulating total mitochondrial copper. It should be noted that only Coa1, Shy1 deletion and no other MITRAC homologs in yeast cause the copper deficiency.

4.2 Iron

Iron enters mitochondria through multiple mechanisms. The most well-studied example of mitochondrial iron import is via MCF proteins MITOFERRIN1 in mammals and Mrs3 and Mrs4 in yeast [60]. MITOFERRIN and Mrs3 have been shown to modulate the transport of iron *in vivo* and *in vitro* while Mrs4 is transcriptionally activated by iron depletion, suggesting a role for homeostasis of iron in mitochondria. Once iron crosses the IM it is used for the synthesis of heme, Fe-S or is bound by other ligands for storage. Genetic experiments suggested that FRAXATIN (or at least the yeast homolog Yfh1) played a role as a chaperone directing iron to produce Fe-S cluster largely based on an iron accumulation phenotype and the activation of Fe-regulated transcription in yeast in *YFH1* mutants [61]. However additional experimental evidence suggests that this is not a required function of Yfh1. The phenotypes of *yfh1Δ* can be bypassed completely by a compensatory mutation in the Fe-S cluster scaffold protein IscU [62]. This combined with other approaches suggested the primary function of Yfh1 is sulfur delivery to form Fe-S clusters. Once iron reaches the iron-sulfur cluster machinery it is synthesized into 2Fe-2S clusters that are then inserted in mitochondrial targets or exported to the cytosol where the cytosolic iron-sulfur assembly (CIA) machinery matures them to 4Fe-4S clusters and/or inserts them into other targets [45]. The other major iron cofactor is heme which is synthesized when iron is inserted into protoporphyrin IX by ferrochelatase [63]. Protoporphyrin IX is synthesized by a series of enzymes that are split between the cytosol and mitochondria with the initial steps of porphyrin synthesis taking place in mitochondria before export of the intermediates for maturation in the cytosol until the final steps occur back in the mitochondrion.

4.3 Zinc and Manganese

The mechanisms of zinc and manganese uptake into the mitochondrial matrix are not well understood. Zinc is required in mitochondria for multiple functions including electron transport chain function (COX), ATP synthesis (Atp32), protection against oxidative stress in the IMS (Sod1), lipid transport (Yme1L), mitochondrial dynamics (Oma1, Yme1L), processing peptidase (MPP), and mitochondrial intermediate peptidase (MIP). A genetic screen to identify a transporter using chemical sensors of available zinc uncovered an unexpected connection between Complex III assembly and mitochondrial zinc. A gene designated *MZM*1 was identified and characterized to show the gene product was required for both zinc maintenance and complex III assembly [64; 65]. However, this did not result in identification of a zinc transporter in mitochondria. Some studies have suggested that zinc can be imported to mitochondria by the calcium uniporter (MCU) [66; 67]. While others have reported dual location of zinc transporters suggesting that zinc entry to the IMS could be facilitated by Zip1 that was shown to localize to the OM [68]. Further still the zinc transporters Zip7 and ZnT7 have also been shown to have dual localization in mitochondria and ER and expression of Zip7 and ZnT7 contribute to cellular zinc exchange between the organelles in certain cell types [69]. Finally Znt9 (SLC30A9) was identified as a zinc exporter in mitochondria [70] and SLC25A25 has been implicated in at least regulating uptake of zinc as deletion can reverse phenotypes induced by SLC30A9 deletion [71]. Perhaps redundancy is the reason for the failure of the yeast genetic screens to yield a single candidate.

Manganese has a single target in mitochondria in the form of superoxide dismutase 2 (SOD2). This matrix localized enzyme is responsible for protection against a subset of the reactive oxygen species generated. Deletion of *SOD2* in mammalian models is embryonic lethal demonstrating its essential role. However, to date no definitive data exists as the identity of the manganese transporter that provides this metal for the matrix for SOD2 assembly and activity. In yeast deletion of the gene *MTM1*, which encodes a MCF, caused decreased activity of Sod2 but did not prevent manganese accumulation [72]. It was subsequently shown that deletion of *MTM1* changed iron availability resulting in mismetallation and inactivation of Sod2 by iron [73; 74]. Critically this is an example of the complex requirements for essential cofactor assembly in mitochondria and highlights the need for additional research on regulatory mechanisms that change availability.

5. Cofactor assembly

Mitochondria are essential for the synthesis of cofactors required for normal cellular function. It is widely accepted that synthesis of Fe-S clusters in mitochondria is the sole essential function for the organelle in eukaryotes. This is because divergent eukaryotes which have lost other mitochondrial function retain an organelle with Fe-S cluster assembly machinery. In addition to Fe-S clusters, mitochondria also house the machinery for assembly of heme and molybdenum cofactor (MoCo). Heme is required for all aerobic eukaryotes and is involved in oxygen metabolism, sterols synthesis, and amino acid biogenesis amongst other processes. MoCo is involved in multiple cellular functions including sulfur, drug, and nucleotide metabolism.

5.1 Heme assembly

Heme or Fe-protoporphyrin IX is an essential cofactor required for a plethora of cellular processes in eukaryotes. In metazoans, the heme biosynthesis is typically partitioned between the cytosol and mitochondria, with the first and final steps taking place in the mitochondrion. Key enzymes in the pathway that are found in the mitochondrial matrix are aminolevulinic acid synthase, which utilizes glycine and succinyl-CoA to produce aminolevulinic acid, and FECH, which utilizes iron and protoporphyrin IX to make heme [75]. Overall, the pathway has been well studied and all the biosynthetic enzymes structurally characterized. Nevertheless, understanding of the regulation of heme synthesis in different cells and factors that influence this process remains incomplete. Some details of the transcriptional regulation in the context of erythroid development are known, but transcriptional regulation in most other cell types or cellular conditions is much less understood [63]. Recent work supports post-translational mechanisms for the regulation of the mitochondrial heme biosynthesis enzymes [76]. Studies have shown protein-protein interactions regulate heme precursor levels [47] and the incorporation or disruption of cofactors into key enzymes [77; 78].

5.2 Iron-sulfur cofactor assembly

Fe-S clusters are essential cofactors for the ETC and many other biochemical reactions and processes [45]. The cofactor assembly systems are required to sequester, chaperone, and regulate delivery of the cofactor. The assembly proteins are not strictly required for building Fe-S cluster, because the clusters will self-assemble in solution, but is thought these proteins are necessary to circumvent the toxicity and indiscriminate reactivity of free iron and sulfide.

Defects in the biosynthesis in Fe-S cluster cause dysfunction in mitochondria, neurodegenerative and cardiovascular disease, genomic instability, and the development of aging and cancer.

Mitochondria assemble not only mitochondrial Fe-S clusters but are also involved in the biosynthesis of Fe-S clusters for proteins in the cytosol and in the nucleus [45]. These cytosolic and nuclear Fe-S cluster proteins with essential functions for example, ABC protein Rli1, which participates in ribosome assembly and ribosome recycling during termination of polypeptide synthesis [79]. Eukaryotic replicative DNA polymerases which also contain a Fe-S cluster in their C-terminal domain [80]. The Fe-S cluster cofactor appears to be indispensable for efficient interaction with their accessory proteins during DNA replication.

5.3 MoCo

Molybdenum is a versatile redox element that is used by enzymes to catalyze diverse reactions. While not a focus of this review we will briefly discuss molybdenum cofactor (MoCo) as it is synthesized in mitochondria and has links to iron and copper. MoCo is an ancient cofactor involved in sulfur, drug, and nucleotide metabolism. MoCo synthesis begins in mitochondria with guanosine 5’-triphosphate which is converted via the two proteins to cyclic pyranopterin monophosphate (cPMP). Both mitochondrial enzymes in humans, MOCS1A and MOCS1B, are encoded by a single gene alternatively spliced to yield either protein. For MOCS1A exon one encodes the mitochondrial localization signal, and for MOCS1B localization is designated via exon 10 [81]. Interestingly MOCS1A can also be localized to the cytosol in primates via exon 1b, and while it appears to be functional its physiological role in the cytosol is

unclear. MOCS1A belongs to the radical SAM enzyme superfamily and has two iron-sulfur clusters [82], specifically 4Fe-4S clusters, thus is connected to iron and iron-sulfur cluster homeostasis. cPMP is then exported across the mitochondrial membranes to the cytosol where it is further modified [83]. In plants this is mediated by ATM3 which is the homolog of yeast Atm1 and mammalian ABCB7. Once in the cytosol MPT synthase transfers the two sulfurs to cPMP to create molydopterin (MPT). This intermediate can bind copper, and the copper may act as a protecting group as the cofactor synthesis proceeds [83]. The next step in synthesis is formation of the of adenylated-MPT before the final step of Mo insertion. Therefore, MoCo synthesis has overlap with copper and iron both utilizing cofactors and sharing a transport pathway.

6. The connection between metals and organization

Mitochondria have a clear role in multiple aspects of metal homeostasis as described above. But many of the experimental approaches and techniques used have been “static” in nature and did not necessarily consider the dynamics of mitochondria. This includes consideration of total content, structure, and localization within the cell in different tissues and organisms. In a “two-way street” we have limited understanding of the impact that alterations in mitochondrial physiology have on metal content and utilization or how dynamic changes in mitochondria alter cofactor synthesis. Rates of fission and fusion, the ultrastructure of the organelle, and rates of mitophagy all have the possibility to impinge on metal homeostasis and cofactor assembly. An emerging area of overlap is between metal homeostasis/cofactor assembly and MICOS. As described previously the coordination of the proteins in larger complexes is a developing theme

and we will discuss two interactions of MICOS with iron and copper proteins that regulate both cofactor assembly and mitochondrial function.

6.1 Ferrochelatase-MICOS

The terminal enzyme of the heme biosynthesis pathway, ferrochelatase, has been identified to be part of a multi-protein complex or metabolon in the mitochondrial matrix. This complex has been studied in both mammalian cells (FECH) [47] as well as yeast (Hem15) [84]. In terms of the mammalian cells, most work has focused on the metabolon in developing erythroid cells, as these cells make a large amount of heme. An interesting finding was that mitochondrial heme metabolon interacted with MICOS proteins including MIC60, Figure 1.3 [84; 85]. Further work in yeast showed that loss of MICOS negatively impacts Hem15 activity, it affects the size of the Hem15 high-mass complex, and results in accumulation of reactive and potentially toxic porphyrins (which arise from the heme intermediates porphyrinogens) that may cause oxidative damage. Restoring intermembrane connectivity using a heterologously expressed protein capable of tethering the IM and OM “artificially” in MICOS-deficient cells mitigates these cytotoxic effects [84].The artificial tether clearly shows the importance of mitochondrial ultrastructure to the function of this enzyme.

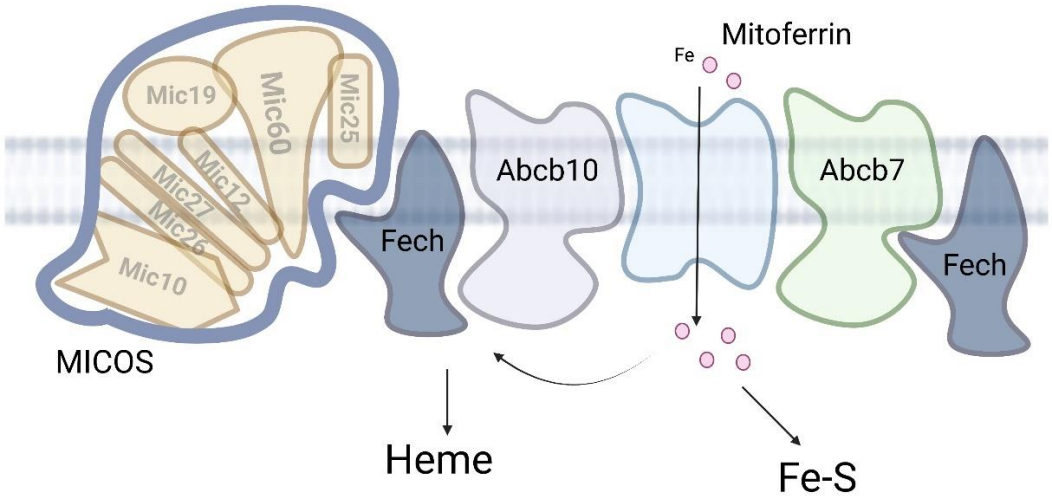

Figure 1.3: **MICOS and mitochondrial iron**

Iron enters mitochondria via the MCF mitoferrin. The iron is then partitioned between heme synthesis, Fe-S assembly, and other iron. Accumulation of the other iron is deleterious in multiple disease states.

The localization of ferrochelatase to MICOS also provides a connection to iron and Fe-S cluster trafficking at these sites. Ferrochelatase was shown to interact with SLC25A37/MITOFERRIN1/Mrs3, ABCB10, and ABCB7 [47; 48; 86; 87]). The connection with MITOFERRIN and ABCB10 is clear as iron is a substrate for the enzyme and thus can be directly trafficked for heme synthesis. The role of FECH and ABCB7 [88; 89] interaction is less clear, except that FECH itself has an 2Fe-2S cluster [90] and thus may regulate or be regulated by Fe-S cluster flux in the matrix. Overall, these data provide new insights into how heme biosynthetic machinery is organized and regulated, linking mitochondrial architecture-organizing factors to heme synthesis for the efficient import of heme precursors and export of heme.

6.2 COX17-MICOS

In addition to the role Cox17 plays in in COX assembly, it also been linked to a copper dependent interaction with the MICOS complex [103; 104]. Cox17 interacts with Mic60 to modulate MICOS complex integrity [104]. This interaction does not involve Sco1 or Cox11. However, the Cox17-MICOS interaction is regulated by copper, Figure 4. Cox17 is therefore a factor involved in maintaining the architecture of mitochondria via the MICOS complex. The regulation of the MICOS complex and therefore IM structure by Cox17 and copper was unexpected. However, it further consolidates a link between the mitochondrial morphology, ETC integrity, and metal homeostasis. It was proposed that Cox17 may be facilitating protein-protein interactions that enhance MICOS stability or that it could be delivering of copper to the MICOS complex [104]. This would require a yet to be identified copper-binding site in the complex. Cryptic sites of copper binding leading to metalloallosteric regulation have recently

been described in multiple well studied processes (lipolysis, proliferation, and autophagy) and therefore a precedent exists to identify these new sites [105]. Another possible model would be that the presence of excess Cu-Cox17 would indicate that COX is maximally metaled and therefore closing of cristae junctions should proceed to allow for enhanced activity of the OXPHOS in the enclosed intercristae space, Figure 1.4.

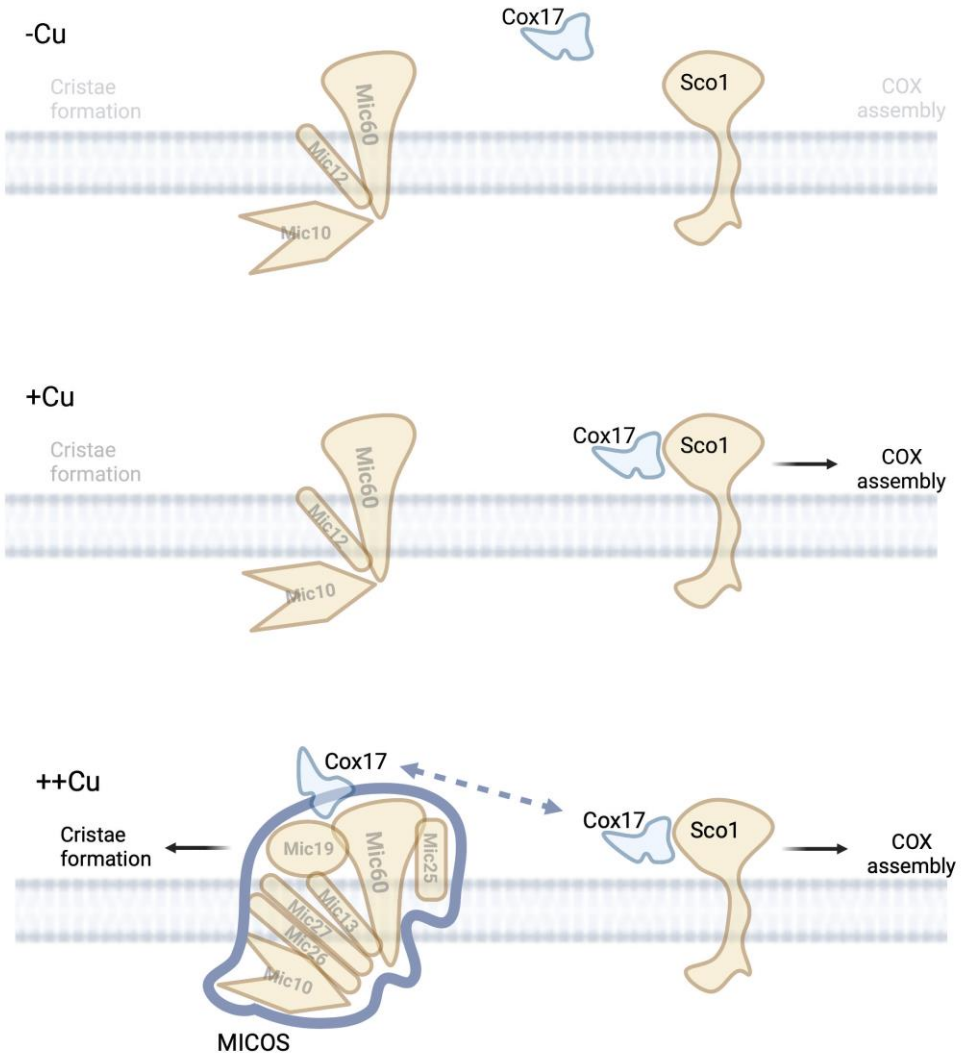

Figure 1.4: **Copper and MICOS complex**

Under low copper conditions no copper is available in the IMS to mediate the assembly of COX or allow for efficient assembly of MICOS suggesting low numbers of cristae in mitochondria as the Cox17 MICOS interaction is copper dependent and required for cristae formation.

6.3 SOD1-MICOS

The regulation of metal homeostasis is important to avoid the potential formation of reactive oxygen species (ROS). Superoxide dismutase (Sod1) is involved in the scavenging of ROS in the cytosol and in the IMS of mitochondria. Sod1 requires zinc and copper ions and an intramolecular disulfide bond to catalyzes the conversion of superoxide into hydrogen peroxide and water. The activation, localization, and retention (in IMS) of Sod1 is largely dependent upon the copper chaperone Ccs1 [106; 107]. Ccs1 is involved in the transfer of copper and disulfide bond formation in the maturation of Sod1. The assembly of Sod1 is also dependent on Mia40. Mia40 is responsible for import and oxidation of Ccs1 to the IMS. Ccs1 is responsible to "fold and trap" Sod1 in the IMS. An additional role for mitochondrial ultrastructure in Sod1 activity was uncovered. Mitochondria that are lacking any components of the MICOS complex can create unusual separations in the IMS. MICOS mutants accumulate a disproportionate amount of oxidized Sod1 [108]. The authors speculate that the reduction of the disulfide bond in Sod1 is prevented by mitochondrial ultrastructure/compartmentalization [108]. The metalation state of Sod1 in the MICOS mutants is unknown and perhaps mitochondrial copper status in MICOS mutations play some role in oxidation states of the Sod1. It is known that decreased mitochondrial copper can change the steady state levels of IMS localized Sod1.

Many mutations in human SOD1 are associated with amyotrophic lateral sclerosis (ALS), a nervous system disease that causes loss of muscle control. This debilitating disease has meant that numerous studies have investigated the role of mitochondrial SOD1 is disease progression.

The accumulation of wildtype reduced Sod1 was dependent on the cristae architecture controlled by MICOS and mitochondria with MICOS defects showed an increase in mitochondrial accumulation of ALS-related reduced variants of Sod1 [108]. This accumulation resulted in the accumulation of toxic superoxide and mitochondrial dysfunction [108]. Further suggesting a role for correct mitochondrial ultrastructure in this disorder.

7. Future directions and unanswered questions

Until now our knowledge of metal homeostasis in mitochondria has been largely based on static measurements and the interpretation of genetic experiments. These have proven to be excellent resources for establishing the components of the pathways and identifying multiple interactors and suppressor that can bypass individual steps. However, recent technological advances in our ability to robustly detect protein complexes and our confidence that these interactions have bona fide physiological consequences have meant that we have learned that maintaining specific complexes in the highly crowded "real estate" of the mitochondrial IM is a recurring theme. The existence of interactions between copper and iron machinery and MICOS, that can serve as "a signpost to the most desirable neighborhood", have triggered many additional questions about how mitochondrial morphology and metals are intertwined.

An outstanding question is what is the role of copper in the Cox17-MICOS interaction? Does this metal bind directly to stabilize a structure or perhaps enhance assembly or is the role of copper in stabilizing a fold of Cox17? The alpha-fold prediction of Mic60 does present multiple cysteine residues (as potential donors of thiol ligands) within 3-6 Å of each other [109]. The roles of these residues in metal binding have not been investigated. There have not been

consistent reports of mitochondria morphology changes in cells lacking the major copper transporters such as CTR1, which restricts all available copper stores. However, this has not been systematically measured. In *SLC25A3* mutant cells, which have a localized copper deficiency in mitochondria, it has been reported that a less interconnected mitochondrial network and a mitochondrial fusion defect exists that is not explained by altered abundance of OPA1 or MITOFUSIN 1/2 or relative amount of different OPA1 forms [110]. This defect could be due to decreased mitochondrial copper which leads to changes in downstream targets such as COX17-MICOS complex. Additionally, yeast with lacking *MDM38,* a membrane-associated mitochondrial ribosome receptor with a role as K+/H+ exchange, have a mitochondrial fragmentation defect that can be suppressed by overexpression of *PIC2* and *MRS3* [111; 112]. The mechanism of that rescue is linked to multiple physiological changes, but it is possible to speculate that *PIC2* and *MRS3* could be contributing to this rescue via copper transport function of these proteins. Further investigation would be required to pinpoint the mechanisms of defects and rescue of morphology in these mutants.

A major challenge for defining additional roles of the copper transporting MCF SLC25A3/Pic2 is the fact it has been identified in many complexes. In fact, SLC25A3 was included in the Contaminant Repository for Affinity Purification (CRAPome) due it is abundance and “stickiness” [113]. So, determining which complexes are “real” requires directed hypotheses. These hypotheses could include phenotypes such as the mitochondrial morphology under multiple conditions. While these experiments can be labor intensive, armed with the knowledge gained to date, it is now reasonable to invest the time. One observation that could be further investigated is SLC25A3 was found associated with MITRAC but not included as an

interactor due to abundance in control samples [59]. The possibility of SLC25A3 interaction to MITRAC complex would form an attractive link between COA1 and SURF1 (MITRAC members with mitochondrial copper defect) and copper. The mechanism of this is under investigation in yeast where *coa1Δ* cells have decreased copper availability[53]. The copper deficit in mitochondria would limit the ability of Cox17 to complete its copper dependent interaction with MICOS raising the possibility of ultrastructure defect in this mutant. Unravelling this connection could further link MICOS to copper.

The interactions between MICOS and SAM and the affects that this could have on IMS content and connectivity to facilitate export of matrix components is understudied in metal homeostasis. The current model for copper and iron is that they are transported into the matrix by MCF proteins localized in the IM [5]. While transporters of iron in the OM have been proposed the directionality of the transport has not been completely resolved. For copper the model has always suggested that porins would act as IMS importers. If porin, or other proteins of the OM, were affected then the source of copper for SLC25A3/Pic2 could be disrupted. In addition, the model suggests that the copper chaperones (Cox17, Sco1, Cox11) are bypassed during import but used during export. Therefore, a link to mitochondrial ultrastructure and compartmentalization would help solve part of this bypass conundrum.

The assembly of heme and Fe-S are critical to aerobic eukaryotes and efforts to understand how the soluble metal is inserted into a hydrophobic molecule like protoporphyrin IX have long been discussed. The observation that this is mediated through a megacomplex answers some of the unresolved questions such as how do the substrates channel to the correct location and in part may also explain how a promiscuous enzyme such as

ferrochelatase, that has been shown to insert multiple metals into the ring, can maintain specificity *in vivo*. Linking cofactor assembly to cristae morphology and therefore mitochondrial health is a way to coordinate investments of energy. If mitochondria are unable to produce heme or Fe-S, then they can be eliminated as by mitophagy or at least reset by increased fission or fusion rates.

Clearly copper and iron are linked to mitochondrial ultrastructure changes. In addition, the uptake of zinc, manganese, and the export of the MoCo assembly intermediates could all depend on specific aspects of the MICOS machinery since all must traverse the IMS during each process. Further understanding of the details of the metal and cofactor trafficking and the interactions with MICOS can provide information as to the regulation of these processes. It is possible that by locating these assembly proteins with a major complex that mediates critical morphology changes in the IM structure, means that metals and the cofactor assembly proteins have positioned themselves in an exclusive neighborhood and therefore are able to monitor, dictate and respond to changes in a dynamic manner to maintain cellular homeostasis and physiology.

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Chapter 2: The involvement of copper in Leigh Syndrome, a fatal neurological disorder

(Unpublished)

Abstract

Copper is an essential yet toxic micronutrient that is required for all eukaryotes to survive. It is primarily utilized as a co-factor for numerous enzymes that are involved in various critical biochemical reactions, such as ETC, iron uptake, and oxidative stress protection. The most important mitochondrial copper enzyme is COX. COX is the terminal electron accepting complex of the electron transport chain. Defects in the assembly of COX can lead to several mitochondrial diseases such as Leigh Syndrome. Leigh syndrome, also known as subacute necrotizing encephalomyelopathy, is a neurodegenerative disease with bilateral lesions in the brain. This condition is characterized by a progressive loss of mental and movement abilities in which these symptoms start as early as three months old and usually results in death within two to three years. Earlier studies in mammalian systems including cell culture and mouse models have reported a defect in cytochrome c oxidase (COX) activity. Here we show the involvement of copper and possible roles that can lead to potential therapeutic mechanisms for Leigh Syndrome.

Introduction

Copper is an essential yet toxic micronutrient that is required for all eukaryotes to survive. It is primarily utilized as a co-factor for numerous enzymes that are involved in various critical biochemical reactions, such as ETC, iron uptake, and oxidative stress protection [3]. Majority of mitochondrial copper is found in the matrix bound by a ligand that maintains an inert protected complex, while the exact identity of the stabilizing ligand is unknown, the localization to the matrix is clear. This matrix copper pool is required for the metalation of Cytochrome *c* Oxidase (COX). Because the inner membrane is sealed to maintain membrane potential, a transporter is required for copper to enter the matrix. Mitochondria have four major classes of transporters in the IM, which includes the mitochondrial carrier family (MCF/SLC25), ATP-binding cassette (ABC) transporters, mitochondrial pyruvate carrier (MPC), and sideroflexins [31]. MCF proteins form the largest family in the IM with 53 members in humans and 30 members in yeast that are collectively responsible for the transport of numerous substrates including various Krebs cycle intermediates, nucleoside di- and triphosphates for energy metabolism and nucleotide replication, and amino acids for degradation or maintenance of the urea cycle [32]. SLC25A3, a member of MCF, carries phosphate and transports copper across the mitochondrial inner membrane into the matrix. Importantly, the insertion of the copper cofactors into COX occurs in the intermembrane space; thus, inner membrane localized carriers are required to move copper both into and out of the mitochondrial matrix [6]. Yet, no transporter has been identified for the export of copper from the matrix to the intermembrane space.

The major Cu enzymes in mitochondria are cytochrome *c* oxidase (COX) which is found in the mitochondrial inner membrane, and Cu, Zn superoxide dismutase (SOD1) which is localized to the intermembrane space (IMS) [21, 22]. The organelle has a suite of protein chaperones dedicated to Cu delivery to both COX and SOD1; the associated Cu transfer reactions occur in the IMS, the compartment between the outer and inner membrane (IM). [23]. The disruption of intracellular copper homeostasis introduces inappropriate biochemical interactions and the presence of free copper within the cells generates damaging reactive oxidative species. SOD1 catalyzes the conversion of the radical in molecular oxygen to hydrogen peroxide (H_2O_2) through the alternate reduction and oxidation of Cu [11]. H_2O_2 is then detoxified independently by catalase and glutathione peroxidase. While >90% of total cellular SOD1 is found in the cytosol, about 5% is localized to the IMS [12]. The mitochondrial localization of SOD1 appears to be dependent on a "fold-and-trap"-like mechanism, as retention of the protein upon import into the IMS requires that SOD1 co-localizes with its cognate chaperone CCS and that its Cu co-factor is available [11-13]. SOD1 lacks a traditional mitochondrial targeting sequence at its amino terminus or an internal amino acid motif that targets it to the organelle. Rather, mitochondrial import of SOD1 has been shown to depend on the activity of the MIA40/ERV1 pathway, which is also responsible for CCS import. To function properly, this pathway requires that MIA40 form a transient disulfide bonded intermediate with CCS before its subsequent resolution and release of the substrate. ERV1 then oxidizes MIA40 so it can catalyze additional import reactions [14]. SOD1 must be metal free and have its essential disulfide bond reduced to translocate across the outer membrane (OM) and interact with the MIA40/ERV1 relay system. Once unfolded, apo-SOD1 enters the IMS, the metallochaperone

CCS facilitates insertion of its Cu co-factor and formation of the disulfide bond, effectively trapping SOD1 in the IMS [15-18].

The relative prioritization and distribution of Cu to COX and SOD1 within the IMS appears to be regulated at least in part by the COX assembly factor CMC1, because manipulating CMC1 expression levels affects the amount of enzymatically active SOD1 [19, 20]. However, the mechanisms that distribute Cu within the IMS and prioritize it for COX assembly or SOD1 maturation are unknown.

The most important mitochondrial copper enzyme is COX. COX is the terminal electron accepting complex of the electron transport chain. The electrons shuttled from reduced electron carriers eventually are passed to oxygen which is reduced to water. This enzyme has 14 subunits; three subunits that form the catalytic core, COX1, COX2, and COX3, are encoded by the mitochondrial genome and 10 subunits remaining, COX4, COX5a, COX5b, COX6a, COX6b, COX6c, COX7a, COX7b, COX7c, and COX8, NDUF4 encoded in the nuclear genome [9]. These subunits are assembled into the mature complex through a complicated pathway with coordinated action of greater than 30 assembly factors. These factors are required to control the translation and insertion of both nuclear encoded proteins and the mitochondrial encoded core subunits and incorporation of the copper and heme cofactors. In the catalytic core, COX3 is involved minimally, COX2 binds the binuclear CuA site that is required for accepting electrons from cytochrome c, and COX1 accepts electrons from COX2 through its cofactors and transfers the electrons to heme a and then to the heme a_3-CuB site where oxygen is bound. Cox1 is the largest subunit that contains a heme *a* and a_3 plus CuB cofactors for oxygen reduction and channels for proton translocation [3,7].

The formation of COX1 is determined by several assembly factors such as COA1, COA3, and SURF1 proteins. SURF1 is the human homolog of the yeast protein SHY1 and is one of many assembly factors involved in the regulation of cytochrome c assembly, specifically the heme a formation of COX1. This protein is a component of the mitochondria translation regulation assembly intermediate of cytochrome c oxidase complex or MITRAC [8]. Interactions within the MITRAC helps to regulate copper within mitochondria. COA1 and COA3 are also a part of the MITRAC complex where in yeast, COA1 binds with SHY1 for heme insertion and COA3 binds with SHY1 for COX1 translation efficiency.

Improper assembly of COX can lead to several mitochondrial disorders. Mitochondrial disorders are characterized as genetic disorders that have defects in oxidative phosphorylation where majority of the dysfunctions are encoded in the nuclear genome. These defects are caused by mutations where each individual mutation shows a tissue specificity. Although some disorders have been determined, there is still much to understand about the other diseases that are not solved. Leigh syndrome, also known as subacute necrotizing encephalomyelopathy, is one of the disorders where much isn't understood on a molecular level. Leigh Syndrome is a neurodegenerative disease with bilateral lesions in the brain. This condition is characterized by progressive loss of mental and movement abilities in which these symptoms start as early as three months old. The usual results end in death within two to three years. There is a small population that do not develop symptoms until adulthood or have symptoms that worsen more slowly [1]. Studies in mammalian systems including cell culture and mouse models have reported a defect in cytochrome c oxidase (COX) activity [2].

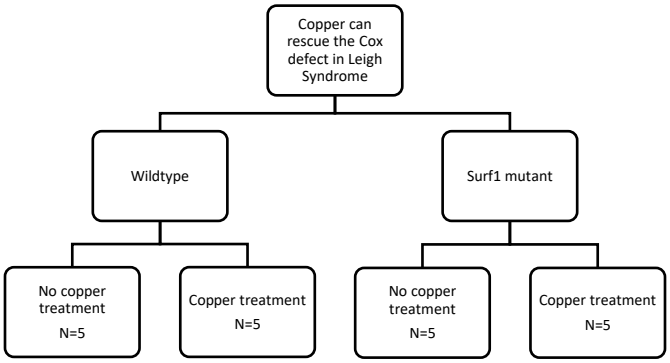
Objective

It has been established that mutations in the gene encoding SURF1 lead to Leigh Syndrome. These mutations generally have resulted in unstable protein that is rapidly turned over making many of these patients essentially null mutants for SURF1. Experiments in yeast have demonstrated that the complete deletion of the SURF1 homolog (*SHY1*) results in decreased mitochondrial copper content and decreased COX activity. In this chapter I used patient cells and cells with *SURF1* mRNA depleted by siRNA to investigate if this copper phenotype is conserved and what if any role this depletion plays in the phenotype. Based on the previous yeast experiments, I hypothesized that the addition of exogenous copper to mammalian cells depleted of *SURF1* will reverse the COX defect demonstrating that the copper defect is a major contributor to this phenotype.

Experimental Design

To test this hypothesis and answer the questions about the role of copper in Leigh syndrome, I used a yeast model and a human cell culture model. In cell culture I used wild-type human patient fibroblast cells and patient derived mutant cells with a *SURF1* defect. Along with the mutant cells, I also mimicked Leigh Syndrome in cells using RNAi to knock down *SURF1*. Cells were treated with the addition of copper to determine if there was a rescue of the defect of COX. With a total of five replicates each, I tested the copper content in the whole cell versus the mitochondria using ICP-OES. Next, I looked at COX activity in the cells using a chemically reduced cytochrome c and monitored the conversion of reduced to oxidized with standard absorbance over time assay. To look at the copper effect on other copper containing enzymes, I

examined superoxide dismutase activity in human cells and superoxide dismutase stability in the IMS of yeast. I then looked at the effects of copper in response to oxidative stress using a hydrogen peroxide and diamide stress assay. Below is a summarized scheme of the approach that I took to conduct this study.



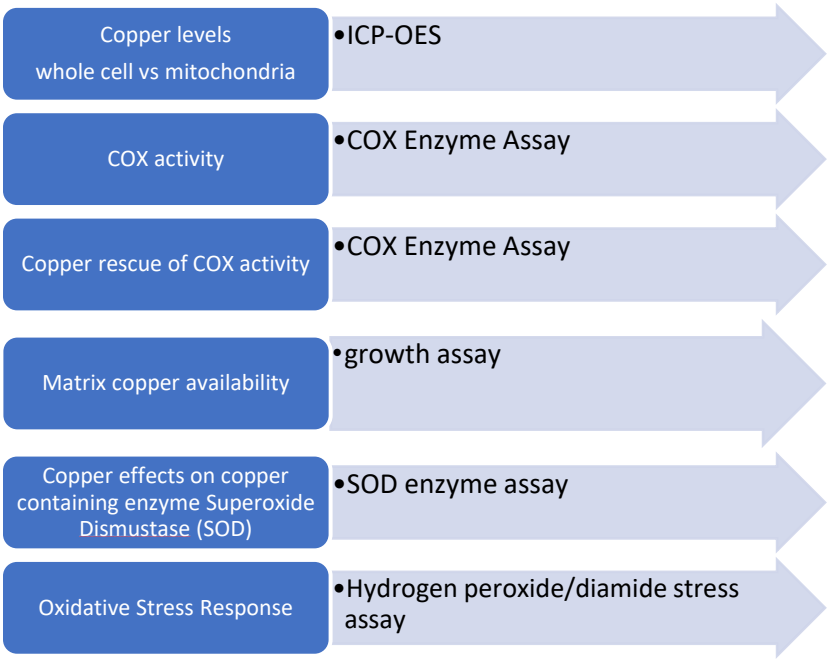


Table 2.1: Experimental Design. Experimental design for majority of experiments includes a comparison of wild-type and mutant cells for total metals and then physiological consequences of copper deficiency**.**

Materials and Methods

Cell culture, Transfection, and Copper supplementation

Cells were maintained in high-glucose DMEM containing sodium pyruvate, 50 µg/ml uridine, and 10% fetal bovine serum at 37 °C at an atmosphere of 5% CO_2 for 24-48 hours. Cells were treated with siRNA for SURF1 and COA1 (Human Silencer Select). All oligonucleotides were transfected into cells at 60–80% confluence using the RNAi Max protocol at 8 nM final concentration (Thermo Fisher Scientific) and allowed to grow for 24–48 h at 37 °C at an atmosphere of 5% CO_2. After incubation, Cu-ionophore complexes (Cu-Atsm and Elesclomol) at 40 µM concentration were added to the cells and incubated for an additional 24 hours. COX deficient patient cells 5092, MCH65 (Montreal Children's Hospital patient #65), and HEK293 (Human embryonic kidney cells) cells were used throughout the study.

Diacetyl-bis(N4-methylthiosemicarbazone) (ATSM) was synthesized as described 5 g of 4-methyl-3-thiosemicarbazide was added to 100 ml of ethanol (100 ml), heated to 60 °C, followed by the addition of 2 ml of diacetyl (2,3-butadione). ATSM was precipitated by addition of concentrated sulfuric acid, then collected by filtration, and washed with methanol. Cu-ATSM was made by dissolving 4 g of ATSM in 100 ml of methanol, followed by the dropwise addition of copper chloride dissolved in water, and stirring the mixture for 30 min. Cu-ATSM was precipitated by adding water; the product was collected via filtration and washed with methanol and water. Cu-ATSM was diluted in DMSO (0.75 g/15 ml) and then added to the culture medium at 40 µM final concentration.

Yeast Culture

BY4741 strains deleted for *SHY1* and *COA1* were used throughout the study. For growth assays, cultures were grown on standard glucose medium, glycerol-lactate medium, and glycerol-lactate-copper medium at 30 °C for 24–36 hours. Matrix copper was measured by growth of *sod2*Δ transformants in Shy1Δ and Coa1Δ in hyperoxia verses normoxia environment. Agar plates were prepared with medium containing 0.4 µM $CuSO_4$, 100 µM $CuSO_4$, or lacking added copper and were incubated in a chamber purged with 100% oxygen for hyperoxia growth or cultured in atmospheric oxygen for normoxia growth. Transformants also included cells harboring human Sod1 targeted to the matrix (m-h*Sod1*).

ICP-OES

To measure total mitochondrial and cellular copper, intact mitochondria extracted through differential centrifugation and whole cell extracts were isolated from yeast and cell culture. Extractions were digested in sealed, acid-washed tubes at 95°C in 150 µl of metal-free 40% nitric acid (Optima). The samples were then diluted to 1 ml into double-distilled water for analysis. Serial dilutions of commercially available mixed metal standards were used to construct a standard curve. Blanks of nitric acid or buffer samples were also digested in the acid-washed tubes for comparison, and spiked controls were analyzed to ensure reproducibility [36].

Enzyme activity

Cytochrome *c* oxidase (COX) and malate dehydrogenase (MDH) activities were measured as described previously using a Shimadzu UV-2450 spectrophotometer [36]. COX activity was determined by monitoring the reduction of 32 µM bovine cytochrome *c* at an absorbance of 550 nm by 5-10 µg of cell extracts (40 mM KH_2PO_4, pH 6.7, 0.5% Tween 20). MDH activity was determined by monitoring the reduction of 6 mM oxaloacetate and 3.75 mM NADH at an absorbance of 340 nm. All activities for COX and MDH were normalized to protein concentration using Bradford reagent, and the average of COX/MDH determine total COX activity. Superoxide dismutase (SOD) activity was measured using a xanthine oxidase-linked assay kit (Cayman Chemical) and absorbance was measured at 460 nm on Cytation 3 plate reader (BioTek). Analysis for SOD activity was determined using the SOD (U/ml) formula provided within the assay kit (Cayman Chemical) and converted to percent inhibition.

Oxidative Stress Assay

Oxidative stress was measured by multiple methods. Cells were grown on a 96 well plate and treated with 1 mM concentration of diamide was added to each well with an incubation period of 2 hours. Cell proliferation was then measured using the MTT cell proliferation assay kit (BioVision) and absorbance was read at 590 nm. Proliferation was calculated as percent viability using the cell viability formula provided with the assay kit and normalized to wildtype. Cu-ATSM was added at a concentration of 80 µM to the cells and incubated for an additional 24 hours. Treatment of diamide and MTT proliferation followed incubation of copper.

A separate set of cells were grown on 12 well plates and treated with 100 μM concentration of hydrogen peroxide with an incubation period of 2 hours. Cell viability was measured using crystal violet and the absorbance was read at 570 nm. Cell viability was calculated using the same formula from the MTT assay and normalized to wildtype. Cu-ATSM was added at a concentration of 80 μM to the cells and incubated for an additional 24 hours. Treatment of hydrogen peroxide and crystal violet viability followed incubation of copper.

Immunoblot Assay

Immunoblot analysis was used to determine the steady state levels of proteins. 10-25 μg of total protein from mitochondria were separated on a 15% SDS-PAGE gel system and electrophoretically transferred onto a nitrocellulose membrane. Membranes were blocked in 1× Rapid-block blocking solution (VWR Life Science) before protein detection. The monoclonal anti-human SOD1 was purchased from Santa Cruz Biotechnology. Loading controls were performed using porin (mitochondria).

Data Analysis

All results are presented as mean with errors bars of standard error of the mean. p values<0.05 were considered significant as assessed by Student's t test or one-way ANOVA.

Results

Total mitochondrial copper in COX mutants

Studies of Leigh Syndrome patient cells with mutation in the assembly factor SURF1 have shown a defect in COX activity and stability [39]. Defects in COX assembly factors result in decreased COX due to proteolytic turnover of the partially assembled enzyme. This enzyme accounts for about 15-25% of mitochondrial copper depending on the conditions and tissue examined. To see if total mitochondrial copper is affected by the defect of the assembly factors, we measured the total amount of mitochondrial copper in COX assembly factor mutants using inductively coupled plasma atomic emission spectroscopy (ICP-OES). Purified mitochondria from yeast mutants included mitochondrially-encoded copper binding structural subunits (cox1Δ, cox2Δ), assembly factors for CuA site in COX2 (*sco1Δ*, *cox17Δ*), assembly factors for Cox1 (including CuB site) (*mss51Δ*, *cox14Δ*, *cox10Δ*, *cox11Δ*, *cox19Δ*) and the copper importer Pic2 (*pic2Δ*) plus the s*hy1Δ* (SURF1-homolog) and *coa1Δ* (interactor with Shy1 in Cox1 assembly) were analyzed (Fig 2.1). Of the tested mutants all had decreased levels of COX but most had no effect on total copper. However, deletion of the copper transporter Pic2, and unexpectedly, the assembly factors Shy1 and Coa1 showed substantially lower amount of total mitochondrial copper (Fig 2.1). This demonstrates that when assembly factors Shy1 and Coa1 are defective, copper is affected in not only COX but also in the matrix and potentially for mitochondrial superoxide dismutase (SOD1).

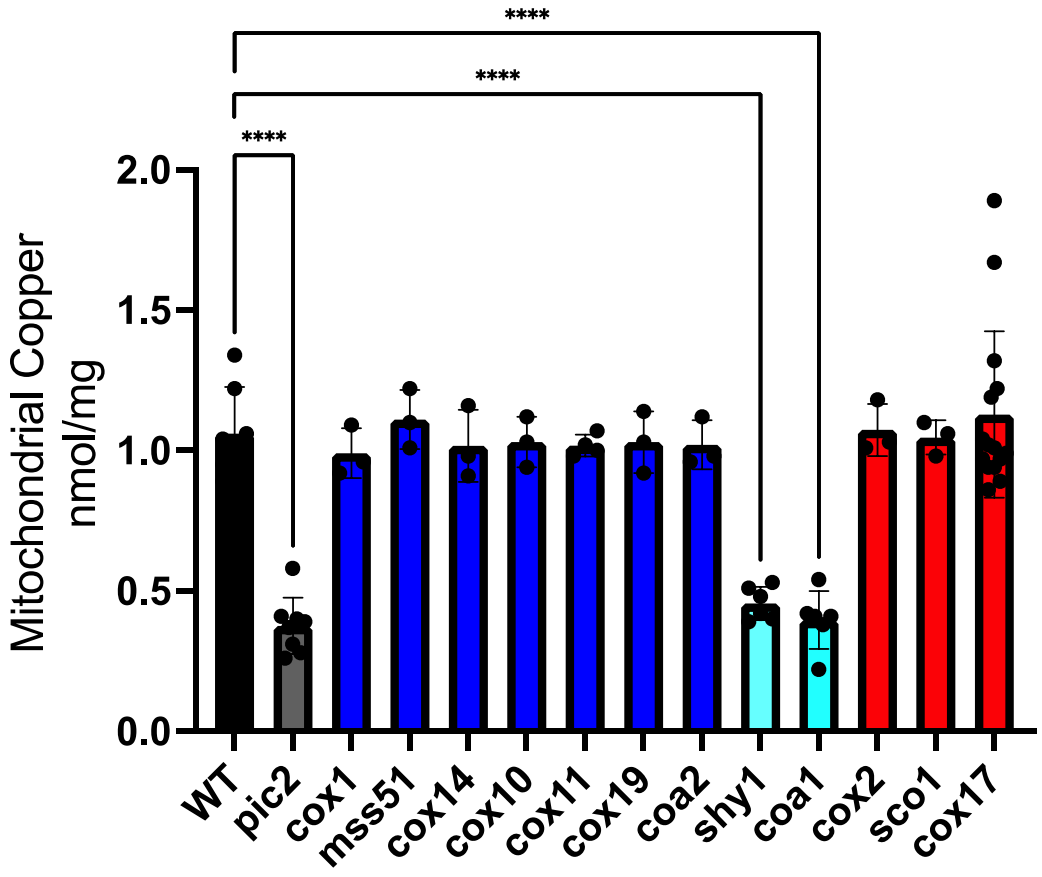


Figure 2.1 ICP-OES analysis of purified mitochondria from various yeast mutants. All mutants have a COX deficiency. The mutants colored blue are involved in the assembly or translation of COX1 while the bars colored red are mutants in genes encoding proteins involved in COX2 assembly. All mutants result in undetectable levels of the core subunits in mitochondria. Pic2 is the mitochondrial carrier family protein required for copper import into mitochondria. Results of one-way ANOVA are shown when $p<0.05$.

Matrix copper availability

To confirm that these mutants have lower bioavailable matrix copper I measured the activation of heterologously targeted human SOD1. SOD2 is the manganese containing superoxide dismutase in the mitochondrial matrix. When SOD2 is deleted, yeast become susceptible to hyperoxia. However, this phenotype can be reversed by expressing a matrix-targeted human SOD1 (m-hSOD1) (Fig 2.2). Activation of this enzyme is dependent on available copper levels and can be modulated by changing available copper in the media. I confirmed the decreased mitochondrial copper in *shy1Δ* and *coa1Δ* by showing the lack of cell growth in a hypoxic environment indicated by the failure to activate m-hSOD1 especially under copper restricted conditions (Fig 2.2). Increasing copper reversed the defect suggesting it was the limiting factor in the experiment.

The stability and retention of yeast superoxide dismutase in the IMS is dependent on available mitochondrial copper. Expression of matrix targeted competitors such as metallothionein reduced IMS Sod1 levels. Immunoblot analysis of cells lacking *COA1* or *SHY1* revealed a similar decreased steady state level of IMS-SOD1 (Fig 2.3) which is an independent indicator of mitochondrial copper deficiency.

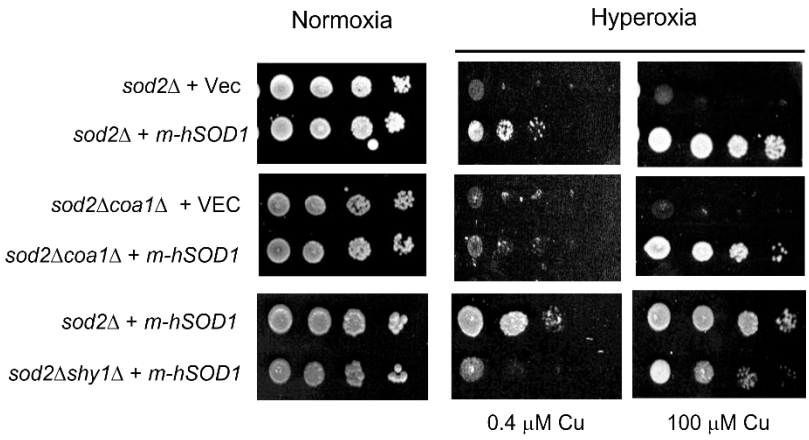


Figure 2.2 Bioassay of matrix copper availability. Sod2 is a manganese containing superoxide dismutase in the mitochondrial matrix. Deletion of *SOD2* makes the yeast susceptible to hyperoxia. However, this phenotype can be reversed by expressing a matrix-targeted human SOD1 (m-hSOD1). Activation of this enzyme is dependent on available copper levels and can be modulated by changing media copper levels or disrupting genes involved in mitochondrial copper homeostasis. Hyperoxia defect of control cells (*sod2Δ*) can be rescued on media with as little as 0.4 μM Cu (*sod2Δ + m-hSOD1*) while deletion of *COA1* or *SHY1* prevents this rescue.

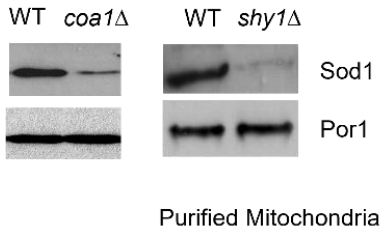


Figure 2.3 Immunoblot analysis of yeast Sod1 in purified mitochondria. Mitochondria were purified from wild-type (WT) or cells lacking *COA1* (*coa1Δ*) or *SHY1* (*shy1Δ*) and western blotted for Sod1 and porin (mitochondrial loading control). The stability of Sod1 in mitochondrial IMS is dependent on available copper.

Copper rescue of growth defect in yeast on non-fermentable carbon sources

Due to the lack of mitochondrial copper in the mutant cells, I wanted to observe if supplemental copper would reverse the COX defect. When yeast grow on glucose rich media, they use fermentation to recycle the redox equivalents needed for production of ATP. When grown on non-fermentable carbon sources such as glycerol-lactate (gly-lac), they are forced to assemble COX and use the electron transport chain to recycle redox intermediates to produce ATP. Both *shy1Δ* and *coa1Δ* grow robustly on glucose rich media but cannot grow on media with glycerol-lactate as the carbon source (Fig 2.4). I predicted that an increase in copper would reverse the COX defect in these mutant cells. Growth was improved when supplemental copper was added to the nonfermentable media.

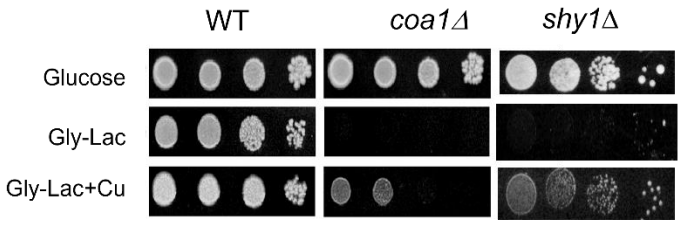


Figure 2.4 Copper rescue of yeast phenotypes. Yeast lacking *COA1* or *SHY1* cannot produce COX and therefore are unable to grow on media containing only non-fermentable carbon sources such as glycerol or lactate. However, this defect can be reversed by the addition of copper to the medium.

Copper levels in COX deficient cell lines

SURF1, the homolog of *SHY1*, has been established as a COX assembly factor that when mutated leads to Leigh Syndrome in humans. To investigate if the copper defects exist in humans, I replicated the experiments from yeast in human fibroblasts in cell culture. I first tested total mitochondrial copper levels in whole cell and isolated mitochondria in human fibroblasts. Mitochondrial copper in the mutant patient cell line (*SURF*$^{-/-}$) was measured via ICP-OES, I observed a decrease in total mitochondrial copper compared to the wild-type (Fig 2.5). I also measured total cellular copper in the mutant cells (Fig 2.5). For comparison I measured cellular copper in *SCO1*$^{-/-}$ that has a similar COX defect but also a copper deficiency that is induced by a signaling cascade that regulates the localization and levels of Cu-transporters, and *ATP7A*$^{-/-}$ the copper exporter in fibroblasts. In contrast to the signaling pathway activated in *SCO1*$^{-/-}$, *SURF1*$^{-/-}$ fibroblasts resulted in a slight increase in cellular copper content despite the decrease in mitochondrial copper.

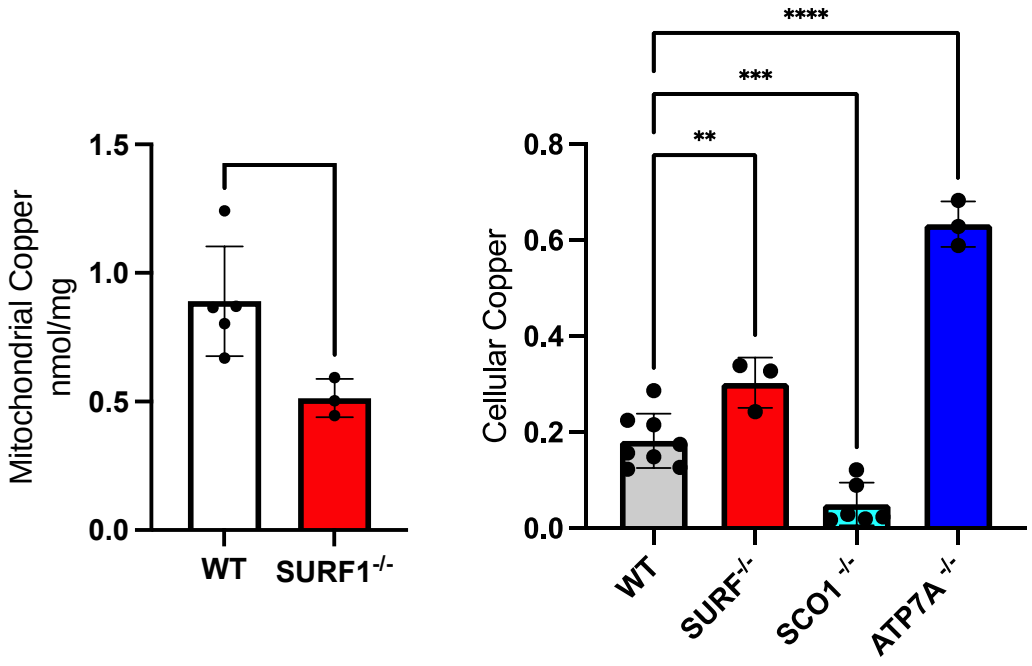


Figure 2.5. Copper analysis of SURF1 mutant. ICP-OES analysis of mitochondrial purified from WT fibroblasts from *SURF1* knock-out fibroblasts. Whole cell copper in WT, SURF1, SCO1 (Cu-deficient) and ATP7A (Cu-loaded) fibroblasts. Results of Student's t-test or one-way ANOVA are shown when $p<0.05$.

COX rescue in mammalian cells

With mitochondria copper being low in human SURF1 cells, I wanted to investigate if copper could rescue the COX defect as in the yeast model. I cultured wildtype and SURF1 mutant fibroblasts in regular media or media supplemented with Cu-histidine. Cu-histidine has been used as a supplement in the treatment of human copper deficiency. SURF1 fibroblasts cultured with supplementation by Cu-Histidine (SURF1 Cu-HIS) showed an increase in cellular copper ~3 fold but this was unable to rescue COX activity (Fig 2.6). This could be due to Cu-HIS not being able to accumulate in mitochondria.

The discovery that ionophores, such as elesclomol, can be used to deliver Cu and restore function in numerous animal and cell models of Cu-related disorders has been a major advance in the field [24]. While formerly tested at much higher concentrations as a cancer therapeutic, the application of exceptionally low concentrations of elesclomol were found to enhance Cu uptake and liberation of internal Cu stores, allowing for rescue of Cu associated defects in a number of subcellular compartments in multiple yeast and mammalian models [24-27]. In addition, direct injection of the Cu-binding ionophore ATSM is in a Phase2/3 clinical trial (clincaltrials.gov: NCT04082832) to partially correct disease symptoms in patients with amyotrophic lateral sclerosis (ALS) [28]. However, all ionophores and related compounds are toxic when used at high concentrations. In yeast, compounds like 2-(6-benzyl-2-pyridyl)quinazoline cause significant cellular and mitochondrial accumulation of Cu resulting in enhanced toxicity [29]. Cu-elesclomol can induce cell death in mammalian systems, and a genome wide CRISPR screen for suppressors resistant to this ionophore identified mitochondrial ferredoxin as a target that can modulate toxicity [30]. This discovery led to the

description of a Cu-elesclomol triggered, ferredoxin-dependent form of cell death termed cuproptosis, which occurs independently of known markers of apoptosis and ferroptosis [30]. In contrast to Cu-His, the COX deficiency can be reversed by the addition of Cu-ATSM to the culture medium. Cu-ATSM is an ionophore that can efficiently cross membranes and has been successfully used to rescue the Cu-modulated COX defect in SLC25A3 (mitochondrial Cu-transporter) knockout cells.

To further establish that copper can reverse the COX defect I used SURF1 RNAi treatment of treated HEK293 cells. The addition of siRNA showed a COX defect relative to control cells but the COX deficiency of HEK293 cells treated with SURF1 RNAi was reversed by the addition of Cu-ATSM (Fig 2.7) and Cu-elesclomol (Fig 2.8). Cu-elesclomol is a new and leading candidate for therapy of Cu-deficiency (with purported decreased toxicity relative to ATSM).

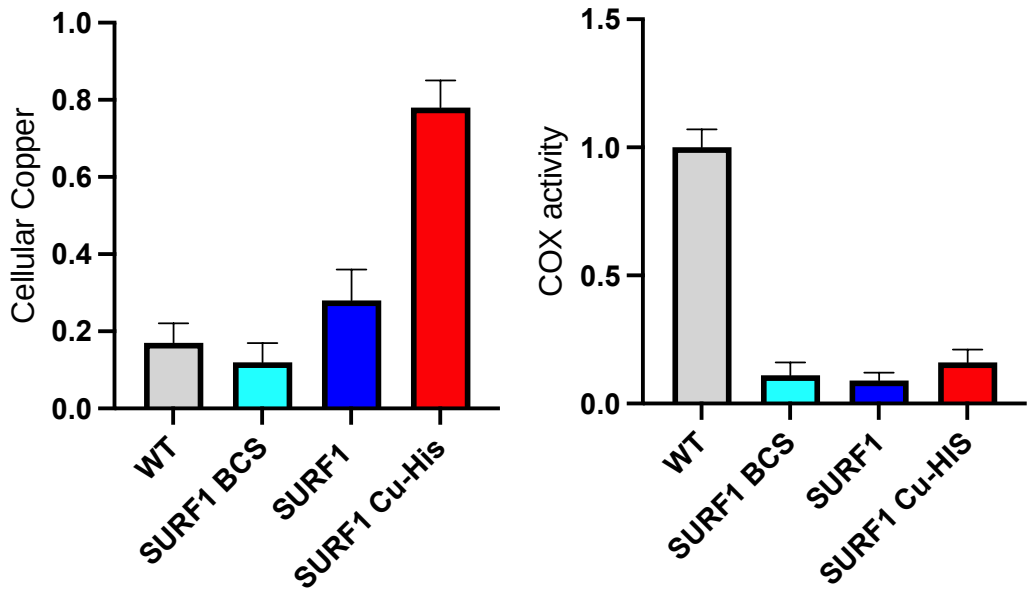


Figure 2.6 Cu treatment of SURF1 mutant cells. Cells grown in either copper limitation (BCS) or Cu-excess (Cu-HIS) were compared for total copper levels via ICP-OES or COX activity. Cu-HIS increased total cellular copper three-fold but did not rescue the COX defect.

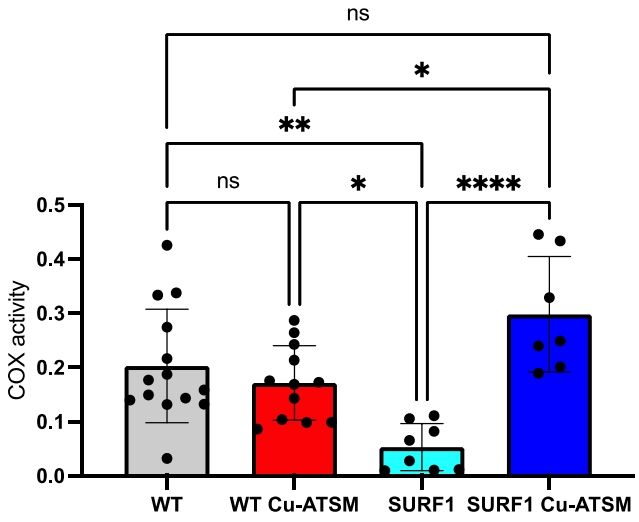


Figure 2.7 Rescue of COX with Cu-ionophores. Immortalized fibroblasts were assayed for COX activity in the presence or absence of supplemental Cu-ATSM. Results of a one-way ANOVA are shown.

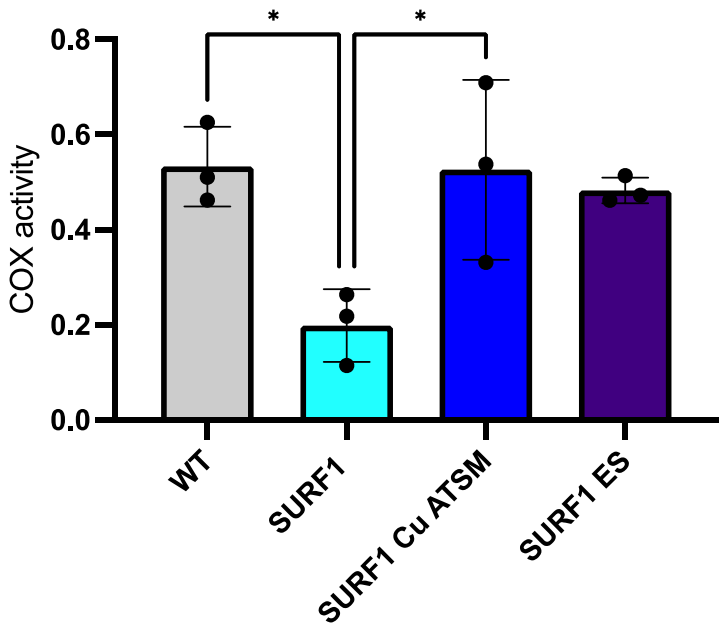


Figure 2.8 SURF1 RNAi knockdowns have a COX defect that can be rescued by Cu-ionophores. HEK293 cells treated with scrambled control (WT) or SURF1-RNAi were assayed for COX activity. The knockdown of SURF1 resulted in decreased normalized COX activity that was rescued by the addition of ionophores. The results of one-way ANOVA are shown when $p<0.05$ (SURF1 vs SURF1 ES was not significant at $p=0.0553$)

SOD1 activity in human fibroblasts

Mitochondrial copper stored in the matrix is used as cofactors for COX and SOD1. After measuring COX activity and copper rescue of COX, I wanted to investigate if there was a defect of SOD1 activity and if copper could rescue the activity. One unit of SOD1 is the amount of enzyme to exhibit 50% dismutation of the superoxide radical. SURF1 RNAi in HEK293 shows a significant loss of total SOD activity, ~38% activity (Fig 2.9). The addition of copper increased the activity of SOD to ~71%.

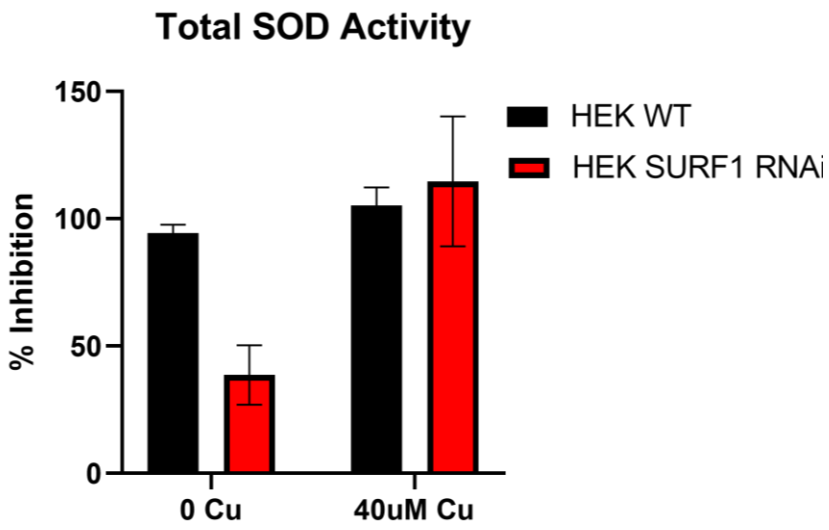


Figure 2.9: SOD activity in fibroblasts
SURF1 RNAi fibroblasts show a significant loss in total SOD activity (n=4 *, $p < 0.05$) that was rescued by the addition of copper (n=4 *, $p < 0.05$). Although there was an increase of cellular copper levels in the mutants, total SOD activity accounts for SOD in the cytosol and in mitochondria. SOD2 activity was not changed by Cu-supplementation

Oxidative Stress in human fibroblasts

Since total SOD activity showed a decrease in the fibroblasts, I wanted to examine sensitivity to oxidative stressors. I looked at both wild-type, *SURF1* mutant fibroblasts, and *SURF1* RNAi treated cells. Cells were treated with 1 mM diamide, which produces oxidation of thiols depleting stress responsive proteins and glutathione to induce ROS production. Control cell growth decreased when diamide was added to the cells presumably due to oxidative stress. However, the *SURF1* mutant cells seemed resistant to this treatment albeit they were growing at a slower initial growth rate (Fig 2.10). I also treated HEK293 cells with and without *SURF1* RNAi with 100 µM of hydrogen peroxide and measured cell viability. I observed the same results for hydrogen peroxide treatment with *SURF1* knockdown showing resistance to stress. Being that mitochondrial copper is decreased in the mutant cells, I wanted to observe if an influx of copper would trigger a different response to oxidative stress. I added 80 µM of Cu-ATSM to the cells for 24 hours then treated the cells with diamide or hydrogen peroxide. The increase in copper caused increased sensitivity in the wild-type and reversed the observed resistance to oxidative stressors in the mutant cells.

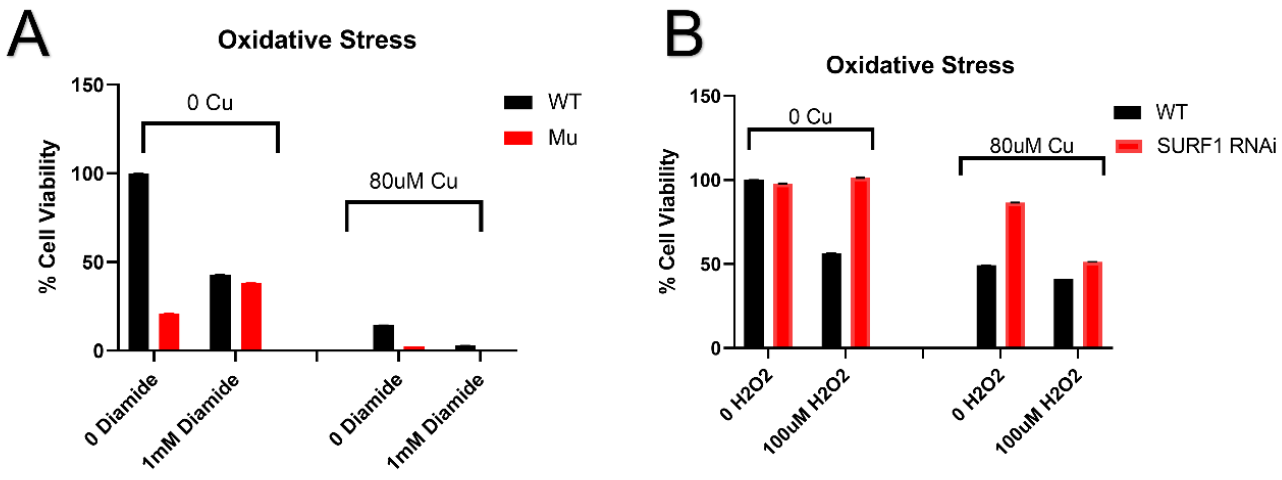


Figure 2.10: Oxidative stress in fibroblasts
SURF1 mutant fibroblasts and *SURF1* RNAi were treated with either diamide or hydrogen peroxide to induce oxidative stress. A) Fibroblast mutants show a resistance to treatment with diamide compared to wildtype cells (n=4 ****, $p < 0.0001$) which was reversed with addition of exogenous copper (n=4; ****, $p < 0.0001$). B) *SURF1* RNAi mutants treated with hydrogen peroxide also showed a resistance to the stress (n=4; ****, $p < 0.0001$) and showed sensitivity to stress with the addition of copper (n=4; ****, $p < 0.0001$).

Discussion

Copper is an essential element required for the proper functioning of cells in mammals. Maintaining copper homeostasis is crucial in preventing defects that lead to diseases. Copper is especially important in mitochondria as it is utilized for the major enzymes, COX for respiration and SOD1 for protection against oxidative stress. COX deficiency caused by mutation in assembly factors is a leading cause of fatal human diseases such as Leigh Syndrome. Leigh Syndrome is defined by a specific brain pathology and can be caused by multiple mutations (75 different locus' recorded) [37] and in this study I focused on *SURF1* which is found in 15% of all cases. Previous studies in yeast showed mutation of *SHY1* (yeast homolog of SURF1 in humans) causes COX to be defect and the Shy1 associates with numerous factors including a protein named Coa1 that has a role in mitochondrial copper metabolism [38]. In fact, when looking at yeast, we were able to show that mutation of the genes encoding Shy1 and Coa1 resulted in total mitochondrial copper being decreased. In this study I endeavored to determine if the role in maintenance of mitochondrial copper was conserved in mammalian cells.

The total levels of copper in mitochondria mostly reflects a storage pool in the matrix. This copper is imported by the mitochondrial carrier family protein SLC25A3 (or Pic2 in yeast). This stored copper is used to build COX and IMS-SOD1 once it is released back to the IMS. However, the regulation of this pool is an open question, and the identity of the exporter is unknown. A decreased level of mitochondrial copper in the SURF1 mutant presumably results in a failure to redistribute copper to the IMS and therefore loss of COX and mitochondrial SOD1. Adding exogenous copper to the cell should compensate for the losses and return copper to normal levels. However, membranes form the greatest barrier to homoeostasis and could

prevent free access of copper to the necessary cellular locations. When copper was added as Cu-HIS cellular copper increased but did not rescue the enzyme activities. However, when adding copper in the form of ionophores that can bypass the membranes COX and SOD1 activities were restored. These data suggest that SURF1 plays a role in copper import and that it cannot simply be overcome by high cytosolic levels of copper further supporting the hypothesis that copper must come from the matrix to metalate COX.

Interestingly total cellular SOD1 levels have been shown to decrease in SLC25A3 mutant cells so perhaps a similar mechanism is present in SHY1 mutants as only 5% of total cellular SOD1 is localized to the IMS, so the 50% decrease reflects greater than mitochondrial SOD1. Increased copper availability in the cytosol does not require ionophore treatment so this activity could be rescued without ionophores. However, there are still additional unknown signals that would be needed to explain the deficiency in cytosolic SOD1 when you have mitochondrial specific defect. The exact nature of this pathway needs to be understood as SOD1 in the cytosol is linked to multiple disorders including amyotrophic lateral sclerosis. Since I showed that total mitochondrial copper was low that resulted in low SOD activity, there should be a susceptibility to oxidative stress in the mutant cells. But I was able to show a resistance to stress in the mutant cells. With the addition of copper, cells became sensitive to the oxidative stress causing a significant decrease in cell viability. This phenomenon may be linked to the assembly intermediates of COX. In an earlier study, *SHY1* mutant cells in yeast were resistant to transient treatments with hydrogen peroxide treatments. The susceptibility of yeast to hydrogen peroxide treatment was linked to a partially assembled COX intermediate. This intermediate exposed a heme A-Cox1 complex that acts as a pro-oxidant [35]. Shy1 was

shown in bacteria (and in this yeast model) to be responsible for the insertion of heme A into this intermediate [38]. Therefore, mutation of SHY1 prevented the accumulation of the pro-oxidant intermediate and led to peroxide resistant cells [35]. Our SURF1 mutant cells showed a resistance to peroxide treatment, supporting a role in heme A insertion in mammalian cells. What I also discovered was that the addition of copper was able to change the resistance of the cells and make them sensitive. This could be due to damage associated with copper in the cytosol via Fenton chemistry or could be due to copper stimulating assembly of COX but the absence of SURF1 results in poorly formed complexes that gain the pro-oxidant activity due to the lack of the SURF1 chaperoning function.

Copper could be important therapeutic in Leigh syndrome. However, the details of how it regulates pro-oxidant intermediates and the delivery of copper to mitochondria are still major questions. Because I was able to bypass the need of SURF1 by using ionophores to get copper through the membranes, I saw that SURF1 has an additional role in regulating copper transport. I will look deeper into how that regulation occurs and if there are other players involved with SURF1.

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Chapter 3: The regulation of mitochondrial copper availability through the interaction of MITRAC components and SLC25A3

(unpublished)

Abstract

Copper is an essential yet toxic micronutrient that is required for all eukaryotes to survive. It is primarily utilized as a co-factor for numerous enzymes that are involved in various critical biochemical reactions, such as ETC, iron uptake, and oxidative stress protection by SOD. The most important mitochondrial copper enzyme is COX. COX is the terminal electron accepting complex of the electron transport chain. These two copper-containing enzymes, COX and SOD, must first be metalated which is mediated by metallochaperone proteins within the IMS to function properly. This makes a specific copper transport pathway necessary. The source of the copper is a labile copper-ligand pool that is localized to the matrix. Studies in yeast and mammalian cells demonstrated that the mitochondrial carrier protein, Pic2/SLC25A3 contributed to copper uptake to the matrix. Mitochondria translation regulation assembly intermediate of cytochrome c oxidase complex or MITRAC is a complex of proteins that bind together to regulate COX1 translation and its insertion in the IM. Interactions within MITRAC helps to regulate copper within mitochondria. Several assembly factors such as COA1, COA3, and SURF1 are a component of MITRAC that determines the formation of COX1. In this study we link copper transporter SLC25A3 with MITRAC members SURF1 and COA1 in the regulation of copper availability and export from the matrix.

Introduction

Copper is important for the proper functioning of the cell. It is required in mitochondria for the function of enzymes involved in respiration, oxidative stress protection, and iron uptake. Maintaining the perfect balance of copper within the cell involves many different mechanisms and factors. Improper regulation of copper can lead to several fatal mitochondrial diseases such as Wilson disease and Menkes disease; therefore, intracellular copper must be properly maintained [1]. The two most important enzymes that need copper are cytochrome *c* oxidase (COX) and superoxide dismutase (SOD). COX and SOD must first be metalated which is mediated by metallochaperone proteins within the IMS to function properly. This makes a specific copper transport pathway necessary [8]. The source of the copper is a labile copper-ligand pool that is localized to the matrix [7]. Studies in yeast and mammalian cells demonstrated that the mitochondrial carrier protein, Pic2/SLC25A3 contributed to copper uptake to the matrix [6].

COX is a copper-heme A terminal oxidase embedded in the mitochondrial inner membrane [2]. It catalyzes electron transfer from reduced cytochrome c to molecular oxygen in a process coupled to the transfer of protons across the inner membrane, thus contributing to the generation of the proton gradient that is used by ATP synthase to drive ATP synthesis. Mammalian COX contains 14 major subunits, two of which bind three redox centers required for electron transfer [3]. The catalytic core consists of the mitochondrially-encoded subunits COX1, COX2, and COX3. COX2 binds the binuclear CuA site required for accepting electrons from cytochrome *c*. These electrons are then transferred to the cofactors of COX1, first to heme *a* and then to the heme *a*3–CuB site where oxygen is bound. COX biogenesis requires more

than 25 auxiliary proteins known as COX assembly factors. Theses assembly factors play an important role related to heme a biosynthesis, copper delivery and homeostasis, transport of nuclear-encoded subunits across the mitochondrial membrane, and translation, membrane insertion and maturation of mitochondrial encoded subunits [4]. Majority of ETC defects that bring about mitochondrial dysfunction and human disease is caused by pathogenic mutations in assembly factors. At a minimum, nine of these factors coordinate the insertion of the copper cofactors that are essential for the catalytic competence of the COX holoenzyme. Studies in yeast has shown that the copper used for COX assembly comes from the matrix and this matrix copper pool is conserved in mammals [3]. Biogenesis of COX occurs in a segmental pathway with different assembly proteins fusing into complexes to promote the merging of structural subunits or cofactor insertion. Early insertion of copper and heme are important because of their location being deep within the core structure of the enzyme. COX1 translation and insertion into the IM are mediated by a suite of specific translational activators and chaperones that form a complex known as MITRAC [5].

Mitochondria translation regulation assembly intermediate of cytochrome c oxidase complex or MITRAC is a complex of proteins that bind together to regulate COX1 translation and its insertion in the IM. Interactions within MITRAC helps to regulate copper within mitochondria. Several assembly factors such as COA1 and SURF1 are a component of MITRAC that determines the formation of COX1 [5]. *SURF1* is the human homolog of the yeast protein SHY1 and is one of many assembly factors specifically involved in heme a insertion into COX1. In yeast, COA1 binds with SHY1 for heme insertion and COA3 binds with SHY1 for COX1 translation efficiency. In this study we will expand our findings in SURF1 from the previous study and

investigate the regulation of copper availability and any potential interactions with other assembly factors and transporters.

Objective

Previous yeast studies have established a connection between *COA1* and *SHY1*. These genes are shown to interact and mutations on these genes had effects on mitochondrial copper content and translation efficiency. Since that study was completed SLC25A3/Pic2 was identified as a copper transporter importing copper to the mitochondrial matrix. Therefore, it is now possible to investigate the mechanistic details of the regulation of copper availability. Critically it is completely unknown and not understood how copper is exported from the matrix to the inner membrane space. I predicted that SURF1 and COA1 proteins form a complex and either in the complex or individually interacts with the copper transporter SLC25A3 to regulate copper transport.

Experimental Design

To investigate the regulation of copper availability, I administered multiple gene silencing of assembly factors *SURF1* and *COA1* and copper transporter *SLC25A3.* I used wild-type cells, patient derived mutant cells, and siRNA silencing of *SURF1*, *COA1*, and *SLC25A3.* I also looked at the overexpression of *PIC2* in yeast cells. I then silenced multiple genes at once to determine any genetic interactions by determining additive phenotypes. The phenotypes that were examined were copper level in the whole cell, copper in mitochondria, COX activity, copper rescue of COX, protein stability using an immunoblot, and ability of copper uptake and oxygen consumption in overexpressed *PIC2* yeast cells. Below is a summarized scheme of the approach that we took to conduct this study.

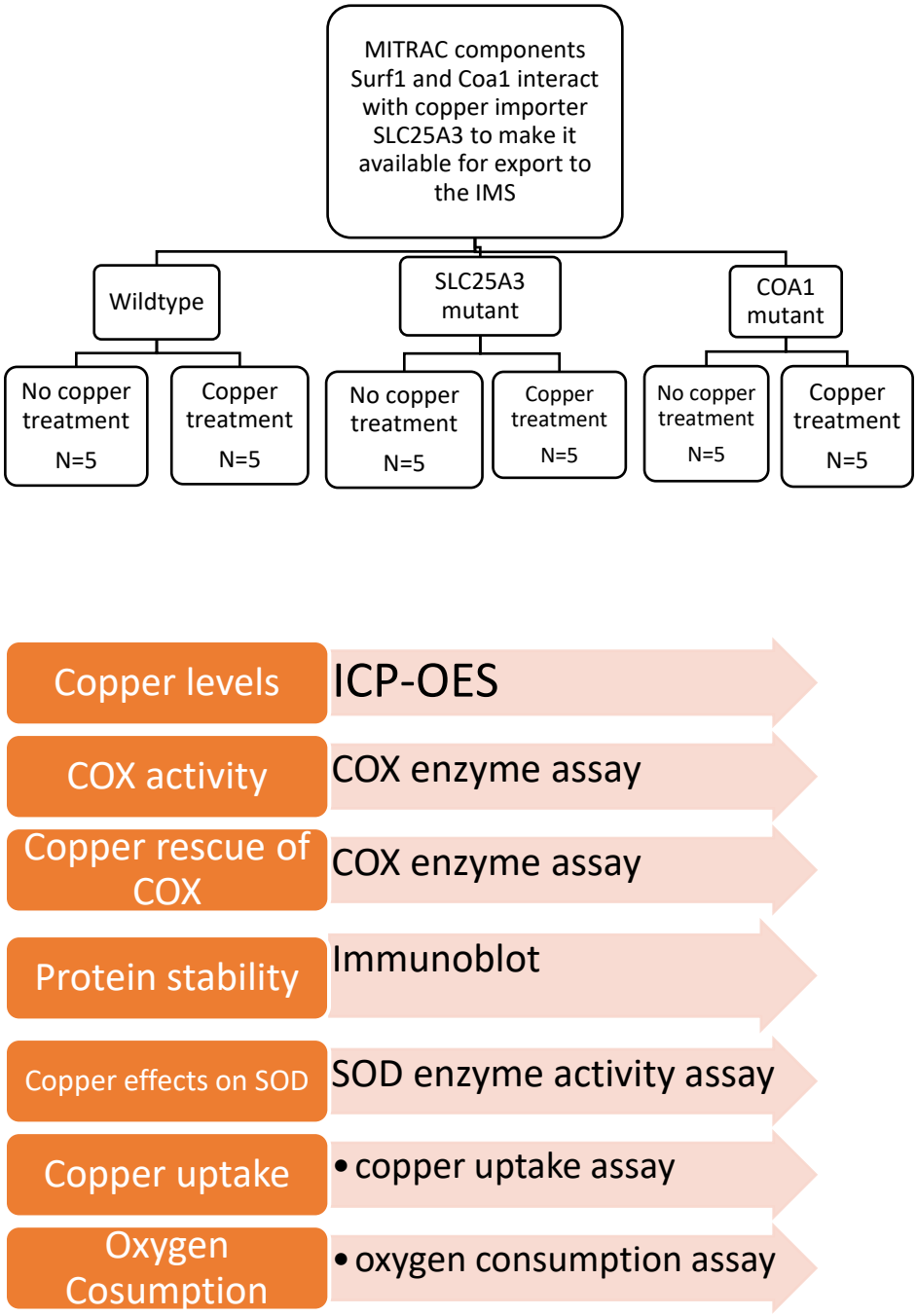

Table 3.1: Experimental design. Experimental design for majority of experiments in this chapter include comparison of wild-type and single and double mutant cells for total metals and then physiological consequences of copper deficiency**.**

Materials and Methods

Cell culture, Transfection, and Copper supplementation

Cells were maintained in high-glucose DMEM containing sodium pyruvate, 50 µg/ml uridine, and 10% fetal bovine serum at 37 °C at an atmosphere of 5% CO_2 for 24-48 hours. Cells were treated with siRNA for *SURF1*, *MITRAC15* (*COA1*), and *SLC25A3* (Human Silencer Select). All oligonucleotides were transfected into cells at 60–80% confluence using the RNAi Max protocol at 8 nM final concentration (Thermo Fisher Scientific) and allowed to grow for 24–48 h at 37 °C at an atmosphere of 5% CO_2. After incubation, Cu-ionophore complexes (Cu-Atsm and Elesclomol) at 40 µM concentration were added to the cells and incubated for an additional 24 hours. MCH65 (Montreal Children's Hospital patient #65), HEK293 (Human embryonic kidney cells), and MEF (mouse embryonic fibroblasts) were used throughout the study. MEF cells were maintained in 15% fetal bovine serum.

Diacetyl-bis(N4-methylthiosemicarbazone) (ATSM) was synthesized as described 5 g of 4-methyl-3-thiosemicarbazide was added to 100 ml of ethanol (100 ml), heated to 60 °C, followed by the addition of 2 ml of diacetyl (2,3-butadione). ATSM was precipitated by addition of concentrated sulfuric acid, then collected by filtration, and washed with methanol. Cu-ATSM was made by dissolving 4 g of ATSM in 100 ml of methanol, followed by the dropwise addition of copper chloride dissolved in water, and stirring the mixture for 30 min. Cu-ATSM was precipitated by adding water; the product was collected via filtration and washed with methanol and water. Cu-ATSM was diluted in DMSO (0.75 g/15 ml) and then added to the culture medium at 40 µM final concentration.

Yeast culture

BY4741 strains deleted for *SHY1* and *COA1* were used throughout the study. For growth assays, cultures were grown on standard glucose medium at 30 °C for 24–36 hours.

Copper uptake

The mitochondrial copper uptake rate was obtained by incubation of isolated mitochondria with copper for 0–4 min at room temperature, centrifugation, and then acid digestion followed by ICP-OES (PerkinElmer Life Sciences 7300-DV). Uptake was reported as the increase in copper over time [11].

ICP-OES

To measure total mitochondrial and cellular copper, intact mitochondria and whole cell extracts were isolated from yeast and cell culture were digested in sealed, acid-washed tubes at 95°C in 150 µl of metal-free 40% nitric acid (Optima). The samples were then diluted to 1 ml into double-distilled water for analysis. Serial dilutions of commercially available mixed metal standards were used to construct a standard curve. Blanks of nitric acid or buffer samples were also digested in the acid-washed tubes for comparison, and spiked controls were analyzed to ensure reproducibility [11].

Enzyme activity

Cytochrome *c* oxidase (COX) and malate dehydrogenase (MDH) activities were measured as described previously using a Shimadzu UV-2450 spectrophotometer [11]. COX

activity was determined by monitoring the reduction of 32 μM bovine cytochrome *c* at an absorbance of 550 nm by 5-10 μg of cell extracts (40 mM KH_2PO_4, pH 6.7, 0.5% Tween 20). MDH activity was determined by monitoring the reduction of 6 mM oxaloacetate and 3.75 mM NADH at an absorbance of 340 nm. All activities for COX and MDH were normalized to protein concentration using Bradford reagent, and the average of COX/MDH determine total COX activity. Superoxide dismutase (SOD) activity was measured using a xanthine oxidase-linked assay kit (Cayman Chemical) and absorbance was measured at 460 nm on Cytation 3 plate reader (BioTek). Analysis for SOD activity was determined using the SOD (U/ml) formula provided within the assay kit (Cayman Chemical) and converted to percent inhibition.

Immunoblot Assay

Immunoblot analysis was used to determine the steady state levels of proteins. 20 μg of total protein from mitochondria were separated on a 15% SDS-PAGE gel system and electrophoretically transferred onto a nitrocellulose membrane. Membranes were blocked in 1× Rapidblock blocking solution (VWR Life Science) before protein detection. The monoclonal anti-rabbit SLC25A3 (Invitrogen and Abcam). Loading control HSP60 was used to measure abundance.

Oxygen Consumption

Oxygen consumption was measured using the 5300A Biological Oxygen Monitor instrument. Cells were extracted and placed in a sealed chamber and an oxygen probe was

place inside the chamber to seal the chamber. Oxygen uptake was recorded by the instrument that computes an oxygen consumption rate over time.

Data Analysis

All results are presented as mean with errors bars of standard error of the mean. *p* values<0.05 were considered significant as assessed by Student's *t* test.

Results

COX activity and copper in assembly factor mutant COA1

I reported previously in yeast that a mutant assembly factor of COX1 subunit, *Coa1*, had decreased total mitochondrial copper and a defective COX that was rescued by the addition of copper. I replicated the experiment done in yeast to mammalian *COA1* by treating HEK cells with an RNAi silencer for MITRAC15 (*COA1*) for 48 hours. After incubation we tested COX activity by measuring the decrease of absorbance at 550 nm of chemically reduced cytochrome c. I showed that COA1 did indeed have a COX deficiency compared to the wildtype cells (Fig 3.1). To investigate if additional copper could rescue the activity, I transfected the cells for 48hrs with the MITRAC15 (*COA1*) silencer and treated the cells with 40 µM concentration of copper for an additional 24 hours. The addition of copper to those cells were able to rescue the activity (Fig 3.1).

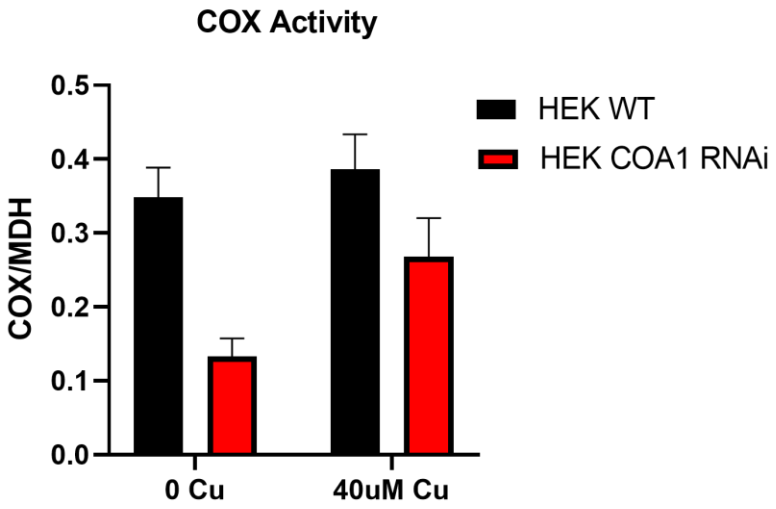

Figure 3.1: COX activity and rescue in COA1 mutants Coa1 mutants in mammalian cells showed a significant decrease in COX activity compared to the wildtype in HEK cells (n=5 **, p <0.01). This deficiency was rescued by the addition of exogenous copper (n=5 *, p < 0.05)

COX activity and rescue in copper transporter

In chapter 2 I showed *SURF1*$^{-/-}$ mutants with a total mitochondrial copper deficiency which affected copper in SOD, COX, and matrix. Copper is imported into the matrix via the phosphate carrier, SLC25A3, that dual substrates of both phosphate and copper [3]. I wanted to test if SLC25A3 knockdowns had the expected effects on COX activity. I found that silencing the transporter does cause a defect in COX activity (Fig 3.2). Next, I wanted to see if adding copper would help reverse the defect (Fig 3.2). However, I was unable to rescue the activity with copper addition in the form of Cu-ATSM at 40 µM. These cells may require higher concentrations of Cu to reverse the phenotype than mouse embryonic fibroblasts with a knockout of *SLC25A3*$^{-/-}$, however, even in those cells, multiple thresholds for rescue of different copper pathways exist (Fig 3.3). The level of copper required to rescue *SLC25A3*$^{-/-}$ knockdown appears to be higher than the requirement for rescue of *COA1*$^{-/-}$ or *SURF1*$^{-/-}$ knockdown in the same cell type.

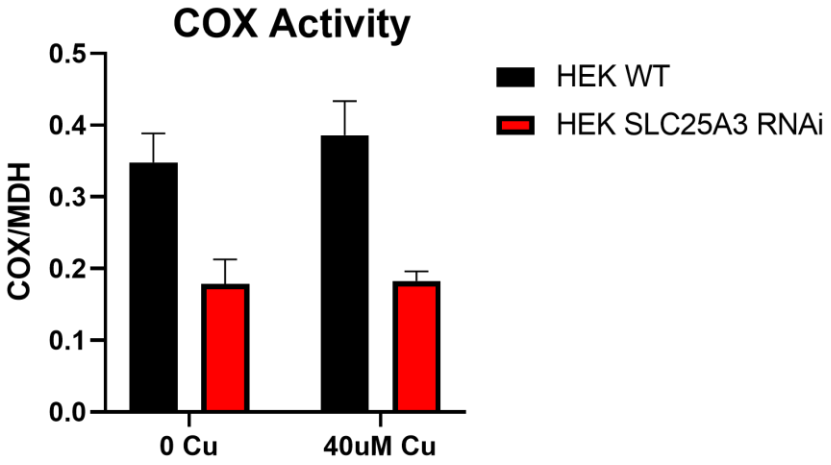

Figure 3.2: COX activity in SLC25A3 mutants Mutant *SLC25A3*$^{-/-}$ in HEK cells, showed a significant decrease in the COX activity (n=5 *, $p < 0.05$) that was not able to be rescued due to the transporter being silenced and copper not being able to reach the matrix.

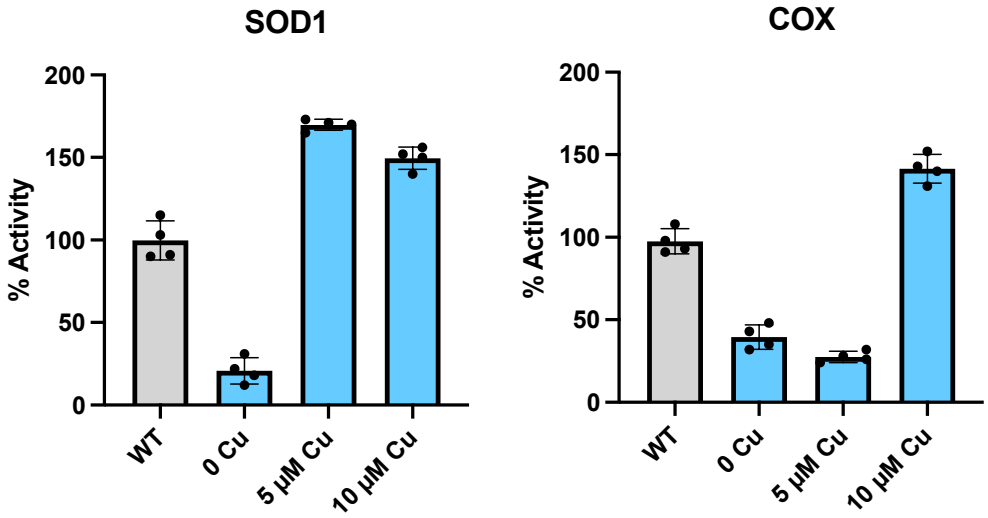

Figure 3.3 SLC25A3 mouse embryonic fibroblasts (MEFS) copper rescue. Wildtype (WT-grey) of *SLC25A3* $^{-/-}$ (blue) MEFs were cultured in no added copper (WT or 0 µM Cu) to establish baseline activities for SOD1 and COX. The mutants showed decreased activity of both enzymes while both could be rescued by addition of copper. SOD1 activity was rescued at lower levels than what was required for COX. It should be noted that the level required for rescue in this cell type is lower than the level in HEK293 cells.

SOD Activity in mutant COA1 and SLC25A3

I previously saw that with low COX activity there followed low SOD activity in *SURF1*$^{-/-}$ mutant cells. I wanted to investigate if there was a similar pattern in *COA1*$^{-/-}$ and *SLC25A3*$^{-/-}$ mutants. I measured SOD1 activity in HEK transfected *COA1*$^{-/-}$ and *SLC25A3*$^{-/-}$ silencer cells using a xanthine oxidase-linked assay kit. I discovered that *COA1*$^{-/-}$ mutants had a small decrease in SOD activity that was not significant while *SLC25A3*$^{-/-}$ continued to have normal SOD activity (Fig 3.4). For *SLC25A3*$^{-/-}$ this result is different to the MEF cells where a deficit is observed and the reason for the difference is unknown.

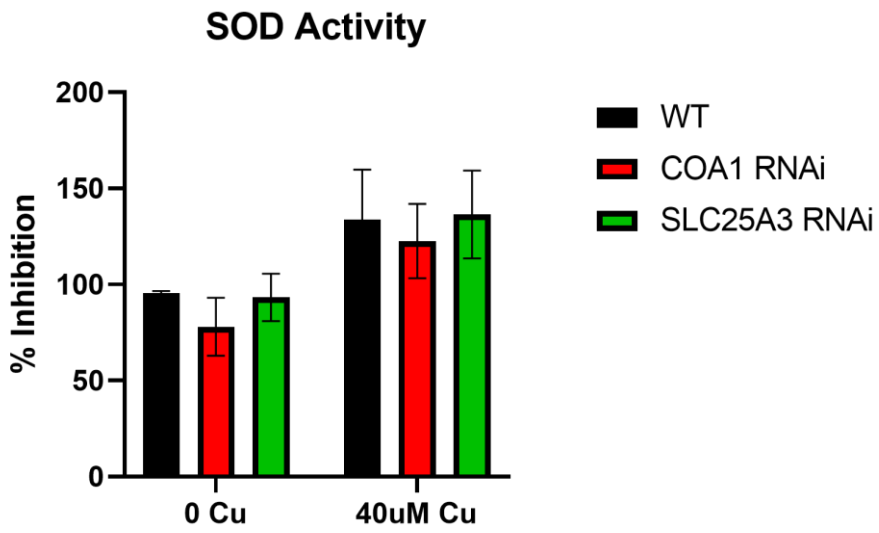

Figure 3.4 SOD activity in COA1 and SLC25A3 mutants *COA1*$^{-/-}$ mutant showed an insignificant decrease in SOD activity (n=4 pvalue:.28) that was enhanced by the addition of copper (n=4 *, $p < 0.05$). For *SLC25A3*$^{-/-}$ mutant, there was no defects to SOD activity (n=4 p-value=0.85) and like *COA1*$^{-/-}$, copper increased the activity (n=4 *, $p < 0.05$).

COX activity in double mutants

The regulation of mitochondrial copper homeostasis and how copper is exported out of the matrix into the IMS is unknown. A hypothesis is that the regulation of total copper levels or import versus export (which would affect total copper levels) is via protein-protein interaction. I suspect interactions between MITRAC components, especially SURF1 and COA1, and the copper transporter SLC25A3, could be an important factor in this regulation. These proteins have been connected in multiple studies. *SLC25A3* is a component of the "contaminant repository" in immunoprecipitation studies suggesting it is unreliable in interactions. However, it is 17-fold enhanced over background in a proteomic study that defined the MITRAC complex and was found as an interactor with *PIC2* in yeast that when deleted decreased the growth of the double mutant in competition assays (Table 3.1).

Table 3.2 Top hits from a yeast screen with pic2Δ deletion. Cells with double deletions of selected inner membrane transporters were screened in a growth competition assay. Genetic interactions were defined as negative when cells were eliminated from the population over the course of the experiment. Scores of -40 were considered inhibited for growth. Deletion of *SHY1* in the presence of *PIC2* deletion results in significant loss in the population suggesting roles in similar pathways. MIR1 is the phosphate transporter in yeast that has overlapping substrate with PIC2 and is considered a positive control in this experiment.

Bait	Hit	Growth scores
PIC2	RPO41	-97.6
PIC2	SCO1	-79.5
PIC2	MIR1	-67.4
PIC2	QCR2	-49.5
PIC2	SHY1	-44.8
PIC2	IDP1	-39.1
PIC2	IDH2	-36.7
PIC2	SAC6	-36.4
PIC2	ADK1	-36.0
PIC2	NDI1	-34.7

I decided to investigate the connections between these factors in cell culture by taking advantage of the fact that knockdowns only resulted in ~50-60% decrease in COX allowing me to look at the effects of multiple proteins being silenced at the same time for additive phenotypes. HEK293 cells were treated with RNAi silencing of a combination of *SURF1, COA1,* and *SLC25A3* protein and then measured COX activity and rescue by copper. The silencing of SURF1+SLC25A3 (SA) in combination did not result in additive phenotype (Fig 3.5) and consistent with the increased requirement for copper supplementation in a SLC25A3 knockdown, 40 µM Cu was unable to return activity (Fig 3.6). The same phenotype was observed for SLC25A3+COA1 (AC), there was a decreased in COX activity (Fig 3.5) and copper addition showed no significant rescue (Fig 3.6). On the other hand, in the SURF1+COA1 (SC) double mutant COX activity was rescued by the addition of 40 µM (Fig 3.6).

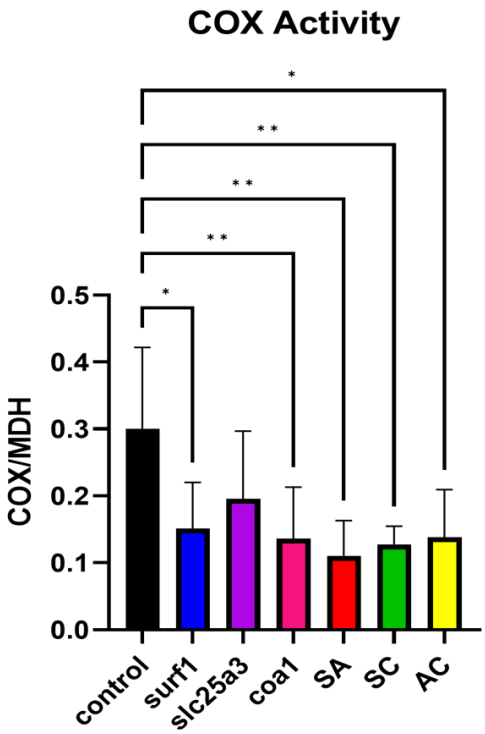

Figure 3.5 Double mutant COX activity Double mutants showed a significant decrease in COX activity compared to wildtype cells. This did not result in any additive phenotypes.

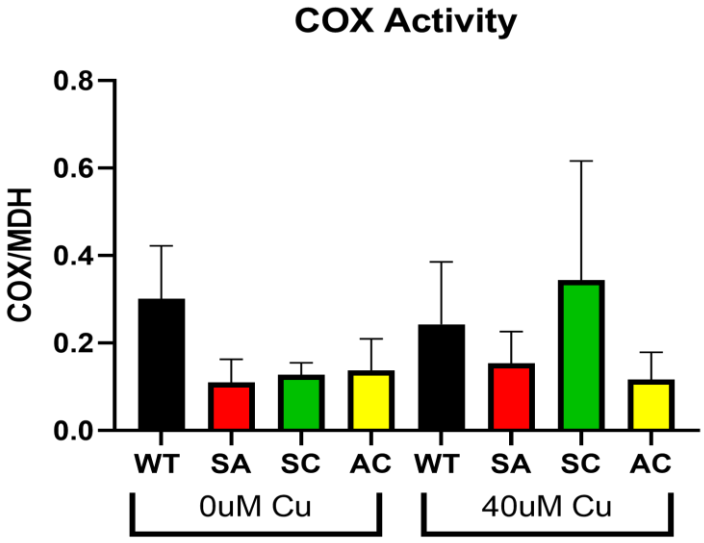

Figure 3.6 Double mutant COX activity with copper addition Although double mutants showed a significant decrease in COX activity compared to wildtype cells, only one of the double mutants, SURF1/COA1 (SC) had COX activity that could be reversed by the addition of copper.

SOD activity in double mutants

To get an idea of the interaction between assembly factors and the copper transporter, we wanted to test SOD activity of the double mutants. In HEK293 cells, *SURF1* and *COA1, SURF1* and *SLC25A3,* and *SLC25A3* and *COA1* were silenced together to create a double mutant through transfection. This experiment resulted in a decrease of SOD activity in the SURF1 + SLC25A3 mutants which was reversed by copper addition (Fig 3.7). However, no change was observed for SLC25A3+COA1 and SURF1+COA1 mutants as SOD activity was maintained with and without the presence of excess copper.

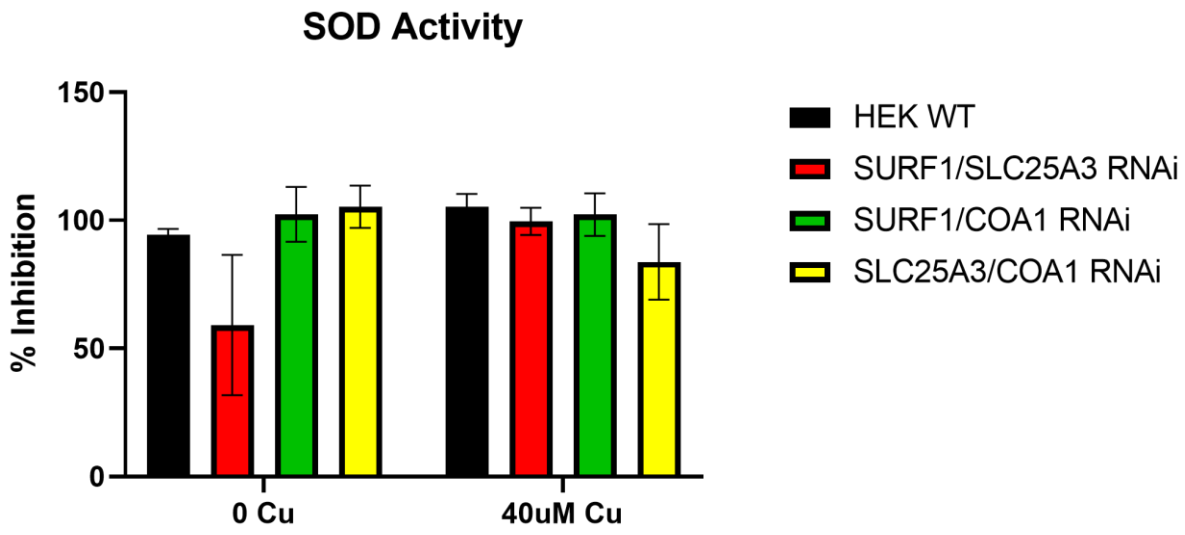

Figure 3.7 Double mutant SOD activity

Total SOD activity measured in HEK double mutants showed a decrease in activity for SA that was reversed by copper addition. SC and AC double mutants SOD activity were not affected by the defect and showed no change when copper was added.

Regulation of SLC25A3/Pic2 in SURF1/Shy1 mutant

The previous results suggest that SURF1 and SLC25A3 act in a pathway as the double mutant does not have additive effects. There are two possible mechanisms for this result; one is the stability of SLC25A3 is compromised in a *SURF1*$^{-/-}$ mutant or that SLC25A3 activity requires SURF1/COA1. I prepared cells for immunoblot of SLC25A3 to determine if the protein was present but non-functional in cell culture (Fig 3.8). The levels of SLC25A3 appear to be normal in the mutant (or even higher given the difference in loading control).

To further test whether SLC25A3 requires SURF1/COA1 for activity I used the yeast model system to test for rescue of phenotypes associated with SURF1/COA1 (*shy1Δ* and *coa1Δ*) deletion. Both *shy1Δ* and *coa1Δ* have a COX defect as discussed earlier so I assayed oxygen consumption in these mutants overexpressing PIC2. One hypothesis is that overexpression of PIC2 would result in increased copper loading in the mitochondrial matrix. However, an important caveat to this prediction is that the cytosolic homeostasis is intact and provides a chelating environment so mitochondria expression may not necessarily increase cytosolic availability to allow this rescue, however, PIC2 expression does rescue copper in *pic2Δ* cells. Overexpression of PIC2 in the *shy1Δ* and *coa1Δ* cells did not reverse the oxygen consumption defect in these cells (Fig 3.9). In addition, the total mitochondrial copper in these cells (Fig 3.10) did not increase nor was overexpression able to rescue the copper uptake defect of *coa1Δ* mitochondria (Fig 3.11).

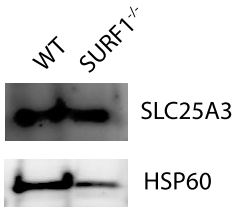

Figure 3.8 Immunoblot of SLC25A3 in human fibroblasts. Western blot of cell lysates from immortalized WT human fibroblasts or SURF1 mutant. 20 µg of total protein was loaded into each lane. SLC25A3 antibody detection suggests that SLC25A3 is present and stable in SURF1 mutants.

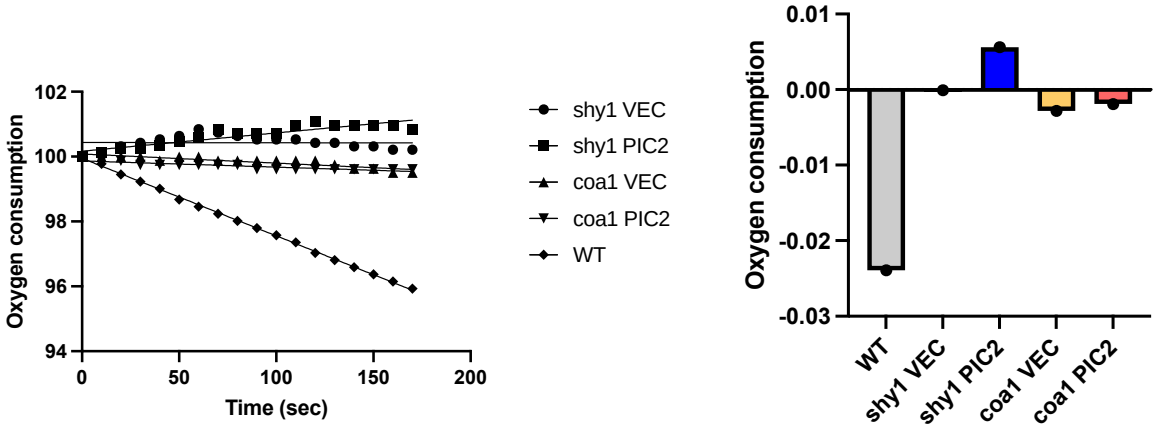

Figure 3.9 Oxygen consumption of coa1Δ and shy1Δ cells overexpressing PIC2. Yeast cells of the specified genotype were grown in rich media with 1% glucose and oxygen consumption measured as rate of oxygen depleted from a sealed cuvette per second. The slope of the line is presented as a rate and shows no rescue of the oxygen consumption defect with high expression of *PIC2*.

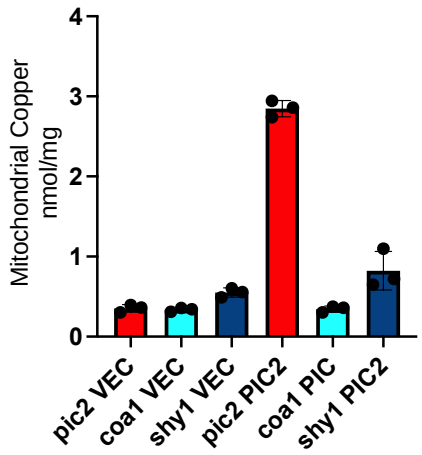

Figure 3.10 Total mitochondrial copper in yeast mutant. Mitochondria isolated from *shy1Δ* and *coa1Δ* expressing an empty vector (VEC) as a control or PIC2. The *pic2ΔPIC2* mitochondria act as a control for wild-type levels of copper in mitochondria. The three mutants all show low levels as shown previously (chapter 2) and only pic2Δ levels are rescued by overexpression of PIC2.

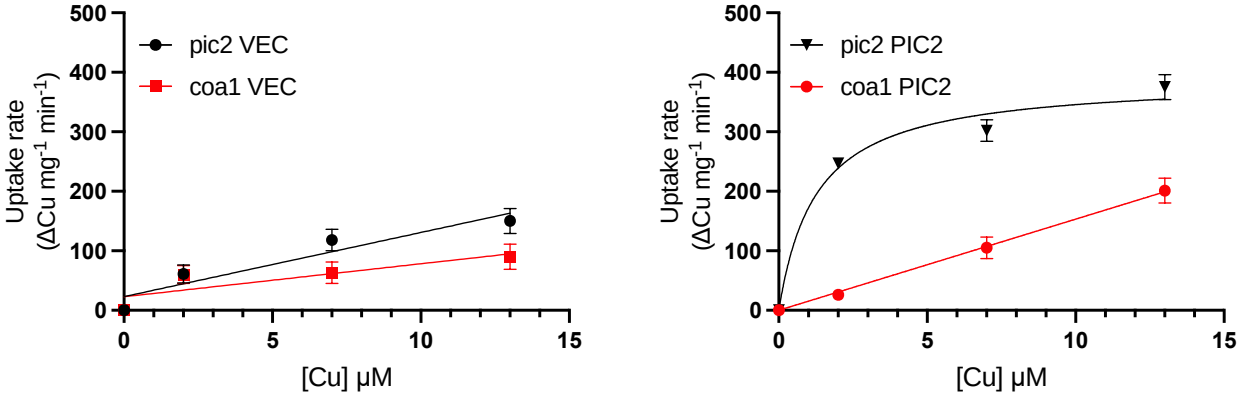

Figure 3.11 Cu-uptake into isolated mitochondria. Mitochondria isolated from *pic2Δ* or *coa1Δ* cells with either empty vector (VEC- control) or *PIC2* were assayed for copper uptake before incubation in different concentrations of copper and first order rate calculated. PIC2 completely reverses the uptake defect in pic2Δ cells but only partially rescues activity in *coa1Δ*.

Discussion

Regulation of mitochondrial copper is important for maintaining proper functioning of enzymes in mitochondria [8,13]. These enzymes, such as COX, must be assembled by many assembly factors and during the assembly process, copper must be readily available from the matrix to be inserted into COX [14, 15]. How copper availability is regulated from the matrix to the IMS is unknown. Herein, I provide potential mechanisms for the regulation of copper availability. I specifically looked at two components of the MITRAC complex and the copper transporter SLC25A3.

Previous work looking has established that *SURF1*$^{-/-}$ causes Leigh Syndrome [16] and some work has suggested a link between copper and SURF1 particularly via interaction partners like COA1 [17,18]. SURF1 is a member of the MITRAC complex that binds with another member, COA1, to insert heme into COX subunit one [19]. I show that copper levels in mitochondria were affected by the loss of *SURF1* and that was linked with low COX and SOD1 activity. These enzyme activities were increased when copper was supplemented in the media leading to increased levels within the cell. This led to the conclusion that SURF1 and COA1 have a role in copper bioavailability which affects COX and SOD1 activities in the IMS. I propose that this activity could be regulating SLC25A3 or potentially another transporter.

In addition, I investigated COX activity in mammalian mutated *COA1*$^{-/-}$ cells and found that like yeast, COX activity was decreased which was rescued by the addition of copper. Total SOD activity was only partially decreased in this mutant and activity was rescued in the presence of exogenous copper. I also established that copper is required for SLC25A3 in the HEK293 cells. I found that a defect in COX activity in the cells lacking SLC25A3 via transient

transfection but adding copper at 40 µM was not able to rescue the phenotype. This is different to the MEF cells that have a complete knockout of *SLC25A3* which needed 10 µM Cu-ATSM to rescue COX and maybe surprising as the transient knockdown presumably has more residual SLC25A3 available [20]. However, no information is known regarding total mitochondrial copper levels in HEK versus MEFs nor how much copper makes it to mitochondria under these conditions.

It is still unknown how copper is exported from the matrix, but multiple experiments have demonstrated that the required copper for IMS enzymes must be cycled through the matrix [12]. It is speculated that this is to allow for coordination of the assembly process [21]. Because SURF1 and COA1 are membrane bound proteins, we tested the hypothesis that SURF1 and COA1 are part of a complex that regulates activity of SLC25A3 [20,12] or another transporter in export. The simultaneous knockdown experiments suggested that the gene products are required in the same pathway, as no additive phenotypes were observed. Therefore, we favor a regulation by binding in a complex or possibly by affecting some other aspect of mitochondrial morphology.

In yeast the experiments suggest that *coa1Δ* mitochondria have decreased import that is comparable to *pic2Δ* yeast. Therefore, it is likely that at least Coa1 is required for uptake activity. We have shown elsewhere that Coa1 protein expressed in a heterologous expression system does not have intrinsic copper transport capabilities so it is most likely regulatory and further we assume that it must form a complex to facilitate uptake. It should be noted however that both SLC25A3 and Pic2 have been purified from expression systems and purified protein have transport activity so there is still some complication in understanding the exact function

[20,12,23]. It is still possible the SLC25A3/Pic2 export copper as well as import but a failure to import prevents accumulation of the substrate and therefore investigation of the export. Additional assays or phenotypes would be needed to fully address this question.

An additional consideration about the complexity of the mechanisms that result in lower mitochondrial copper is that COA1 and the mitochondrial copper chaperone COX17 have been implicated in interactions with components of MICOS [24,25]. The MICOS complex is critical for the structure of the inner membrane cristae [26,27] which could have potentially to affect transport of mitochondrial copper. To test this hypothesis, we attempted several experiments addressing mitochondrial morphology. C*oa1Δ* cells show decreased numbers of mitochondria and morphology suggestive of increase fission (or decreased fusion). However, *shy1Δ* cells do not show the same phenotype. In addition, *COA1* overexpression appears to partially rescue growth phenotypes associated with a loss of MICOS. While this data suggests a possibility, our attempts at investigating mitochondrial morphology in cell culture were unsuccessful and future studies could address these links.

The data presented suggest that COA1 and SURF1 are required for the in vivo activity of SLC25A3. The implications of this have recently been extended by the discovery that a novel cell death pathway exists in cells that is induced by mitochondrial copper causing the aggregation of lipoylated proteins [28,29], therefore understanding the regulation of how this pool is maintained could have additional implications beyond the diseases associated with COX deficiency.

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Chapter 4: Concluding Remarks/Future Directions

Copper is a vital redox cofactor that is involved in many different pathways [3]. Years of elegant experiments have defined a role in electron transport chain in cytochrome c oxidase, removal of oxidative species in superoxide dismutase, and iron homeostasis in multi-copper oxidases. While these enzymes are critical to cellular physiology, major unanswered questions still exist about regulating copper distribution and potentially new targets of copper binding within the cell [3]. Some years ago, it was suggested that cytosolic copper was limited to almost zero implying that incredible fine-tuned chelation and sequestration mechanisms existed [4]. These incredible chelation conditions required that chaperone proteins were needed to deliver copper to targets within the cell [5]. Importantly these mechanisms were entirely consistent with restricting free copper to prevent toxicity. Copper toxicity is primarily due to the destruction of Fe-S clusters with smaller components contributed by mis-metalation of proteins and oxidation of important redox regulating molecules such as glutathione [6]. One important observation that was recently made is that cells lacking all cytosolic chelation power due to mutations of all the metallothioneins can only be rescued by expression of ATP7A (or compensatory expression of ATP7B) showing that membranes were the most efficient barrier to control copper levels [1]. Importantly, many questions remain unanswered about how priority of delivery of copper is established, how mitochondria maintain a constant supply of copper (inside an impermeable membrane barrier in the matrix), and how copper is exported from the matrix into the IMS for use in COX. The gaps in this knowledge limit our ability to make all-encompassing hypotheses to explain the physiology observed in normal and disease

associated states. My work in this dissertation primarily focused on whether copper was involved in Leigh Syndrome physiology. It is well established that Leigh Syndrome is a neurological disorder related to mitochondrial function and that SURF1 mutations are prevalent and result in COX deficiency [2]. However, no therapies have been developed.

Understanding the key players and mechanisms can lead to better knowledge of many diseases like Leigh Syndrome and treatments for better survival rate. Gaining the understanding of the smaller picture can direct an improvement of the bigger picture. I presented the possibility of a potential drug treatment, using elesclomol [8,9,10], for Leigh syndrome patients with a SURF1 defect. I was successful in showing the use of copper ionophores to increase cytochrome c oxidase activity. Future testing will be required to determine the toxicity of these ionophores especially with disease states. However, it was recently shown that elesclomol toxicity requires respiration [11] therefore applied in a therapeutic window in respiratory deficient cells could be possible, then lower maintenance doses could be applied. This drug has been used in cancer trails and is FDA approved but significantly more testing will need to be needed before it would be possible to consider pushing from animal trials (limited by the unsuccessful recapitulation of the human physiology in mouse [12]) and then the next phase of clinical trials in humans. Since I looked specifically at SURF1 mutations, further testing of other mutations on the different assembly factors and the effects of copper on them should also be investigated. This would help in determining which patients with Leigh syndrome could be included in the cohort. However, given data suggesting this is related to *SURF1* and *SLC25A3* interaction rather than the final COX activity this would most likely be a treatment for *SURF1* patients only.

To further understand the regulation of copper in mitochondria, I showed further genetic interactions between the copper importer *SLC25A3* and MITRAC members *SURF1* and *COA1*. These interactions likely control import of copper into the matrix. However, it is also possible that these interactions have a significant change in export to IMS [6]. One possibility is that SLC25A3 may have a dual function as a copper importer and exporter where it undergoes a conformational change for export. This conformation change could be via interactions with MITRAC members [13]. Investigating each part of MITRAC, their structure, and properties can help with understanding the complex as a whole and any interactions with SLC25A3. I have only tested two components of MITRAC that have shown promising results of genetic interaction with SLC25A3, further experiments should complete protein-protein interactions as well as expand to other parts of MITRAC such as COA3 (which I collected preliminary data for but did not complete those studies).

A third possibility is that copper availability and export is linked to a well-studied complex, MICOS. The MICOS complex organizes mitochondrial architecture for processes on mitochondrial membranes [14]. Data that was not presented showed a loss of mitochondria and morphology in yeast *COA1* mutant cells, and the overexpression of *COA1* was able to rescue growth associated with mutants that caused MICOS. These data suggested a genetic interaction between *COA1* and MICOS. A key physiological outcome of the COA1-MICOS interaction was a mitochondrial morphology defect. An attempt to replicate this phenotype in human cell culture was unsuccessful, however, further work is required to establish whether it was technical or biological difference between yeast and human cell culture models. Critically this interaction may be regulated by copper or could be regulating copper. I speculate that the

membrane curvature that would be lost in MICOS mutants (or *COA1* deletions in yeast) would have significant effects on transporter properties of the MCF proteins. Therefore, further morphology and protein-protein interaction experiments need to be carefully investigated to understand these relationships.

Leigh syndrome lacks a reliable animal model [12]. Experiments in mouse were plagued by complications or the lack of "human-like" phenotypes. Even when COX was found to be affected the animal models for *SURF1* deletions the phenotypes were not obvious, this severely limits the ability to test for therapies like the one described above. I attempted experiments in *Drosophila melanogaster* to use an organismal model. The interaction between organs and systemic regulation of copper is critical to help paint the picture of copper availability and export. Due to technical difficulties, user error, and subsequent limitation of time no results were included. Although, promising data in wild-type flies showed a potential role of copper in regulating *SURF1* stability. Future work with *Drosophila* will be needed to investigate copper treatment in Leigh syndrome, an additional role of copper in the mitochondria, and a genetic approach to the regulation of copper availability and export.

The final figure (Fig 4.1) demonstrates the ever-growing list of assembly proteins involved in COX assembly and includes my contributions showing SURF1 and COA1 interacting with SLC25A3 in the regulations of mitochondrial copper content and therefore COX activity.

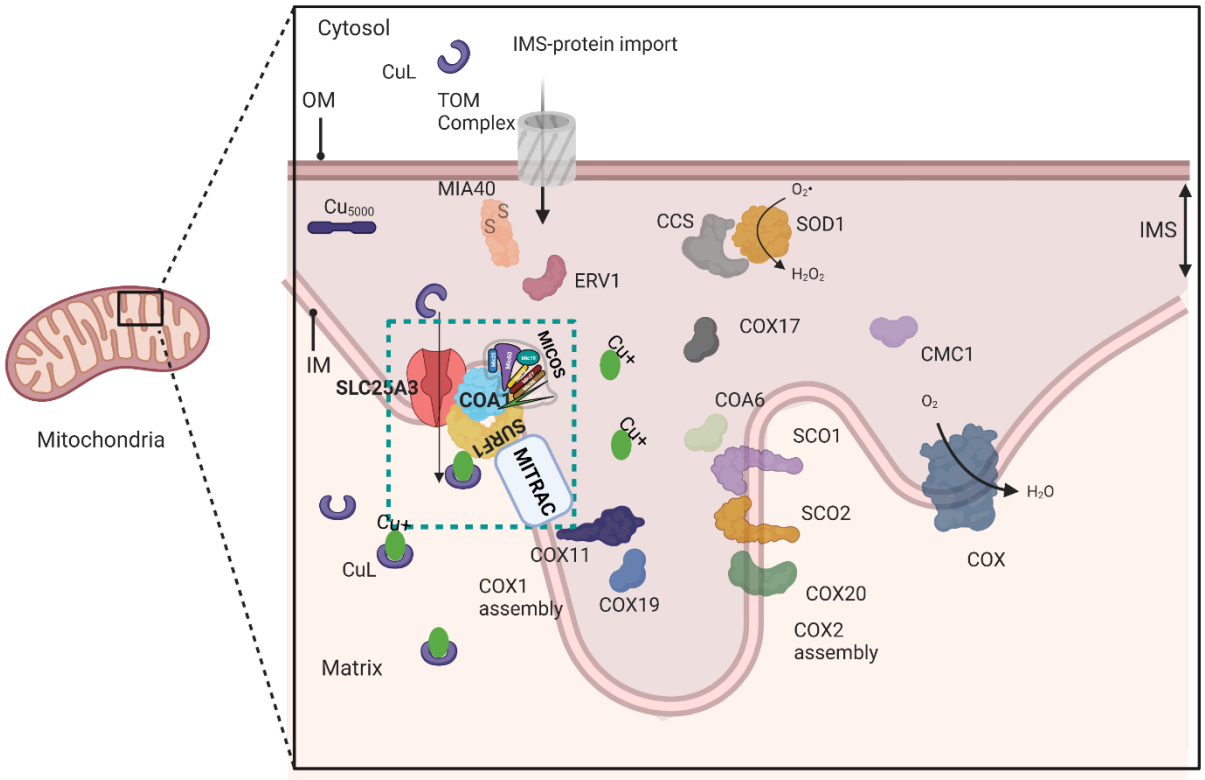

Figure 4.1 MITRAC, SLC25A3, and MICOS interactions

Copper availability and export involves the interaction of several proteins. SLC25A3, COA1, and SURF1 bond interact to make copper available for export. The bond also interacts with MICOS for inner membrane structure change for the export of copper. Other possible members of MITRAC may be involved in this interaction.

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