Mitochondrial Copper Transport in *Saccharomyces cerevisiae*

by

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Abstract

Saccharomyces cerevisiae contains a copper pool within the mitochondrial matrix that is required for assembly and activity of cytochrome *c* oxidase. Matrix copper exists bound to an anionic, fluorescent molecule known as the copper ligand (CuL). The CuL is imported into isolated mitochondria in a time dependent, saturable manner. Using silver as a copper competitor, we screened mitochondrial carrier family mutants and identified *PIC2* as a candidate transporter. The *pic2*Δ mutants grew poorly on copper-deficient, non-fermentable medium supplemented with silver, a phenotype that was exacerbated by expression of a matrix targeted competitor protein. Copper and copper uptake was deficient in mitochondria from *pic2*Δ, and Pic2 expressed in *Lactococcus lactis* mediated uptake of copper given both as the CuL and as copper salts. Therefore, we propose that Pic2 mediates copper import into the mitochondrial matrix. Here, it is shown that deletion of *PIC2* caused a defect in expansion of the mitochondrial CuL pool and uptake of the CuL complex as measured by thin layer chromatography (TLC). Additionally, a physical interaction between Pic2 and the CuL was demonstrated using fluorescence anisotropy. Taken together, these data suggest that Pic2 mediates import of the entire CuL complex. While deletion of *PIC2* caused a decrease in mitochondrial copper and CuL, it was not completely depleted. We looked to another mitochondrial carrier family gene, *MRS3*, previously identified as being involved in high affinity iron uptake, as potential member of the copper import pathway. While deletion of this gene led to copper related growth phenotypes and a decrease in mitochondrial copper expansion, it did not affect mitochondrial

copper uptake. Mrs3 expressed in a heterologous system, though capable of importing iron, did not mediate copper import. Therefore, *MRS3* is a modifier of *PIC2* and has some indirect effect on mitochondrial copper import.

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List of Abbreviations

IM	Inner mitochondrial membrane
OM	Outer mitochondrial membrane
IMS	Inter-membrane space
ETC	Electron transport chain
CcO	Cytochrome c oxidase
BCS	Bathocuproine disulfonic acid
HPLC	High pressure liquid chromatography
TLC	Thin layer chromatography

Chapter 1: Literature Review

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1. Introduction

Copper is an essential element in aerobic eukaryotes. It is required in mitochondria as a cofactor for the enzymes important for utilizing oxygen and protection against the deleterious radical species that come as a consequence of an oxygen-requiring lifestyle. Cells meticulously account for copper and regulate its distribution to prevent uncontrolled redox chemistry (1). To achieve this regulation, copper is bound by various biological ligands that include cuproenzymes, copper chaperones, metallothioneins and a range of small molecule chelators. The enzymes that utilize copper in eukaryotes are generally using its redox properties to carry out oxygen-related reactions (e.g. cytochrome c oxidase, superoxide dismutase, various monooxygenases). Copper is targeted to these enzymes by metallochaperone proteins. If copper becomes free in the cell due to high concentrations or protein turnover it is bound into inert complexes by metallothionein proteins or small molecules such as glutathione or other unidentified ligand complexes. In addition, membranes form a critical barrier for the control of copper concentration and availability in the cell. Concerted action of transport proteins and modulation of the biological ligands determine the balance of copper and its subcellular localization (1).

The mitochondrion is a double membrane-bound organelle best recognized for its function in energy metabolism and cofactor assembly (Figure 1.1) (2). Mitochondria have an

outer membrane (OM) that provides a barrier to proteins but allows for gated diffusion of metabolites and small molecules from the cytoplasm and also a more convoluted inner membrane (IM) that is folded with invaginations that increase the surface area. This inner membrane is sealed to allow formation of the proton motive force required for energy production. It has the highest ratio of proteins to lipids in eukaryotic cells; these include the electron transport chain complexes and ATP synthase. The IM is organized to form cristae with junctions formed by proteins. The area between the two membranes is known as the inter-membrane space (IMS), which has its own unique proteome. The matrix compartment that is surrounded by the IM accounts for the bulk of the volume of mitochondria. This compartment houses the mitochondrial genome, mitochondrial ribosomes, the enzymes of the tricarboxylic acid cycle and essential proteins required for the formation of iron-sulfur clusters (3).

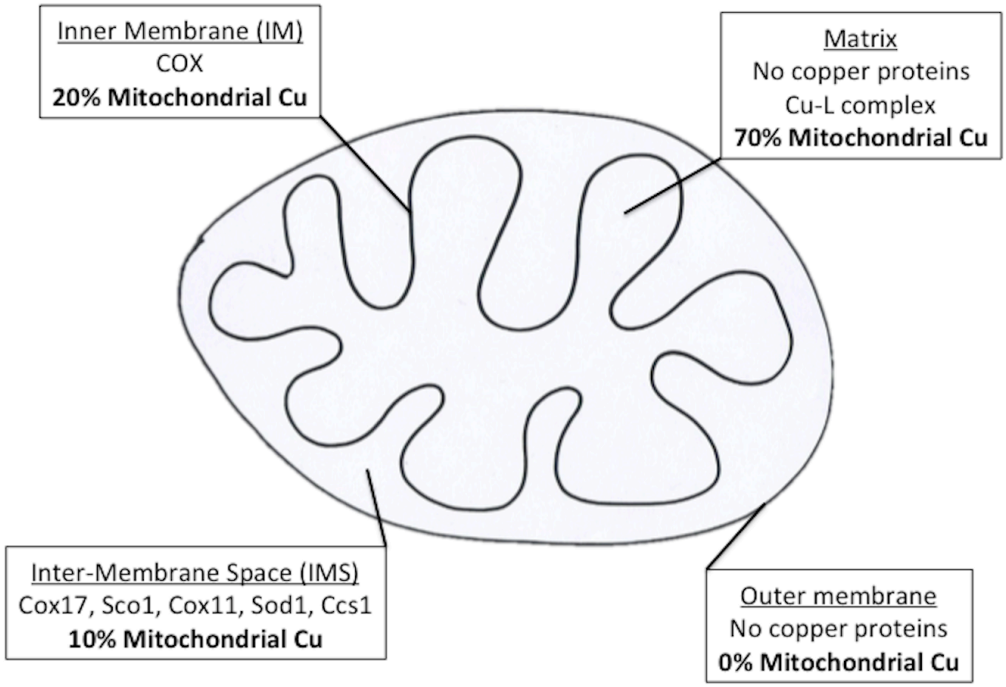

Figure 1.1: Mitochondrial Organization

Mitochondria are organized into distinct compartments with unique proteomes. The outer membrane (OM) is porous, allowing for exchange of contents with the cytosol. This membrane contains no known copper enzymes. The inter-membrane space (IMS) is the compartment enclosed between the OM and the inner membrane (IM). It contains the copper enzyme Sod1, the copper chaperone for Sod1 (Ccs1), and the chaperones for assembly of cytochrome c oxidase (Cox17, Sco1, Cox11). A number of other copper binding proteins with similar folds to Cox17 have been identified in this compartment. Based on biochemical fractionation this compartment accounts for less than 10% of copper in mitochondria. The IM houses the electron transport chain, including cytochrome c oxidase (COX). COX accounts for between 20-30% of copper in mitochondria. The matrix has a unique proteome and metabolome. While no copper enzymes or proteins are found in this compartment it accounts for ~70% of mitochondrial copper.

The mitochondrial proteome is estimated at up to 1000 proteins but only a small number are encoded by the mitochondrial genome. Therefore the majority must be imported into mitochondria without disrupting the membrane potential required for ATP synthesis. The import of unfolded mitochondrial proteins is mediated by a dedicated series of complexes that facilitate transit across the membrane and chaperone proteins that direct them to their correct location. The translocase of the OM (TOM) complex allows passage across the outer barrier; proteins must be sorted either into the outer membrane using a beta-barrel protein sorting and assembly machinery (SAM), or they can be inserted into the inner membrane via the translocase of the inner membrane complex containing TIM22. The targeting to either the SAM or TIM22 complexes is based on protein targeting sequences and requires the presence of small chaperone proteins known as the small TIMS. Soluble proteins found in the mitochondrial matrix or proteins that span the IM, typically with single transmembrane domains, have a dedicated system for translocation via the TIM23 complex. Tim23 complex is closely associated with the TOM complex allowing for efficient translocation to the matrix. Finally, cysteine-containing proteins destined for the IMS use an oxidative folding pathway named the Mia40 pathway (Figure 1.2). A critical component of these systems is the necessity to unfold the proteins for import into mitochondria. Therefore cofactor insertion must occur after import (3).

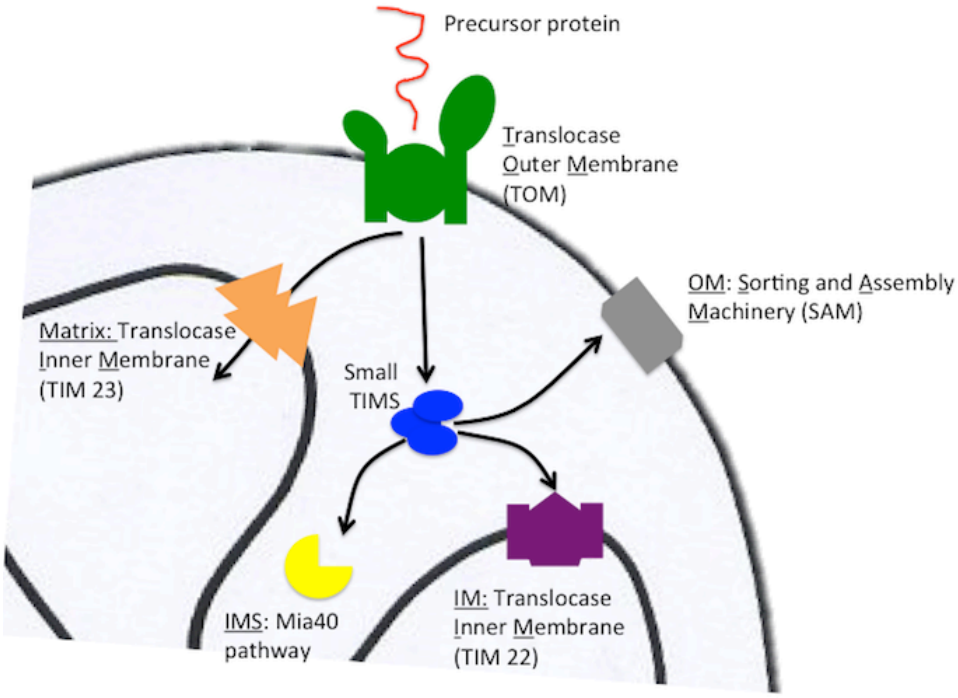

Figure 1.2: Generalized Mitochondrial Import Pathways

Mitochondrial proteins must be imported as an unfolded precursor state. The machinery that permits transit across the outer membrane is called the TOM complex. Pre-proteins are then sorted based on their final localization. The small TIMS deliver outer membrane proteins to the SAM complex, while inner membrane proteins are chaperoned to the TIM22 complex for insertion. The TIM23 complex transports proteins destined for the matrix and IM proteins with a low number of transmembrane domains. The TIM23 complex often works in close association with the TOM complex.

Copper is found in each of the three compartments of the mitochondrion: the IM, IMS and the matrix. The main enzymatic sink for copper (~20%) is cytochrome c oxidase (CcO), an IM-localized multi-subunit complex that converts oxygen to water as the final step in the eukaryotic electron transport chain. Copper in the IMS is bound to superoxide dismutase (SOD) and to the copper chaperones for CcO and SOD. The majority of mitochondrial copper (~70%) is localized to the mitochondrial matrix where it is bound in a biochemically defined but unidentified ligand complex (4). Here we will present an overview of the relevant mitochondrial copper pools and the proteins that bind and distribute copper within the IMS (Figure 1.1). Throughout this dissertation we will move between examples of yeast and mammalian genes and proteins. The nomenclature for *Saccharomyces cerevisiae* will be three letters with one number. Gene names are in capital italics (e.g., *GEN1*), small italics for mutants of that gene (e.g., *gen1*Δ) and sentence case for proteins (e.g., Gen1). For other eukaryotes, capital italics (eg. *GENE*) will be used for gene names and all capitals for protein names (GENE).

2. Copper in the Inner Membrane (IM)

2.1 Cytochrome *c* oxidase

Cytochrome *c* oxidase (CcO) is embedded in the IM and accepts electrons from cytochrome *c* in the IMS and shuttles them to molecular oxygen, reducing it to water. This reaction results in proton pumping from the matrix to the IMS, therefore contributing to the membrane potential. The transfer of electrons from cytochrome *c* to oxygen is dependent on copper and heme cofactors in CcO (5).

The mammalian enzyme complex is made up of 13 subunits that are assembled in stages into semi-defined intermediate complexes in the IM (5). The three largest subunits (Cox1, 2 and 3) are encoded by the mitochondrial genome and make up the hydrophobic core of the complex.

The remaining 10 subunits are encoded by nuclear genes and are imported into the mitochondrion after translation by cytosolic ribosomes. The mitochondrial-encoded core subunits (Cox1 and 2) bind the two copper centers, a binuclear Cu_A site and a copper-heme a_3 Cu_B site (Figure 1.3). The solvent-exposed Cu_A site is bound by Cox2 and is located on the IMS side of the complex. It is a mixed valence site that accepts electrons from cytochrome *c*, reducing the copper before transfer to the cofactors of Cox1: first to the heme *a* cofactor and finally to the Cu_B site. The Cu_B site is a heterometallic site that has a copper ion coordinated with a specialized heme a_3. This is where oxygen binds and is reduced to water. This site is buried in the IM, limiting its accessibility (Figure 1.3) (5). The other subunits of CcO surround this catalytic core and provide stability, sites of regulation, and potentially protection for the cofactors.

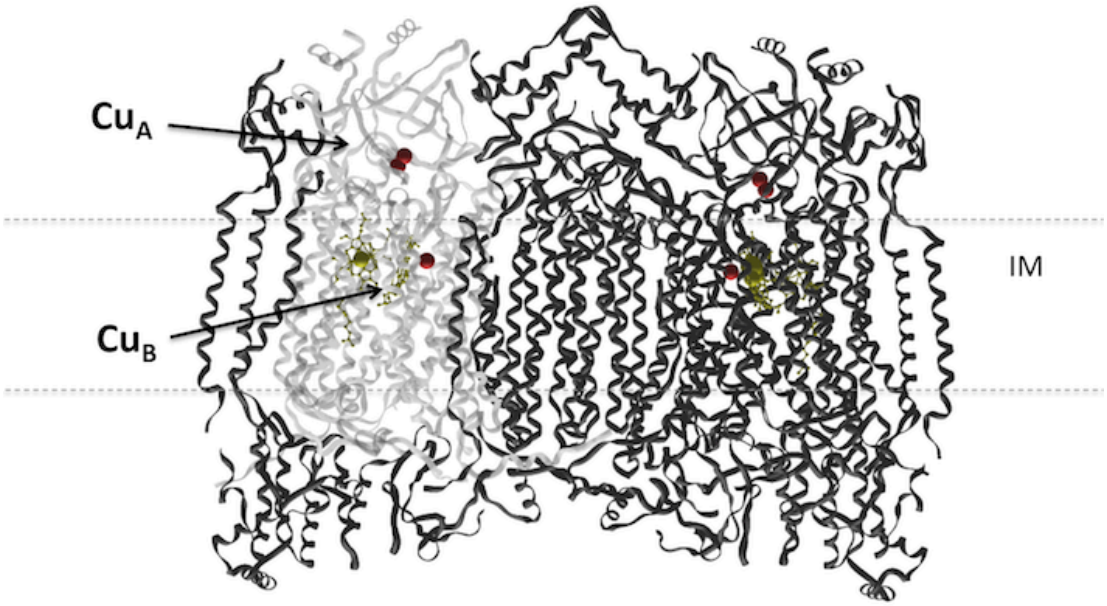

Figure 1.3: The Structure of Cytochrome c Oxidase

Ribbon structure of the assembled bovine COX as a dimer (5). The Cu_A site is positioned above the IM while the Cu_B site is buried 13 Å below the surface of the IM. The copper ions in Cu_A and Cu_B are shown in red. Heme cofactors are shown in yellow. Select subunits are colored light gray in one monomer to highlight the copper and heme co-factors. (Structural coordinates from RCSB PDB ID: 1occ)

To assemble CcO, cells use the concerted action of a number of protein factors. In fact, 20 of the 30 complementation groups related to CcO deficiency are due to mutations in genes encoding assembly factors (6). The stepwise formation of intermediates is largely controlled through protein-protein interactions and formation of transient complexes. These assembly intermediates appear to exchange members as CcO moves towards its final complex. Incorrect or inappropriate complexes are removed by degradation. In *S. cerevisiae,* it is very difficult to isolate partially assembled CcO as degradation rapidly removes inappropriate/incorrect subassemblies. However in humans the degradation is slowed, or prevented, as stable partially assembled intermediates along this pathway have been used to define CcO assembly steps and defects (2).

Numerous assembly factors are required to coordinate the translation of the mitochondrial-encoded subunits with the import of nuclear components and the availability and modification of cofactors. Specific activators modulate the translation of Cox1, 2 and 3 from their spliced transcripts (6). These translational activators can also act as chaperones that stabilize the nascent protein before insertion into the IM. After translation and concomitant with membrane insertion the required copper and heme cofactors must be inserted. The heme molecules that are inserted into CcO are modified from heme *b* to make heme *a.* Heme *a* has a farnesyl tail, enhancing binding and protein packing in CcO, and a modification of a methyl group to a vinyl group to modulate its redox potential (7). In addition to these copper and heme cofactors, CcO assembly factors must also insert zinc, sodium, and magnesium ions into the assembling enzyme.

An intact electron transport chain is required for *S. cerevisiae* to grow on non-fermentable carbon sources but not for fermentative growth. This phenotype has been critical in

identifying the genes involved in the assembly process of CcO (8). The ability to grow under fermentative conditions with mitochondria lacking specific proteins has allowed for the biochemical dissection of these defects. The important role copper plays in CcO assembly has meant that growth defects on non-fermentable carbon (particularly those that can be affected by manipulation of copper levels) have also become a reliable tool for determining multiple aspects of copper homeostasis in eukaryotes.

3. Copper in the Inter-membrane Space (IMS)

3.1 COX17: Soluble Cx_9C IMS copper chaperone

Cox17 functions in the IMS to deliver copper to the IM-bound chaperones Sco1 and Cox11, which insert copper into CcO. *COX17* was identified in a genetic screen for CcO accessory factors. The *COX17* mutant was novel as it was suppressed by the addition of supplemental copper to the medium and the translated product had a dual localization in cytosol and IMS (Figure 1.4) (9). Cox17 is a relatively small soluble protein with a primary sequence that contains cysteine residues organized into twin Cx_9C motifs (10). Multiple conformations of this protein have been isolated from heterologous expression systems, but a conformer that adopts a coiled coil-helix-coiled coil-helix fold stabilized by two disulfide bonds and binds a single copper atom seems to be the biologically relevant species (11,12). Mutational analyses have shown that copper coordination and Cox17 function are not dependent on the two disulfide crosslinks but these crosslinks do provide stability to the fold.

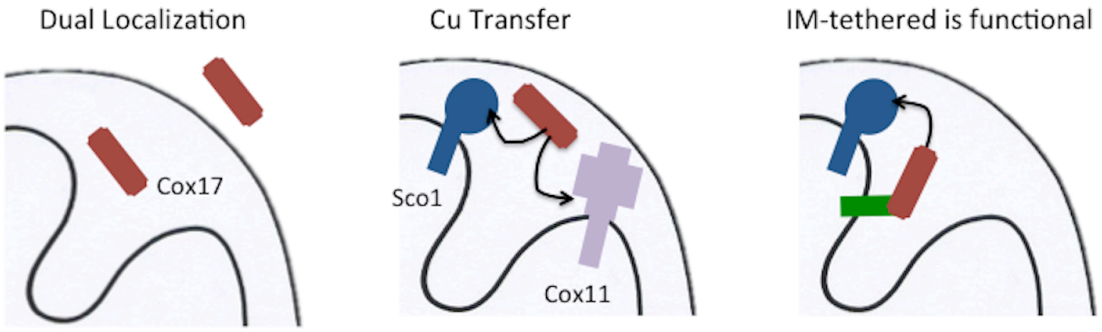

Figure 1.4: The Role of Cox17 in Assembly

Cox17 was proposed to fulfill a copper delivery function based on its dual localization in the cytosol and IMS and its copper binding characteristics. The protein has been shown to deliver copper cargo to both Sco1 and Cox11 and a tethering experiment that localized Cox17 exclusively to the IMS showed this to be the dominant role of Cox17 in vivo.

Cox17 is part of a larger family of Cx_9C proteins that have been implicated as copper-binding proteins with loosely defined functions in CcO assembly (10). These include Cox19, Cmc1, Cmc2, and Cox23; while these proteins are required under different conditions in various models, further investigation is needed to place them definitively in the pathway of copper delivery to CcO (13-15). One feature they all share is that their import into the IMS is dependent on the Mia40 oxidative import pathway, indicating that they are imported and subsequently oxidized to fold into their active conformations.

Deletion of the *COX17* homolog in a mouse model results in embryonic lethality (16). This embryonic development phenotype is similar to that caused by deletion of the high-affinity copper transporter at the plasma membrane (*CTR1*). The similarity in phenotypes reflects the critical role of COX17 in making copper available in the IMS and also the major role that membranes play in controlling copper concentrations in cells. To date no pathogenic alleles of human *COX17* have been described.

The dual localization and copper-binding abilities of Cox17 made it a candidate for delivering copper to the mitochondrion. However, it does not require the dual localization for function, as an IM-tethered Cox17 was able to rescue CcO activity in a cell lacking an endogenous *COX17* gene (Figure 1.4) (17). Therefore Cox17 is not the primary means of copper delivery to the mitochondrion. Instead, it appears that the major function of this protein is to deliver IMS copper to the globular domains of Sco1 and Cox11 for assembly of the Cu_A and Cu_B sites of CcO. The mechanism of the interaction and copper transfer between Cox17 and Sco1 or Cox11 will be discussed below.

3.2 SCO1: The Cu_A metallochaperone

SCO1 was identified in yeast as a being required for CcO assembly, specifically the post-translational stability of Cox2 (Figure 1.5) (18). The translated protein is localized to the IM. This tether is required for function, hinting at a possible role in insertion of Cox2 in the membrane. Subsequently, it was shown that *SCO1* and a highly homologous gene, *SCO2,* were multi-copy suppressors of the non-fermentable growth defect in *S. cerevisiae COX17* mutants (19). Although *SCO2* is highly similar in sequence and structure, and in spite of the fact it could rescue the *COX17* mutant, it was unable to complement in *SCO1* mutants. This demonstrates that the SCO proteins are not redundant in yeast. The ability of *SCO1* to rescue mutants of *COX17* was a critical discovery in assigning a copper-related function to SCO proteins and has also been exploited to understand the mechanisms of Sco1 and Cox17 interactions (Figure 1.5).

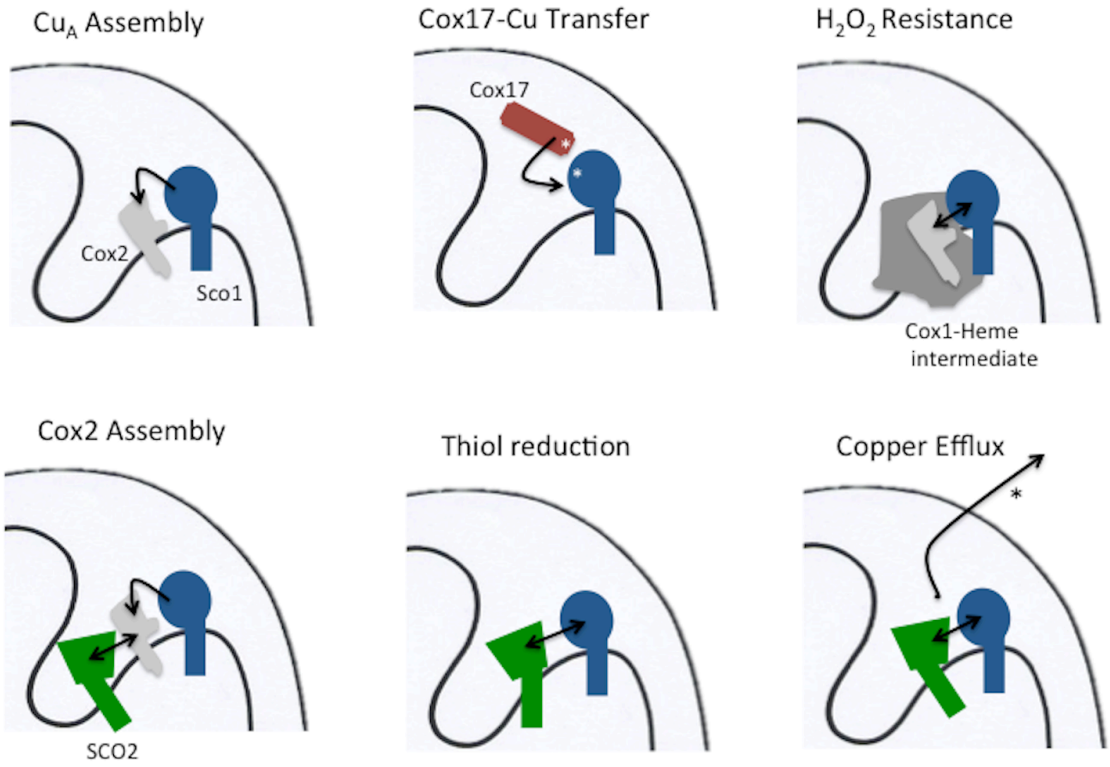

Figure 1.5: The Roles of Sco1 in Mitochondria

In yeast (and humans) Sco1 is required for Cu_A assembly; it receives copper from Cox17. The asterisk represents the position of Cys57 in Cox17 and Pro153 (P174 in humans) in Sco1; mutation of these residues affects the protein-protein interaction and copper exchange. Sco1 plays a role in stability of Cox2 that allows for capping of a Cox1 intermediate that can have pro-oxidant activity. Human SCO1 and SCO2 are required for COX assembly; SCO2 regulates the thiol reduction of SCO1. The interaction of these proteins also signals cellular copper efflux.

Humans also have two *SCO* genes, and despite their similarity and ubiquitous tissue expression, they fulfill independent functions in CcO assembly. Mutations in either gene result in distinct clinical presentations (20). The discovery of a pathogenic allele of human *SCO2* (E140K) that causes fatal cardioencephalomyopathy and COX deficiency confirmed the conserved of role of these proteins in assembly (21). This pedigree and subsequent isolation of a mutant of *SCO1* (P174L) that causes neonatal liver failure and ketoacidotic coma has been crucial to uncovering the details of the role for SCO proteins in assembly of Cu_A in Cox2 (Figure 1.5) (22,23). The striking differences in disease phenotypes has led to a series of structural and protein chemistry studies to try and uncover the difference in function of the SCO proteins. It should be noted that, while numerous studies have shown that humans require both SCO1 and SCO2 for overlapping but independent functions, yeast requires only Sco1 under all conditions reported.

SCO proteins are conserved and have been found in a wide variety of organisms including bacteria that lack cytochrome oxidase-like proteins (24). The fold of the SCO proteins is highly similar to that of peroxiredoxins (25). This fold presents the conserved CxxxC motif on the surface of the protein, making it accessible as a copper-binding site or as a redox active site (26). Indeed this motif seems to fulfill both these functions in different organisms.

Information derived from the solution structures of SCO proteins has led to significant advances in understanding the mechanisms of copper transfer and delivery related to both the interaction interfaces and also oxidation and reduction reactions between the partner proteins (these will be discussed later section 3.4). These studies have been primarily performed with truncated versions of SCO consisting solely of the globular IMS domain. It should be mentioned that truncations of Sco1 that lack the IM tether are nonfunctional in yeast, so information

regarding the important roles for the transmembrane and/or matrix domains are restricted to in vivo observations. Perhaps future structures will include these domains. The truncation studies have been complemented with one study of purified intact Sco1 from yeast mitochondria that demonstrated copper binding (27). The soluble variants bind either Cu(I) or Cu(II) using the cysteine sulfurs of the CxxxC motif and a nitrogen ligand from a conserved histidine residue (28). While this is the full set of ligands for Cu(I), Cu(II)-binding requires an additional oxygen ligand from the carboxyl group of a conserved aspartic acid residue. The relevance of the ability to bind either Cu(I) or Cu(II) is unknown, as the Cu_A site should be capable of accepting either Cu(I) or Cu(II) in the assembly process. The in vivo CcO assembly defects observed in the absence of aspartic acid and histidine residues strongly suggests that copper binding is absolutely required for the function of SCO proteins. In addition to the copper binding assays implicating Sco1 in assembly of Cu_A in Cox2, an early biochemical experiment showed that Sco1 could interact with Cox2 in a pull-down assay, suggesting a physical interaction to allow for the copper exchange reaction (29).

The role for SCO in redox chemistry was initially proposed due to the structural similarity to the peroxiredoxin family (25). This family of proteins participates in antioxidant signaling pathways via modification of active site cysteine residues. Observations of hydrogen peroxide sensitivity in yeast lacking *SCO1* suggested a role in general oxidative stress in mitochondria (30). However, subsequent studies have shown that this sensitivity is due to a misassembled CcO intermediate that has the heme *a* moiety bound and acts as a pro-oxidant (Figure 1.5) (31). This sensitivity to hydrogen peroxide could be suppressed with alleles of *SCO1* lacking the cysteine motif and therefore any redox activity. The hydrogen peroxide growth assay was used to define Sco1-Cox2 interaction. The interaction of Sco1 and Cox2 presumably affects

a Cox1 early intermediate and prevents the pro-oxidant activity (31). This growth assay, coupled with in vitro copper transfer assay with Cox17, was used to determine that the residues of Sco1 present in a loop adjacent to the CxxxC motif were required for interaction with Cox2 but dispensable for interaction with Cox17 (32).

The solution structures of human SCO in multiple conformations provided evidence that the protein can exist in an oxidized state, even while maintaining metal binding, at least in a nickel-bound form (33). The most detailed data demonstrating a role for the eukaryotic SCO protein as a thiol-oxidoreductase come from experiments in human cell culture (34). Mutations in *SCO2* or changes in its expression level affect the ratio of oxidized and reduced cysteine residues in SCO1. This result supports a model where SCO2 can act on SCO1 to modulate its redox state. (Figure 1.5) (35). The active role SCO2 plays to modify SCO1 explains in part the non-redundant but overlapping roles of these proteins in CcO assembly in humans.

Perhaps the most surprising result related to SCO proteins is the role these proteins have in regulating mammalian cellular copper status (Figure 1.5) (36). Immortalized fibroblasts from patients with mutations in SCO1 or SCO2 have a severe copper deficiency as a result of increased export of copper from the cell. The mutations in SCO present in these patient cells trigger a signal that results in inappropriate export of copper from the cell. This trigger can be activated in other CcO assembly mutants without mutation in SCO1 or SCO2, suggesting that the signal plays a role in normal cellular copper homeostasis. Importantly, the copper export signaling in fibroblasts with mutations in genes other than *SCO* can be reversed by expression of SCO proteins (or chimeric SCO proteins). The reversal of this phenotype by increased expression of SCO demonstrates its specific role in this pathway (36). This signaling cascade and

the intermediate molecules involved are still under investigation; one or more of the Cx_9C proteins may be logical candidates.

While the copper-binding characteristics and redox roles described here solely represent data from eukaryotes (except for structures), many elegant and informative studies have been completed in bacterial homologs of SCO proteins. Results from these studies have provided the platform for determining the presumptive mechanisms of copper delivery and thiol-oxidoreductase properties (24, 37-45).

3.3 Cox11: The Cu_B metallochaperone

Deletion of the *COX11* gene results in CcO deficiency in yeast; the initial phenotype appeared similar to that of mutations of genes involved in heme *a* biogenesis (46). In fact, these phenotypes are closely linked because of the role of Cox11 in assembly of the heterometallic heme a_3-linked Cu_B site in Cox1 (Figure 1.6). Cox11 is anchored to the IM by a single transmembrane domain with a large globular domain protruding into the IMS. Experimental data on a homologous protein from *Rhodobacter sphaeroides* demonstrated that Cox11 was required for insertion of copper into the Cu_B site (47). Subsequent in vitro experiments showed that yeast Cox11 could bind copper via cysteinyl sulfur ligands (48). These in vitro experiments were complemented with yeast growth assays that showed that Cox11 required the cysteine residues for function. Further structural evidence from a *Sinorhizobium meliloti* Cox11 homolog revealed a unique immunoglobulin-like fold and showed that the copper-bound form exists as a dimer with cysteine residues arranged at the dimer interface to form a Cu(I)-thiolate cluster (49). The position of this copper-binding site and proximity to the transmembrane domains appear to be optimal for the insertion of copper into the buried Cu_B site.

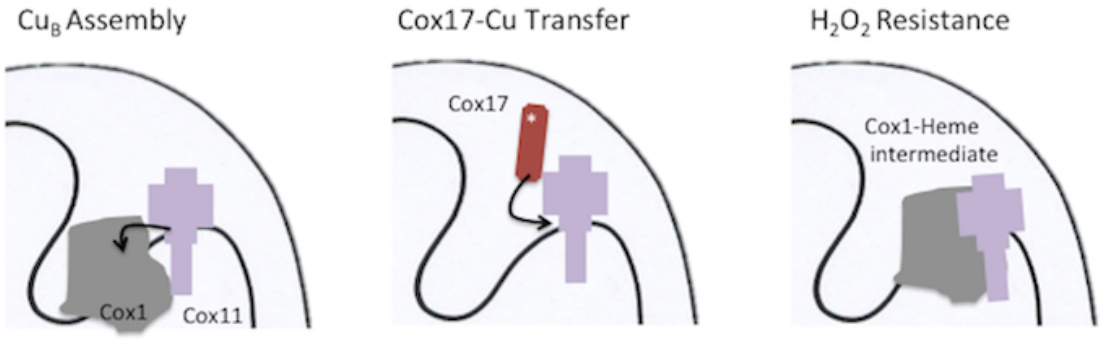

Figure 1.6: The Roles of Cox11

Cox11 delivers copper to Cox1 to form the Cu_B site. It receives its copper from Cox17. The interaction appears independent of the Cys57 containing interface (asterisk). This early step in assembly blocks formation of the Cox1 pro-oxidant intermediate.

Insertion of copper and heme a_3 must occur at an early step in the CcO assembly process due to their buried position in the final complex. Yeast Cox11 has been shown to interact with the mitochondrial ribosome, which could localize the proteins at the sites where nascent Cox1 polypeptide is being inserted into the membrane (50,51). In fact, in *Schizosaccharomyces pombe* Cox11 is fused to a mitochondrial ribosomal protein; the *S. cerevisiae* Cox11 protein can function when fused to the equivalent ribosomal protein. However, multiple studies have demonstrated that this interaction is dispensable for function and that the matrix domain appears to be necessary only for targeting and localization (51,52).

Cox11 transiently interacts with Shy1 (SURF1 homolog in yeast) which is required for heme a_3 insertion into Cox1 (53). This interaction and the role that Cu_B has in closing the assembly step for Cox1 was uncovered by the peroxide sensitivity that is induced by deletion of *COX11* (Figure 1.6). The peroxide sensitivity is due to a pro-oxidant intermediate of Cox1 that contains the heme *a* (31). These data show the degree of coordination required to form the early core intermediates of CcO that bind the copper cofactors. CcO quality control is mediated by proteases that turn over those intermediates which fail to progress. The quality control mechanism may be hyper-activated and assembly factors could have an additional role in protecting the intermediates to allow them to progress to the final complex (54,55).

3.4 Copper transfer: Cox17 to Sco1 and Cox11

Copper availability in the IMS appears to be limited; this is an extension of the situation in the eukaryotic cytosol where transport and sequestration limit the available copper, leading to a demand for copper chaperones (1). In the IMS, Cox17 serves the role of presenting bioavailable copper to Sco1 and Cox11 (Figure 1.4, 1.5, and 1.6).

The Cox17-Sco1 copper transfer reaction is the most thoroughly studied copper transaction of the IMS. Cox17 transfers copper to the exposed CxxxC site on Sco1 through an interaction face that is perturbed by the pathogenic human mutation *SCO1* P174L (Figure 1.5) (22,23). In vitro transfer reactions have suggested that like other copper chaperone-target pathways, a transient interaction of the two proteins results in exchange of copper. In vitro studies with multiple conformers of Cox17 isolated from heterologous expression systems support this interaction, and mass spectroscopy and NMR studies have shown that the copper transfer reactions proceed from a Cu-loaded, partially oxidized conformer of human COX17 to SCO1 (11,56). NMR dynamics studies showed that the mixture of Cu(I)-COX17 conformer, which binds a single copper and has two disulfide bonds, with apo-SCO1 in the oxidized state resulted in an interaction that yielded fully oxidized COX17 and Cu-SCO1 (56). This reaction is specific as it did not occur with the highly homologous SCO2. These in vitro structural observations are complementary to the results from co-expression of COX17 and the SCO proteins in the cytosol of *S. cerevisiae*. In yeast cytosol, a chelating environment, SCO1 metallation was dependent on COX17 but SCO2 copper binding could be independent of COX17 (57).

The final data that support this copper delivery pathway include a combination of in vitro observations and genetic suppression experiments. The phenotype of the *COX17*^{C57Y} allele can be suppressed by over-expression of *SCO1*[19]. Additionally, defects observed in *cox17*Δ are reversed by expression of *SCO1* with the addition of copper[22]. Both of these results support the notion that Sco1 receives copper downstream of Cox17. These studies were further complemented by data showing that the *COX17*^{C57Y} allele could not be rescued by yeast *SCO1* with a P153L mutation (equivalent to the human pathogenic SCO1 P174L mutation). In this

case, the combination of two crippled alleles that disturbed the interaction interfaces of both proteins prevented assembly of CcO in yeast (Figure 1.5).

The delivery of copper to Cox11 has not been as thoroughly studied. However, Cox11 is a target of Cox17. Copper transfer occurs via a separate interaction interface than that used for Sco1, as the copper transfer to Cox11 is not disrupted with the Cox17 C57Y variant (Figure 1.6) (57). These biochemical data may explain in part the ability to rescue this *COX17*^{C57Y} allele with lower levels of copper than what is required to rescue a *COX17* deletion. That is, a complete deletion of *COX17* abrogates not only the metallation of the exposed Sco1 copper-binding site but also the buried Cox11 binding site while in the *COX17*^{C57Y} allele only the exposed site in Sco1 would be affected. In general these data reinforce the idea that limited copper availability and specific protein-protein interactions are required for the correct metallation of cuproenzymes. The source of the copper supplied to Cox17 to facilitate these reactions appears to be located in the mitochondrial matrix.

3.5 Superoxide dismutase

The IMS also houses 1-5% of the total cellular superoxide dismutase (Sod1). Sod1 contains both copper and zinc cofactors and functions in the disproportionation of superoxide to hydrogen peroxide and oxygen (58). The activity of this protein in the IMS is to protect a distinct class of mitochondrial proteins that are not protected by the matrix-localized, manganese-containing superoxide dismutase (Sod2). Ccs1, the copper chaperone for superoxide dismutase, inserts copper into Sod1 in the IMS. Ccs1 activates Sod1 by inserting copper into newly imported apo-protein in the IMS and then catalyzing the formation of an essential disulfide bond (59). Ccs1 has three defined domains: a βαββαβ folded domain with a CxxC motif, a central Sod1-like domain and a carboxy-terminal domain (60,61). Ccs1 interacts with Sod1 as a

heterodimer via the Sod1-like domain that is also required for the activation (62). Mitochondrial localization of Ccs1 is dependent on the Mia40 import pathway that uses a disulfide relay exchange system to fold and trap proteins in the IMS. Import is dependent on a pair of cysteine residues present in the βαββαβ folded domain that are not part of the exposed CxxC motif (63). Sod1 accumulation in the IMS is dependent on the presence of Ccs1 in this compartment. The copper used in these reactions for Sod1 in the IMS originates from the soluble pool in the mitochondrial matrix.

4. Copper in the Matrix

Quantification of total copper in the mitochondria suggests that copper is found in excess of that required for COX and Sod1 (4,64). In fact mitochondrial copper levels do not change even when these proteins are absent. The levels of matrix copper change in response to changes in global cellular copper; increased copper availability leads to increases in the matrix pool (4,65). However, no conditions have been identified that limit matrix copper to undetectable levels.

Depletion of the cytoplasmic metallothionein in yeast allows for significant expansion of the mitochondrial matrix copper pool. This result implies that copper must avoid being intercepted on route to mitochondria by sequestration molecules and other copper chaperones in the cytosol. The modulation of mitochondrial copper levels by chelators such as metallothioneins is not consistent with the characterized cytosolic protein-mediated copper chaperone pathways. A current model suggests that this copper is recruited to mitochondria by a non-proteinaceous trafficking system (Figure 1.7) (66). Biochemical and analytical experiments have been able to uncover certain aspects of this copper complex but the exact identity of this molecule and the pathways used to make it are not yet known. Using the chromatographic properties and a

fluorescence signature it has been proposed that the Cu-ligand (CuL) exists in the cytosol in a metal-free form where it binds copper and is recruited to mitochondria. The eventual matrix import of the CuL complex must require transport proteins to facilitate crossing the impermeable IM. The existence of the pool has been confirmed in cell culture with X-ray fluorescence techniques and metal specific chelators and is supported by independent spectroscopic assays that used specific signals to compare total mitochondrial copper levels relative to the levels of copper estimated based on COX-specific heme levels (64,65,67).

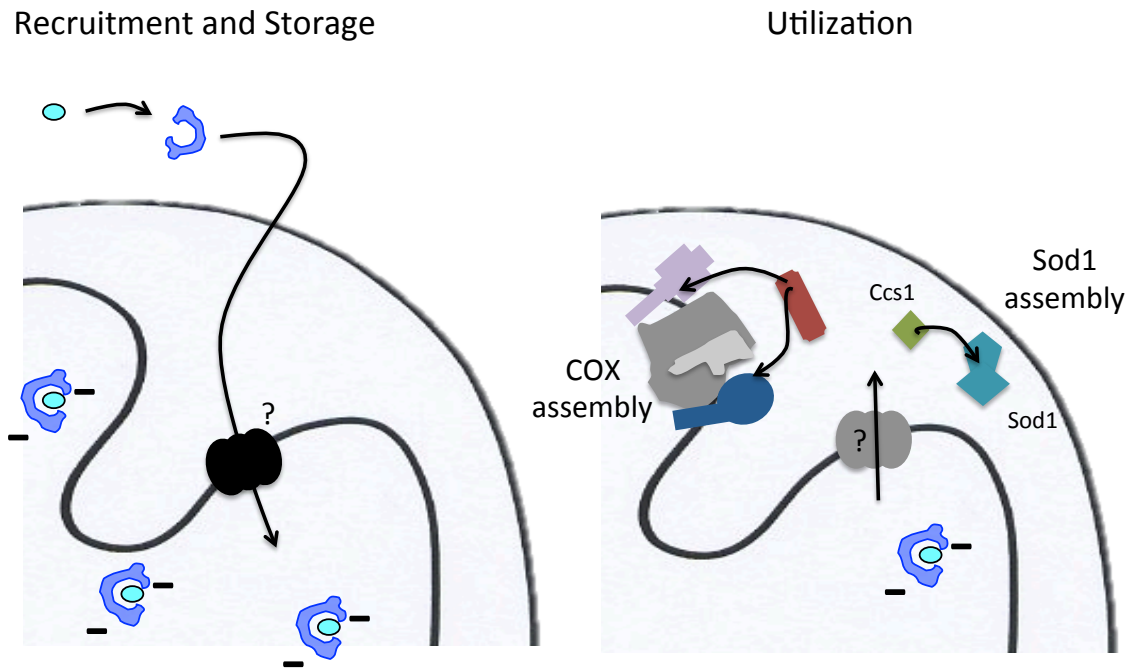

Figure 1.7: Mitochondrial Matrix Copper Pool

The current model suggests copper is recruited in the cytosol by a non-proteinaceous ligand that is translocated across the inner membrane by an unidentified transporter. The matrix CuL complex is anionic and soluble and appears to be the source of copper for the IMS copper chaperones, Cox17 and Ccs1 for the activation of COX and Sod1.

This model and the existence of the matrix copper pool has been biochemically and genetically interrogated in the eukaryotic model *S. cerevisiae.* Mutants of *S. cerevisiae* that lack *SOD2* cannot propagate in high oxygen conditions, but this growth defect can be rescued by targeting the copper-dependent human Sod1 to the matrix, showing that bioavailable copper exists in the matrix (4). This is consistent with the presence of a stable, non-proteinaceous copper complex. Manipulation of available copper in the matrix via expression of heterologous copper-binding competitor molecules prevents assembly of CcO and partially depletes Sod1 from the IMS (68). This phenotype can be reversed by supplementation of the medium with copper. Finally, matrix-targeted copper-binding competitors prevented an IM-tethered human *SOD1* from complementing the growth defects (auxotrophy for lysine and sensitivity to oxygen) of *sod1*Δ or *ccs1*Δ. Activation of this heterologous cuproenzyme should be independent of endogenous regulation (mitochondrial translation or availability of assembly factors) and so these experiments address copper availability while removing other variables. These data also support the model that copper must enter the matrix before being redistributed to the IMS.
One aspect of the matrix copper pool that has been recently suggested is that the localization inside the matrix protects this copper from export in the cytoplasm (34,65). SCO1 patient fibroblasts are profoundly copper deficient, yet the mitochondrial matrix pool is maintained, as measured by a matrix targeted copper-dependent fluorescent probe (65). A second observation in the same setting also supports the maintenance of the matrix pool and further supports the role of matrix copper as the source of copper for the assembly of CcO. The P174L allele of *SCO1* acts as a dominant signal inducing copper deficiency even in the presence of wild-type *SCO1*. In this cell line (*SCO1*P174L expressing WT SCO1) the CcO deficiency is completely reversed.

Therefore the pool of copper required to assemble CcO is intact in *SCO1* patient fibroblasts in spite of the global copper deficit.

The only example of depletion of the matrix copper pool without heterologous expression of competitors relates to assembly factors for Cox1 (69). The assembly factors Coa1 and Shy1 cooperate in early stages of the Cox1 assembly. These proteins regulate the complexes involved in *COX1* translation and also recruit other complex members to facilitate heme *a* binding and prevent degradation of the intermediates. Surprisingly, mitochondria isolated from *S. cerevisiae* lacking either, *COA1*, *SHY1,* or both are copper deficient. The relevance of this copper deficiency is unknown, however copper supplementation to the medium can reverse the growth defect on non-fermentable carbon sources in strains lacking *COA1* or *SHY1*. These data hint at a coordination of copper availability with the translation of a copper target (Cu_B in Cox1). However despite extensive proteomic and genetic experiments with tagged *COA1* or *SHY1,* or mutant yeast strains, no connection has been identified with a candidate transport protein.

5. Conclusions and Future Considerations

A copper requirement for mitochondrial function has been recognized for decades, however even long after the discovery that cytochrome c oxidase is a cuproenzyme many unanswered questions remain. The number of identified mitochondrial proteins with copper-binding capacity continues to grow, but the requirement for so many potentially redundant partners seems perplexingly exaggerated. This disproportionate existence of assembly factors is contrasted by the lack of knowledge regarding trafficking of copper to mitochondria and the transporters involved in transit across the IM. In part this paucity of knowledge could be due to overlapping pathways in the synthesis of the biological ligand for delivery to mitochondria (Cu-ligand) or redundancy or limited specificity in the transporters in the IM.

Mitochondrial copper is proposed to transport into the matrix before redistribution to the IMS for loading of copper enzymes. This is reminiscent of the copper-handling strategies utilized by gram-negative bacteria, where copper enters the cytoplasm before being transported back across the plasma membrane to the periplasm (70). The endosymbiotic theory of eukaryotic origin states that a gram-negative, α-proteobacterium was engulfed by another cell and became the mitochondrion in early eukaryotes (3). While the biosynthesis of iron-sulfur clusters is the sole essential function of modern mitochondria, initial stages of selection of the endosymbiont could have depended ATP production. If this were a copper-dependent process, it would be advantageous for the endosymbiont to maintain a stable copper pool even when the host became deficient. The observations made in *SCO* fibroblasts support this type of model as copper is retained during induced cellular deficit. Therefore the complicated cycling of copper to the IMS via the matrix guarantees availability and may have been important for retention of an original endosymbiont.

Why is copper recruited by a non-proteinaceous ligand? This apparent break from the metallochaperone paradigm in eukaryotes could be due to the requirement of unfolded proteins for mitochondrial import. Metabolites are routinely transported across the IM so this ligand may have been selected for to provide protection against the potential toxicity of free copper. Free ionic copper is transported by P-type ATPases at the plasma membrane and in the trans-Golgi. However no P-type ATPases are found in the IM. The mitochondrial IM does have multiple types of transporters to exchange small molecules and mineral elements. These include a family of proteins that are responsible for metabolite and nucleotide exchange, the mitochondrial carrier family (MCF), ATP binding cassette-transporters, and most recently multimeric associations of single transmembrane domain proteins that allow transport of pyruvate, potassium, or calcium

(71-73). Perhaps the MCF proteins are the best fit as they would be ideally suited to transport a metabolite-like complex. They have also been previously implicated in mitochondrial iron homeostasis (74). High affinity iron uptake into the matrix in *S. cerevisiae* is disrupted under iron starvation conditions by double deletion of MCF proteins encoded by *MRS3* and *MRS4* (75). Mtm1, originally identified due to a lack of Mn-Sod2 activity, has been shown be required for correct iron handling to prevent mis-metallation of Sod2 with iron (74). In addition, Ggc1, a GTP/GDP exchanger, is also required for correct iron handling. Deletion of *GGC1* causes a defect in Fe-S and heme that can be reversed by normalization of mitochondrial GTP levels through expression of a nucleoside diphosphate kinase (77). Therefore a strong precedent exists for multiple MCF proteins to be involved in transport/regulation of redox metals.

The current model of non-proteinaceous recruitment to mitochondria is also consistent with the strategy that methanotrophs use to recruit copper for the cuproenzyme particulate methane monoxygenase (pMMO). These bacteria recruit copper from the environment using methanobactin, a modified peptide-based molecule that acts similarly to iron siderophores (78). When required these bacteria produce methanobactin, export it to the extracellular milieu, and then recruit it back to the cell to extract the copper for use in pMMO (79). The pMMO is loaded in convoluted membrane invaginations reminiscent of the mitochondrial IM cristae. Finally, the non-proteinaceous ligand recruitment model is also consistent with the recent discovery of a eukaryotic intracellular iron siderophore (80). This intracellular siderophore appears to be required at least indirectly for mitochondrial iron import as mutants in yeast appear to have decreased heme synthesis.

A non-proteinaceous ligand suitable for transport by a MCF protein could be produced via multiple intertwined synthesis and degradation pathways. As components of the system are

identified perhaps they can be crippled to uncover the mechanism of transport and synthesis, similar to the important role partially functional alleles of *SCO* in humans have played in defining roles of protein components of mitochondrial copper homeostasis. Identification of the transporters and ligand synthesis pathway will remove one of the rate limiting steps in our understanding of the regulation and mechanisms of distributions of copper in the mitochondria.

6. References

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Chapter 2: Copper Import into the Mitochondrial Matrix in *Saccharomyces cerevisiae* is Mediated by Pic2, a Mitochondrial Carrier Family Protein

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Abstract

Saccharomyces cerevisiae must import copper into the mitochondrial matrix for eventual assembly of cytochrome *c* oxidase. This copper is bound to an anionic, fluorescent molecule known as the copper ligand (CuL). Here, we identify for the first time a mitochondrial carrier family protein capable of importing copper into the matrix. *In vitro* transport of CuL into the mitochondrial matrix is saturable and temperature-dependent. Strains with a deletion of *PIC2* grow poorly on copper-deficient, non-fermentable medium supplemented with silver and under respiratory conditions when challenged with a matrix targeted copper competitor. Mitochondria from *pic2*Δ cells have lower total mitochondrial copper and exhibit a decreased capacity for copper uptake. Heterologous expression of Pic2 in *Lactococcus lactis* significantly enhanced CuL transport into these cells. Therefore, we propose a novel role for Pic2 in copper import into mitochondria.

Introduction

Metals are essential nutrients that pose a management quandary for cells. They must be directed to the correct proteins and organelles through a maze of cellular components and

opportunistic metal binding sites (1). Failure to control their delivery results in cellular stress, presumably due to inappropriate interactions and oxidative damage. Cells have adopted a protein-mediated delivery mechanism for copper within the cytosol. In the budding yeast *Saccharomyces cerevisiae*, copper enters the cell via specific (Ctr1) and non-specific transporters (e.g. Fet4), and is then trafficked to points of utilization by copper chaperone proteins (2). Copper is used as a cofactor in three major enzymes: the multi-copper oxidase Fet3 required for high affinity Fe uptake (3); Sod1, a Cu, Zn superoxide dismutase required for protection against oxidative stress and regulation of glucose signaling in yeast (4); and cytochrome *c* oxidase (CcO), the terminal enzyme complex of the electron transport chain (5). Atx1 is the copper chaperone responsible for delivering copper to the trans-Golgi vesicles via Ccc2, a P-type ATPase (6), while Ccs1 serves as the copper donor for Sod1 and acts as a post-transfer modifying enzyme by facilitating the formation of an essential disulfide bond within the enzyme itself (7). Though cytosolic copper trafficking has been well characterized, the pathway that delivers copper to mitochondria in yeast and in other eukaryotes is completely unknown.

CcO is a multimeric protein complex that contains two copper centers, a binuclear Cu_A site and a heme *a3*-Cu_B site. A number of assembly factors act in concert to build both of these sites. The soluble intermembrane space (IMS) protein Cox17 delivers copper to both Sco1 and Cox11, which are integral inner membrane (IM) proteins that donate copper to the assembling holoenzyme (8,9). Additionally, the IMS protein Cmc1 has been implicated in the control of copper flow within the IMS, potentially by directing copper to the Cox17-mediated CcO assembly pathway (10).

Organellar fractionation experiments showed that greater than 70% of mitochondrial copper is present as a soluble, anionic complex contained within a matrix-localized, bioavailable

pool (11). This complex has been defined as the copper ligand (CuL) and its existence and localization have since been confirmed by X-ray fluorescence imaging and copper chelation studies (12,13). Copper-dependent human Sod1 localized to this mitochondrial compartment is able to rescue a range of phenotypic defects associated with *SOD2* deletion, demonstrating the accessibility of this pool (11). Expression of matrix-targeted Sod1 or Crs5, a copper-binding metallothionein, results in a specific loss of CcO activity that can be rescued by the addition of copper or decreased expression of the competing cuproprotein (14). These observations led us to propose that the matrix copper pool is redistributed to the IMS where it is made available to CcO. Although the exact structural identity of the CuL is unknown, we have characterized many aspects of its *in vivo* function. We propose that the ligand exists in the cytosol in a metal-free form where it binds copper and delivers it to mitochondria, providing a non-proteinaceous trafficking system for copper delivery to the organelle.

The IM is impermeable to most ions and molecules, so transporters must exist that facilitate matrix import of the CuL complex and its subsequent redistribution to the IMS. However, transporters required for the movement of copper across the IM have yet to be identified, and remain a fundamental gap in our understanding of the mechanisms that provide for the assembly of CcO. Data from our previous studies suggest that CuL is the molecule that is transported into the matrix, and that this complex may resemble a metabolite or nucleotide. Therefore copper transport across the IM may proceed through one or more of the mitochondrial carrier family (MCF) proteins. These proteins transport diverse metabolic substrates such as oxaloacetate, citrate, GTP and ATP into and out of the matrix (15).

MCF proteins have previously been implicated in metal ion homeostasis (16). High affinity iron uptake into mitochondria of *S. cerevisiae* is disrupted by simultaneous deletion of

MRS3 and *MRS4* (17). Studies of the Mrs3/4 homologs in vertebrate systems have demonstrated the conserved function of these proteins (18-22). Other members of this family have also been associated with iron transport with varying levels of specificity (23-26). Therefore a precedent exists for the involvement of multiple MCF proteins in modulating mitochondrial metal ion homeostasis. Herein, we present evidence that the MCF protein Pic2 transports copper across the mitochondrial inner membrane, allowing for its accumulation within the matrix.

Materials and Methods

Yeast Strains, Culture Conditions, and Standard Methods

The yeast strains used in this study were BY4741 (*MATa, leu2*Δ*, met15*Δ*, ura3*Δ*, his3*Δ) and the isogenic kanMX4- containing mutant from Invitrogen. The *ccs1*Δ*::IMhSOD1* was created in the Y7092 background (*MAT*α, *can1*Δ*::STE2pr-Sp_his5 lyp1*Δ *his3*Δ *leu2*Δ *ura3*Δ *met15*Δ) (27). All cultures were grown in YP (1% yeast extract, 2% peptone) medium or synthetic defined media (with selective amino acids excluded) with the appropriate filter sterilized carbon source added. Metal concentrations were varied by using Bio101 yeast nitrogen base (Sunrise, Inc) plus added 0.1 mM ferrous chloride to give copper-deficient conditions. If required, further copper chelation was achieved by adding bathocuproine disulfonic acid (BCS). Exogenous copper was provided by adding $CuSO_4$. All of the growth tests were performed at 30 °C with 1:10 serial dilutions of pre-cultures grown under permissive conditions.

Vector Constructs

Matrix-targeted Crs5 was described previously (14). The *PIC2*/*YER053C* open reading frame (ORF) plus 300 base pairs upstream (to include the endogenous promoter) was cloned into pRS415. The *PIC2* ORF was also cloned into pNZ8148 (Mobitech) under control of the nisin-

inducible promoter. The fidelity of each construct was verified by dideoxynucleotide sequencing prior to use.

Isolation of CuL and AgL from mitochondria

Intact mitochondria were prepared and the resultant soluble contents were fractionated as described previously (11). Anionic fractions for reverse phase (RP) chromatography were prepared by adding DEAE (Whatman) resin in batch. The resin was washed with 25 bed volumes of 20 mM ammonium acetate, pH 8.0, and eluted with 5 volumes of 1 M ammonium acetate, pH 8.0. The samples were loaded directly onto a Phenomenex C18 column. Unbound fractions were removed with 50 mM ammonium acetate, pH 5.0 (or 0.1% trifluoroacetic acid to isolate the apo ligand). A 60-min gradient to 100% acetonitrile was used and 1 ml fractions were collected. The final fractions were analyzed for copper by ICP-OES (PerkinElmer Life Sciences 9300-DV) and for fluorescence (PerkinElmer Life Sciences LS55 fluorimeter). Excitation and emission scans of copper-containing fractions used an excitation maximum of 220 nm and an emission maximum of 360 nm using 5 nm slit widths.

Pic2 expression in Lactococcus lactis

L. lactis cells transformed with vector (pNZ8148) alone or carrying the *PIC2* gene were grown overnight at 30˚C in M17 medium with 0.5% glucose and 10 µg/mL chloramphenicol. Cells were diluted into fresh medium at an OD_{600} of 0.1, grown to an OD_{600} of 0.4, and induced using 1 ng/mL nisin for five hours. Protein expression was confirmed using SDS-PAGE followed by SYPRO staining or immunoblot for Pic2.

Copper uptake assays

Isolated mitochondria suspended in 0.6 M sorbitol were incubated with CuL for 30 second intervals and removed from solution by centrifugation. Uptake was measured by ICP-OES as an

increase in copper over time. Copper uptake was assayed in *L. lactis* using a modified method where whole cells were resuspended in soluble matrix copper, purified ligand, or copper salts in either water or potassium phosphate buffer, pH 7.5. Cells were incubated for different time points at room temperature, removed by centrifugation, washed in water, and total metals were measured by ICP-OES. Uptake was reported as the increase in copper over time.

Enzyme Assay

CcO and malate dehydrogenase (MDH) activities were measured as described previously using a Shimadzu UV-2450 (11). Superoxide dismutase (SOD1) activity was measured using a xanthine oxidase-linked assay kit (Sigma Life Science).

Miscellaneous Methods

The monoclonal anti-human SOD1 was purchased from Santa Cruz Biotechnology. Antisera to cytochrome c oxidase subunit 2 (Cox2) and Porin were purchased from Invitrogen. Antisera for yeast Pic2 was raised against a synthetic peptide consisting of the 20 amino-terminal residues (Genscript).

Results

Copper transport into isolated mitochondria

We assume that the transported form of copper into mitochondria is the CuL complex. To assess transport characteristics into yeast mitochondria, we isolated the soluble matrix copper contents using anion exchange resin and incubated purified intact mitochondria with variable concentrations of the stable CuL complex. Mitochondria were removed by centrifugation and then assayed for their total copper content by ICP-OES. CuL was imported into mitochondria in a time-dependent manner (Figure 2.1A). The observed increase in copper content was not due to membrane association as lysis of mitochondria via sonication before assay prevented

accumulation (Figure 2.1A), and lysis after uptake released the copper into the soluble fraction (80 ± 5 % soluble). Increasing concentrations of the CuL complex saturated the initial rate of copper uptake (Figure 2.1B). Half maximal transport was observed at approximately 15 μM CuL complex. Uptake was temperature-dependent as incubation of mitochondria at 4°C prevented CuL accumulation (Figure 2.1C), and identical initial rates were obtained in mitoplasts lacking the outer membrane, suggesting that the transport occurred at the inner membrane (Figure 2.1C). Addition of the uncoupling ionophore carbonylcyanide m-chlorophenylhydrazone (CCCP) did not affect the initial rates of uptake (Figure 2.1C). Therefore we conclude that the mitochondrial inner membrane has a saturable, temperature-dependent copper-transport system.

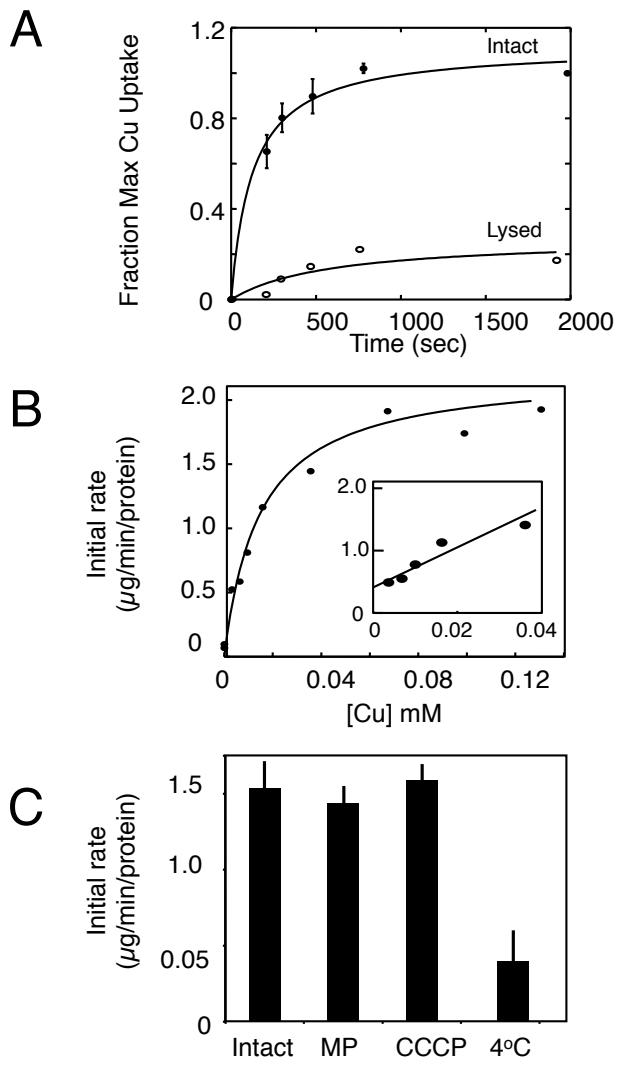

Figure 2.1: *In vitro* CuL Uptake in Purified Mitochondria

A) Intact mitochondria were incubated with purified CuL then isolated by centrifugation and Cu was measured by ICP-OES. Fraction of maximal uptake is plotted versus incubation time for an average of three independent experiments with wild-type mitochondria. Error bars represent standard deviation. B) Initial rates of uptake across a range of CuL concentrations. Data is fit by a hyperbolic curve. Inset is initial rate of uptake over a 0-50 μM range of CuL, and is fitted by a linear regression. C) Initial rate of CuL uptake measured by ICP-OES in intact mitochondria, mitoplasts (MP) prepared by hypotonic lysis, mitochondria incubated with the uncoupler CCCP or mitochondria incubated at 4°C. The averages with standard deviation are shown for three independent experiments.

The silver-ligand complex acts as a competitor for copper uptake

External chelators are often used to deplete the medium of copper. However, we sought to find a competitor that could more directly affect mitochondrial Cu. Silver (Ag) shares similar electronic properties to Cu and it is often used as a toxic mimetic of Cu in biological systems (28,29). We therefore isolated intact mitochondria from yeast grown in glucose containing medium with or without 185 µM $AgNO_3$. Mitochondria from cells grown in the presence of Ag accumulated 17±0.4 mmol Ag/ mol S and contained 3.3±0.2 mmol Cu/ mol S, while mitochondria from untreated cells contained 5.4±0.1 mmol Cu/ mol S. While the mitochondrial copper content was reduced in Ag-treated cultures, the other mineral element concentrations were comparable to untreated cultures (Figure 2.2A). The decrease in copper was associated with a decrease in CcO activity and oxygen consumption (Figure 2.2B). Separate cultures were grown in media containing either 150 µM Ag or 150 µM Cu. Under these identical conditions Ag accumulated to ~5 fold higher concentrations in mitochondria than did Cu (data not shown). Moreover, addition of 185 µM Ag to rich medium containing a non-fermentable carbon source limited the growth of wild-type cells (Figure 2.2C). Mitochondria from these Ag-treated cells were fractionated into soluble and insoluble components and the soluble contents were separated by anion exchange chromatography (Figure 2.2D). The fractions containing CuL also contained an anionic Ag complex (AgL).

Anionic AgL was used for *in vitro* uptake assays. Like CuL, the AgL complex was imported into mitochondria in a time- and concentration-dependent manner (Figure 2.3A). Based on the *in vivo* observation that Ag affected copper accumulation in mitochondria, we attempted to mimic this *in vitro*. AgL complex was added to mitochondria in 10-fold excess as compared to the CuL and the initial rate of uptake was monitored. The excess AgL acted as a competitor,

greatly decreasing CuL uptake (Figure 2.3B). Conversely, a 10-fold excess of CuL slowed uptake of the AgL into mitochondria (Figure 2.3C).

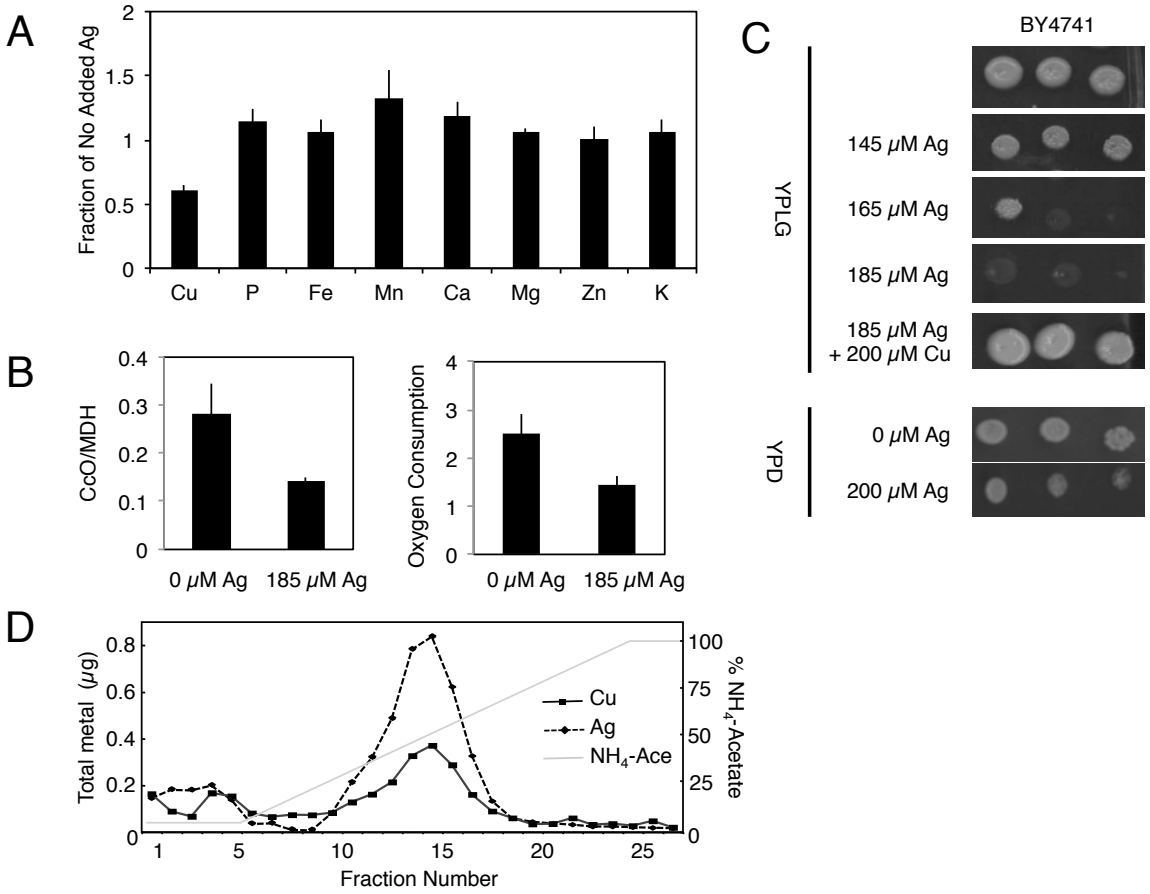

Figure 2.2: Ag Accumulates in Yeast Mitochondria

A) Mineral element content of purified mitochondria from BY4741 yeast cultured in the absence or presence of 185 µM Ag was quantified by ICP-OES, and expressed as a ratio (i.e. +Ag/-Ag). The averages with standard deviation of three independent cultures and mitochondrial preparations are shown. B) CcO activity of purified mitochondria from A) and oxygen consumption of whole cells cultured in YP-galactose medium with 0 or 185 µM Ag added. C) Serial dilutions of BY4741 yeast grown on rich medium with a fermentable carbon source (glucose; YPD) or with a non-fermentable carbon source (lactate-glycerol; YPLG) in the presence of the cell-impermeable copper chelator BCS and increasing Ag concentrations. The growth defect was reversed by adding Cu to the BCS- and Ag-supplemented plates. D) Anion exchange fractionation of soluble contents from purified mitochondria isolated from cultures grown in 185 µM Ag. Cu and Ag were measured by ICP-OES, and are shown as solid line with closed squares for Cu and dashed line with diamonds for Ag.

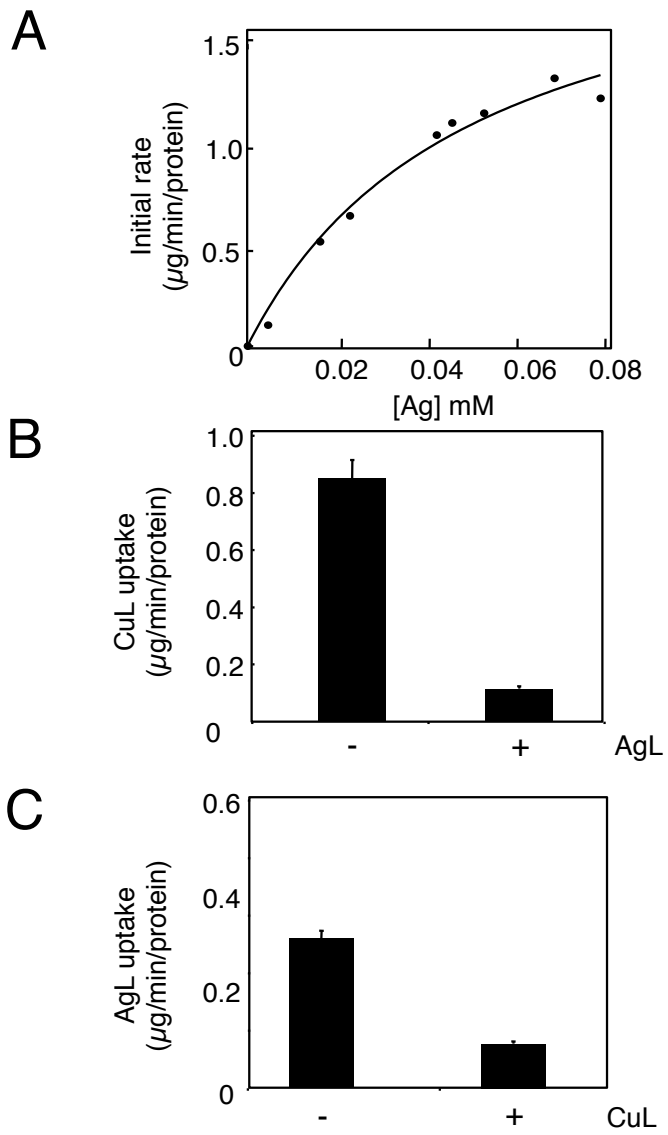

Figure 2.3: *In vitro* AgL Uptake Into Purified Mitochondria

A) Intact mitochondria were incubated with purified AgL, then isolated by centrifugation and Ag measured by ICP-OES. Initial rates of uptake were plotted against a range of AgL concentrations. A hyperbolic curve is fitted. B) Initial rate of CuL uptake at 10 μM CuL measured by ICP-OES in intact mitochondria in the presence of 100 μM AgL (n=5). C) Initial rate of AgL uptake with 10 μM AgL measured by ICP-OES in intact mitochondria in the presence of 100 μM CuL (n=5).

Deletion of PIC2 results in Cu-related phenotypes

Yeast lacking *PIC2*, which encodes a MCF protein, had a growth defect on non-fermentable medium in the presence of the cell-impermeable copper chelator BCS. This defect was exacerbated by addition of Ag to the medium and reversed upon the addition of copper (Figure 2.4A). The decrease in growth was accompanied by a Ag-dependent depletion of Cox2 protein in these mitochondria (Figure 2.4B). The *pic2*Δ strain also showed a defect on copper-limited synthetic medium with a non-fermentable carbon source, without the addition of Ag (Figure 2.5A). In agreement with the growth phenotype, we observed a 50% reduction in CcO activity and oxygen consumption in the *pic2*Δ mutant (Figure 2.5B). To exaggerate the growth defect in the *pic2*Δ strain, we transformed the cells with a matrix-targeted copper metallothionein (m*CRS5)* that we have used previously to biochemically deplete the bioavailable matrix copper pool (14). The *pic2*Δ strain expressing *mCRS5* exhibited a greater growth defect than the *pic2*Δ strain alone on copper replete non-fermentable medium, a defect that became more severe upon depletion of available copper from the medium by the addition of increasing BCS concentrations (Figure 2.5C).

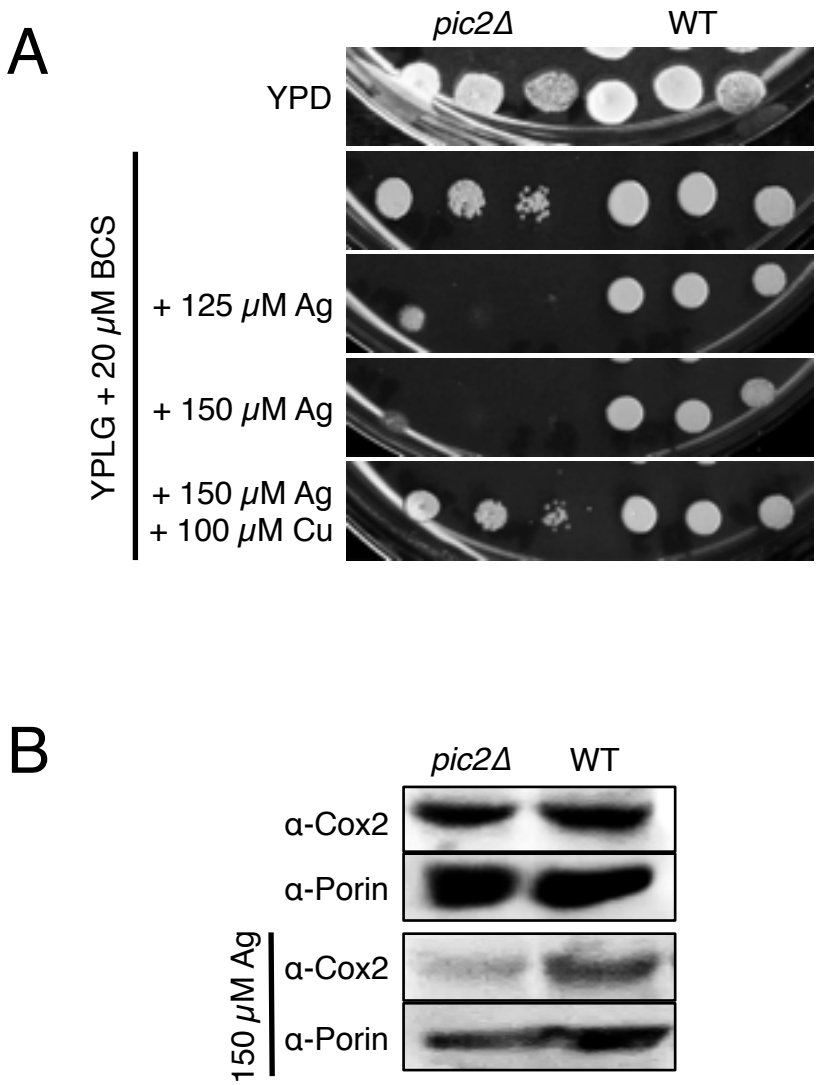

Figure 2.4: Ag Related Growth Phenotypes of *pic2*Δ Yeast Strains

A) Serial dilutions of BY4741 and *pic2*Δ strains grown on rich medium with a fermentable carbon source (glucose; YPD) or with a non-fermentable carbon source (lactate-glycerol; YPLG) in the presence of the cell-impermeable copper chelator BCS and increasing Ag concentrations. The growth defect was reversed by adding Cu to the BCS- and Ag-supplemented plates. B) Western blots for Cox2 in mitochondria isolated from cells grown in rich medium with a fermentable carbon source (top) or in identical medium supplemented with 150 µM Ag (bottom). Porin served as an internal loading control.

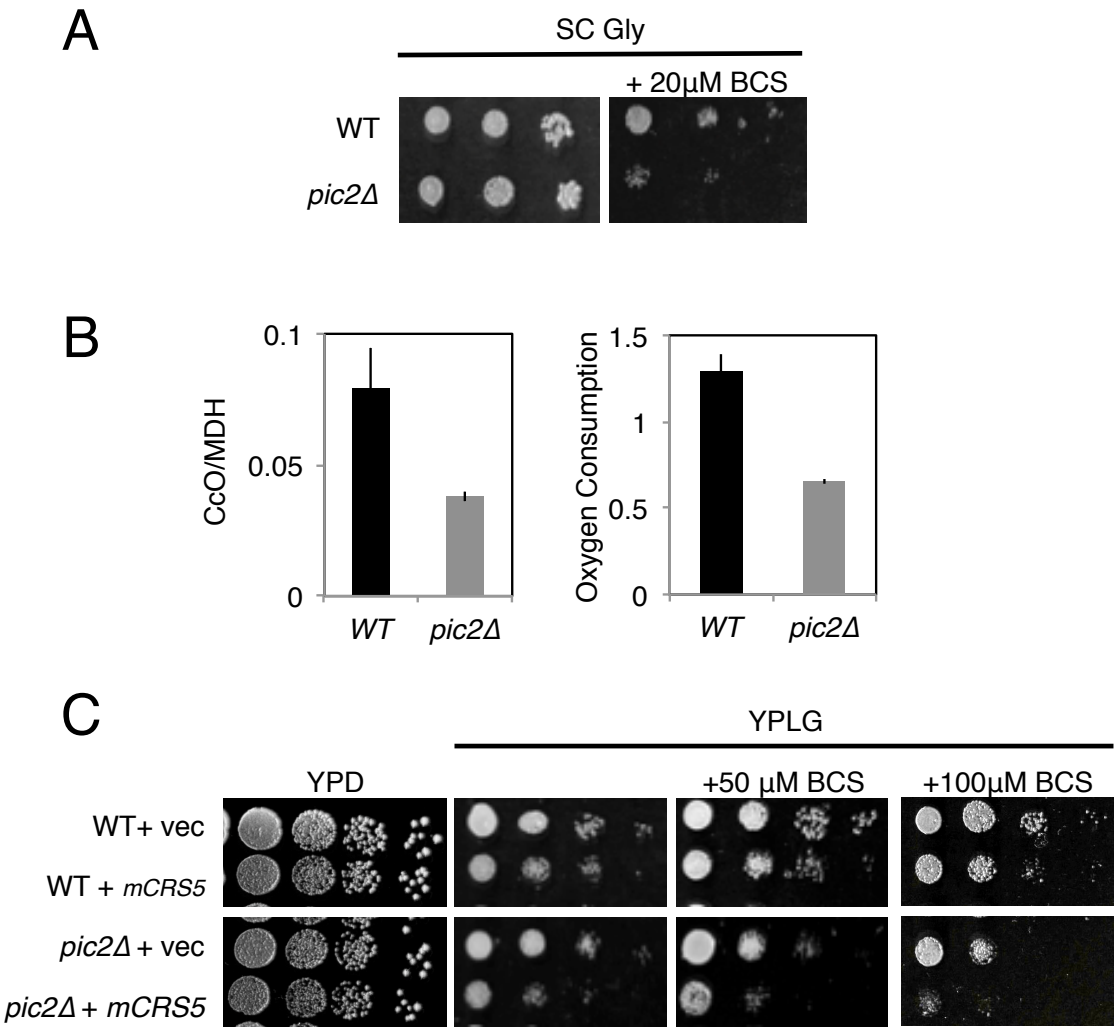

Figure 2.5: Growth Phenotypes of *pic2*Δ Yeast Strains Under Copper Depletion

A) Serial dilutions of BY4741 and *pic2*Δ strains grown on synthetic medium with a non-fermentable carbon source (glycerol; SC Gly) or in SC Gly with 20 μM BCS. B) CcO activity in mitochondria isolated from parental and *pic2*Δ strains grown in synthetic medium containing galactose as a carbon source, and oxygen consumption, read as % air/OD600/s of whole cells from each strain grown in galactose-containing rich medium (n=3). C) Serial dilutions of parental and *pic2*Δ strains transformed with either empty vector (vec) or *mCRS5* on rich medium with a fermentable carbon source (YPD), non-fermentable carbon source (YPLG) or YPLG with limited copper availability (+ 50 μM BCS, + 100 μM BCS).

To monitor mitochondrial copper homeostasis without confounding factors related to translation or the activity of other chaperone proteins, we used the previously described Cu-IMS biomarker strain (14). In this biomarker strain, the gene for the copper chaperone for SOD1, *CCS1,* has been deleted, rendering the yeast SOD1 inactive and causing a lysine auxotrophy. An IM-tethered human SOD1 (*IM-hSOD1*) is then stably expressed, and rescues the SOD1 deficit of the *ccs1*Δ strain in a manner that is dependent on matrix copper being made available within the IMS. Although deletion of *PIC2* did not change the steady state levels of IM-hSOD1 (Figure 2.6A), SOD1 activity in BCS-supplemented medium was decreased to 39% of the parental strain (Figure 2.6B). Supplementation of the medium with Ag progressively decreased the activity of IM-hSOD1 in *ccs1*Δ mitochondria, and deletion of *PIC2* exacerbated this defect (Figure 2.6C). These data strongly suggest that mitochondrial copper required for the metallation of IMS cuproenzymes is a limiting factor in *pic2*Δ cells.

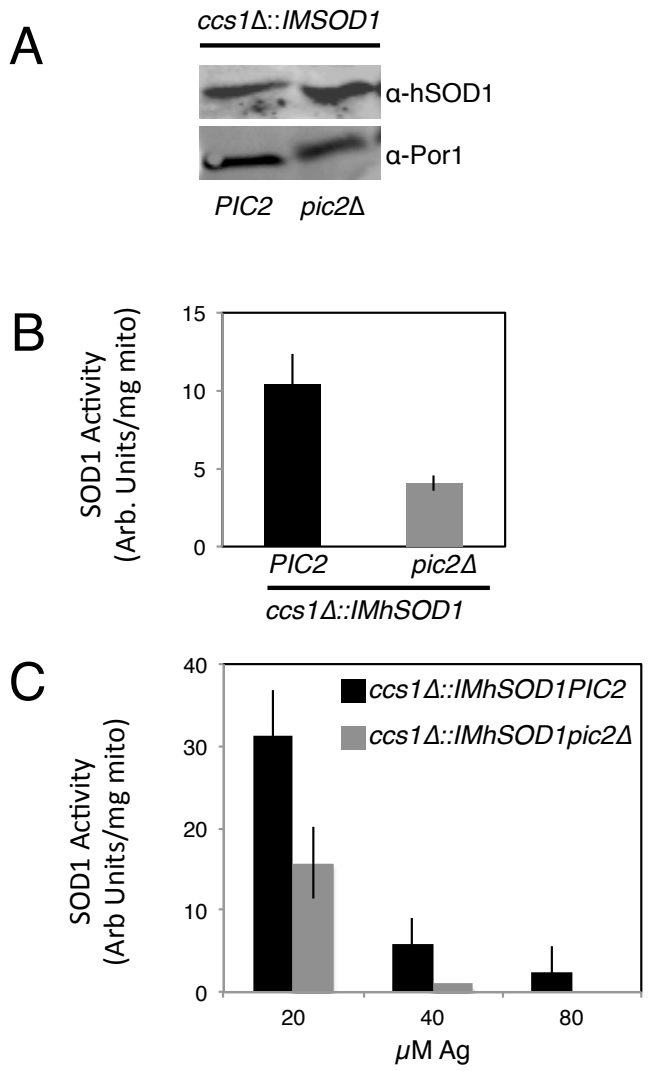

Figure 2.6: Deletion of *PIC2* limits hSOD1 activity in a *ccs1Δ::IMhSOD1* reporter strain

A) Western blot analysis of mitochondrial extracts from *ccs1Δ::IMhSOD* and *ccs1Δ::IMhSOD pic2Δ* strains, probed with human SOD1 antibody and porin as an internal loading control. B) Activity of SOD1 in isolated mitochondria from cells grown in synthetic media with fermentable carbon source supplemented with 50 μM BCS as measured by xanthine oxidase/tetrazolium salt assay (n=3). There was no detectable SOD1 activity in mitochondria from *ccs1Δ*. C) Activity of SOD1 in isolated mitochondria from cells grown in synthetic media with a fermentable carbon source supplemented with increasing Ag concentrations (20, 40 and 80 μM).

Biochemical characterization of pic2Δ mitochondria

Mitochondria isolated from *pic2*Δ cells grown in synthetic medium were analyzed for total metals by ICP-OES and showed a mild decrease in copper to 0.7-fold of the parental strain (1.9 ±0.2 mmol Cu/mol S parental versus 1.2 ± 0.2 mmol Cu/mol S *pic2*Δ). To exaggerate this phenotypic effect, *pic2*Δ cells were grown in synthetic medium with added 0.5 mM $CuSO_4$ and the mineral profile of mitochondria compared to that of parental yeast cultured under the same conditions (Figure 2.7A). While both strains had increased mitochondrial copper under these conditions, the *pic2*Δ mitochondria accumulated less Cu than the parental strain (~0.4-fold). There was no observable change in the content of P, Fe, Mn, Ca, Mg but a ~1.5 fold increase in Zn and K. These data suggested a specific defect in copper uptake in *pic2*Δ mitochondria (Figure 2.7A).

Intact mitochondria from *pic2*Δ cells were incubated with purified CuL complex and assayed for uptake. Initial rates of CuL uptake were decreased in *pic2*Δ mitochondria relative to those from parental cells (Figure 2.7B). Moreover, *pic2*Δ mitochondria showed lower maximal rates of uptake. This change in saturation suggests a decreased capacity for CuL uptake in the *pic2*Δ strain, consistent with the lower total copper accumulation. This uptake defect was evident across a range of CuL concentrations (Figure 2.7B). Because Pic2 has been previously identified as a secondary phosphate carrier, mitochondria from *pic2*Δ cells were assayed for phosphate uptake using an established swelling assay (30). Consistent with previous observations, no defect in phosphate uptake was detected in *pic2*Δ mitochondria (data not shown).

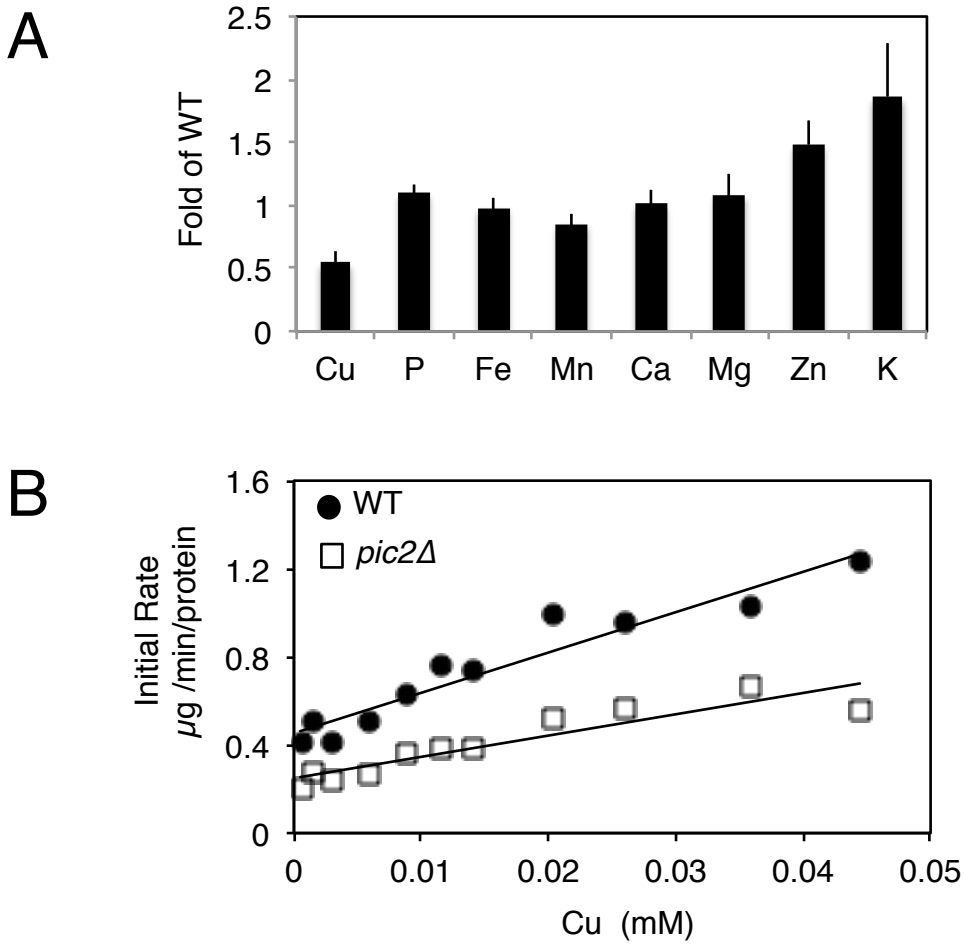

Figure 2.7: Total mineral element profile and uptake in mitochondria from *pic2Δ*.

A) Overall mineral profile of purified intact mitochondria from *pic2*Δ cells assayed by ICP-OES and compared to that of parental cells. Both strains were grown in medium containing 500 µM Cu. The value for each mineral represents the average of ICP-OES analysis of 10 independent mitochondrial preparations and bars represent the standard error of the mean. B) Isolated mitochondria from parental or *pic2*Δ cells assayed for *in vitro* uptake of the CuL. Initial rates of uptake are plotted versus variable CuL concentrations. The line is fit by a linear regression.

Heterologous expression of Pic2

To account for possible indirect effects of other proteins contributing to the observed defects in mitochondrial copper uptake, we cloned *PIC2* into a nisin-inducible vector for expression in the bacterium *Lactococcus lactis*. MCF proteins expressed in *L. lactis* are folded correctly in the cytoplasmic membrane and transport can be assayed directly in whole cells (31). The presence of Pic2 in *L. lactis* induced with nisin was confirmed by Western blot using a Pic2-specific antibody (Figure 2.8A). Expression of *PIC2* enhanced CuL uptake into *L. lactis* compared to cells that carried the empty vector (Figure 2.8B and C). Addition of a ten-fold excess of phosphate in the form of potassium phosphate buffer did not significantly inhibit copper uptake in the *PIC2*-expressing cells (data not shown). *PIC2* expression in *L. lactis* enhanced the cellular uptake of $CuSO_4$ (Figure 2.8D) and Cu-acetonitrile (data not shown), but did not enhance the uptake of Fe presented as $FeSO_4$ (data not shown).

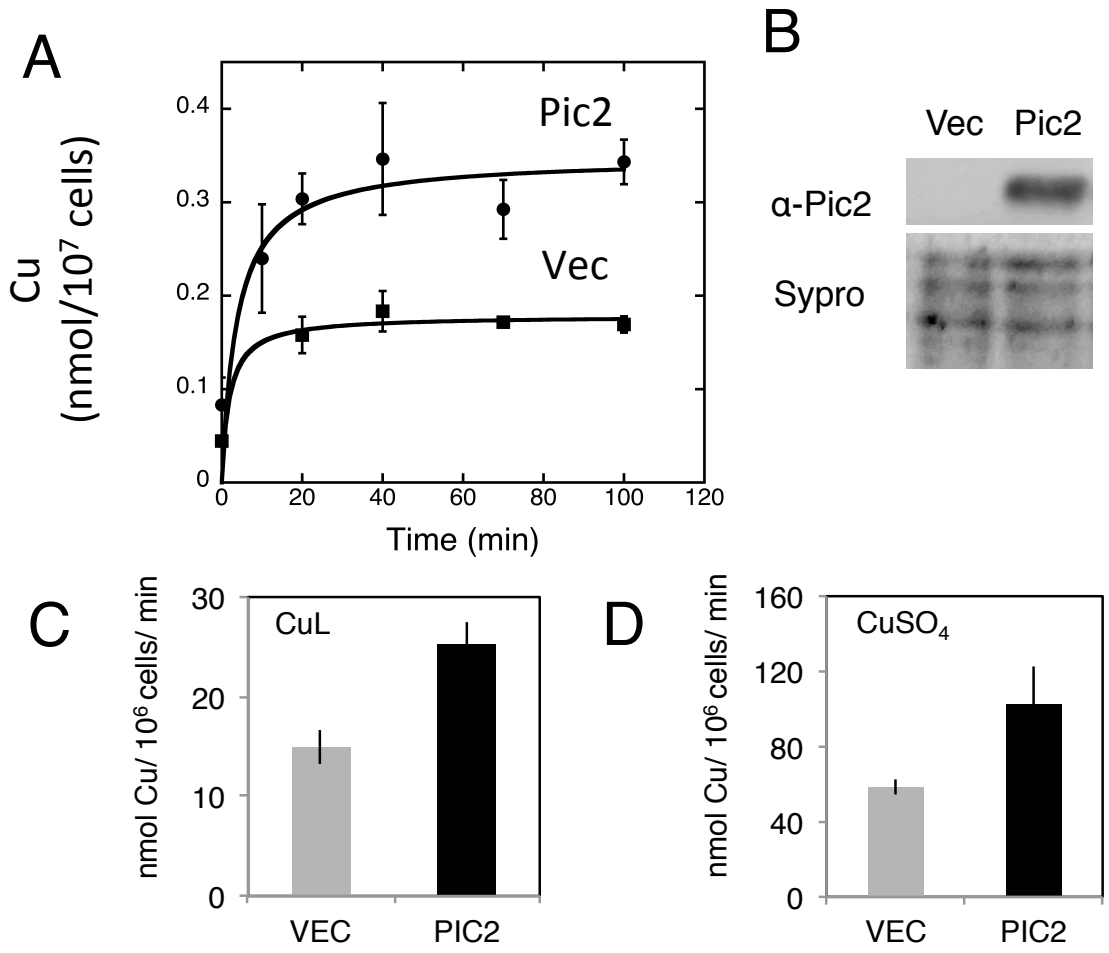

Figure 2.8: Pic2 expressed in *Lactococcus lactis.*

A) Uptake of the CuL by intact cells transformed with an empty vector (VEC) or *PIC2* (PIC2) incubated at room temperature over time with 20 µM CuL (n=4). Error bars represent standard error of the mean. Data is fit by a hyperbolic curve. B) CuL uptake in cells with either VEC or PIC2 incubated with 10 µM CuL (n=3). Error bars represent standard error of the mean. C) Cu uptake in cells with either VEC or PIC2 incubated with 10 µM $CuSO_4$ (n=3). Error bars represent standard error of the mean.

Discussion

Assembly of fully functional CcO depends on a non-proteinaceous pool of labile copper in the mitochondrial matrix (14). Here, we identify Pic2 as the first protein involved in matrix copper import. Yeast cells lacking *PIC2* have copper-related growth defects and a deficit in total mitochondrial copper content. Intact mitochondria from these cells show decreased rates of CuL uptake *in vitro*, and heterologous expression of Pic2 in *L. lactis* supports copper uptake. Taken together, these data support a novel role for Pic2 in mitochondrial copper homeostasis in *S. cerevisiae.*

Total copper in the mitochondria of *S. cerevisiae* is approximately 50 and 200 μM under fermentative and respiratory growth conditions, respectively (32). Previously, we reported that matrix copper content increases at least 6-fold in response to copper salt supplementation in the medium (11). Therefore the *in vitro* estimation of 15 μM for half-maximal transport of copper in wild-type mitochondria is likely biologically relevant.

The data presented here suggest that Pic2 mediates CuL import, and under normal physiologic conditions we believe CuL to be the major substrate for uptake of the metal ion into the organelle. Apo-ligand appears to be underrepresented in the matrix, suggesting that only the CuL complex is transported across the IM (14). We therefore use CuL as the source of copper in most of our assays, and this complex is extremely stable under all conditions we have tested, including boiling and organic extraction (14). However, we cannot exclude the possibility that copper dissociates from the ligand for its transport across the IM, and rebinds the ligand upon entry into the matrix.

MCF proteins are predicted to contain three alpha helices that fold over into a tripartite structure with six membrane-spanning domains (33). Within these helices are conserved

[P]X[DE]XX[RK] motifs which are proposed to narrow the pore of the carrier and form salt bridges to lock it in a state that is closed to the matrix (15,33,34). Substrate binding disrupts these salt bridges and allows for translocation, changing the carrier from an IMS facing state to a matrix facing state. Analysis of the sequence of MCF proteins to identify the salt bridges formed within these motifs has been used to predict whether particular family members act as uniporters or strict exchangers (34). The Pic2 salt bridge network in the IMS-facing conformation appears to be stronger than it is in the matrix-facing conformation, consistent with the idea that Pic2 is capable of uniport transport (34). According to the proposed mechanism, it is the greater relative strength of the IMS salt bridge network that drives the protein to revert back to this original conformation after substrate translocation (34). Further experiments with isolated Pic2 will be required to assess the residues required for CuL transport and the exact mechanism of transport.

The phenotypes observed here are most consistent with Pic2 acting as an importer of CuL, supporting the steady-state level of CuL in the matrix that is required to activate subsequent transport by an exporter. The expression of matrix-targeted copper competitors does not affect the total levels of copper, but does decrease the available CuL and this induces respiratory defects (14)**.** It is assumed that these competitor copper-binding proteins are able to out-compete the ligand complex for copper binding in the matrix. In *pic2*Δ this phenotype is exaggerated, presumably because decreased CuL import shifts the equilibrium in favor of the competitor protein(s) whose expression remains constant. To date, we cannot exclude the possibility that Pic2 is capable of bidirectional transport. Analysis of mitochondria from MCF deletion mutants grown under copper supplemented conditions did not reveal an obvious candidate that significantly over-accumulated copper (data not shown). Therefore any potential mechanism by which copper import and export may be linked remains unknown.

Defects in transport of ionic Cu and Fe were previously observed in sub-mitochondrial particles prepared from mutants of the high affinity Fe-transporters Mrs3 and Mrs4 (16). Similarly, we observe that Pic2 in *L. lactis* can transport $CuSO_4$. Although free copper should not be encountered in the IMS during normal physiologic conditions, this result could explain previous observations of copper accumulation in mitochondria from cells with compromised copper homeostasis. Deletion of *ACE1,* a copper regulated transcription factor, in yeast prevents the increase in metallothionein expression normally seen in response to copper supplementation. In this mutant mitochondrial copper is expanded more dramatically than it is in the parental strain with active Ace1 (11)**.** This increase in copper not only expands the soluble matrix pool but also leads to the accumulation of an insoluble fraction within this mitochondrial compartment, the identity of which has not been investigated. Similarly, mitochondria isolated from a rat model of Wilson disease show accumulation of large quantities of insoluble copper that leads to severe membrane damage (35). It is possible that once copper is no longer bound to the CuL, it can react indiscriminately with various mitochondrial components.

Pic2 has previously been implicated in phosphate transport in yeast as multi-copy *PIC2* could reverse a *mir1*Δ*pic2*Δ growth defect on a non-fermentable carbon source, and rescue phosphate transport in *mir1*Δ*pic2*Δ mitochondria as assayed by mitochondrial swelling (30,36). *PIC2* has also been identified as a high copy suppressor of the mitochondrial K^+/H^+ exchanger mutant (*mdm38*Δ) of yeast (37). The authors concluded that *PIC2* was suppressing the defect via rescue of proton motive force rather that K^+ transport. Interestingly, *MRS3* has also been implicated in transport of ionic copper, and also acts as a suppressor of the K^+/H^+ exchanger mutant (37). Whether the role of Pic2 in phosphate and potassium transport have any affect on copper, or vice versa, remains to be investigated.

The cumulative *in vivo* and *in vitro* data argue that Pic2 is a major component of the mitochondrial copper homeostasis machinery. This initial discovery provides a straightforward genetic strategy whereby this null strain can be used to identify additional transporters involved in organellar copper handling. It may also prove to be a useful tool in defining the pathways involved in synthesis of the ligand that binds Cu, as a *pic2*Δ strain should be sensitized to modest changes in total CuL content.

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Chapter 3: Pic2 Mediates CuL Transport Across the Inner Membrane

Abstract

The budding yeast *Saccharomyces cerevisiae* contains a pool of copper in the mitochondrial matrix that is required for metallation of the inner membrane enzyme cytochrome *c* oxidase, used for respiration, and the inter-membrane space enzyme Cu,Zn superoxide dismutase, which removes toxic oxygen radicals. Copper in this pool is bound to an anionic, fluorescent metabolite known as the copper ligand (CuL). The exact components responsible for intracellular copper transport from the plasma membrane to mitochondria in *Saccharomyces cerevisiae* are unknown. Current data suggests a model where binding to specific components of the polydispersed buffer form the CuL complex. Here, we show an improved purification scheme with increased resolution, which revealed unique characteristics including a novel fluorescent signature. This fluorescence was used to show interaction of CuL with the transport protein Pic2 and import of the complex into isolated mitochondria. This demonstrates that the entire ligand complex is imported into mitochodria via the Pic2 pathway.

Introduction

Copper is an essential, yet toxic metal due to its ability to cycle between different oxidation states. Copper cofactors are used by enzymes in a variety of metabolic processes including iron acquisition, respiration, and removal of reactive oxygen species. Cells tightly control copper levels by using protein chaperones and small molecule chelates. In the event of excess intracellular copper, cells express metallothioneins, cysteine-containing copper binding proteins, for detoxification by sequestration. In fact, the concentration of free copper within the cell has been calculated to be less than one copper atom per cell (1).

Copper is imported by the high affinity transporter Ctr1 or by non-specific lower affinity carriers (2). It must then be carried to its various targets within the cell. The chaperone Atx1 carries copper to the trans-Golgi network for assembly into secreted cuproenzymes, including the multicopper oxidase Fet3, which is critical for high affinity iron import (3). Ccs1 is responsible for copper insertion into the Cu,Zn superoxide dismutase (Sod1), which occurs in both the cytoplasm and in mitochondria (4, 5). In addition to metallochaperones, components of the cytosolic polydispersed buffer are responsible for contributing to specificity of metal delivery systems. In particular, copper trafficking to mitochondria appears to be mediated by components of the polydispersed buffer. Copper in the mitochondrion is used by cytochrome *c* oxidase (CcO) in the electron transport chain and by a portion of total cellular Sod1.

CcO is a multi-subunit complex that contains proteins encoded by both the nuclear and mitochondrial genomes (6). The mitochondrial-encoded catalytic subunits contain two copper sites. The mixed-valence, binuclear CuA site in the Cox2 subunit is exposed to the inter-membrane space and receives electrons from cytochrome *c* (7). The Cu-heme a_3 CuB site, contained within the Cox1 subunit, is buried deep within the enzyme complex and houses the

terminal electron acceptor (8). Two chaperone proteins, Sco1 and Cox11, are tethered to the IM but face the IMS. These assemble the CuA and CuB copper sites concurrently with Cox translation, membrane insertion, and CcO complex assembly (9–11). The soluble IMS protein Cox17 donates copper to Sco1 and Cox11 (12, 13). This protein was originally thought to be involved in copper trafficking to the mitochondrion, but retains its function even while tethered to the IM (14). Also in the IMS is a pool of Sod1, which accounts for 1-5% of total cellular Sod1, and its chaperone, Ccs1 (5). Other proteins exist in the IMS that have CxxC copper binding motifs and have been shown to bind copper. Most notably, the IMS protein Cmc1 has been implicated in distribution of copper between Sod1 and CcO in the IMS (15).

The majority of mitochondrial copper exists in a bioavailable pool within the matrix (16). This pool exists bound to an anionic, fluorescent molecule known as the copper ligand (CuL). Biochemical depletion of the CuL by expression of a matrix-targeted copper binding protein significantly decreases copper delivery to both CcO and IMS Sod1 (17). Therefore, matrix copper is the pool from which the CcO and Sod1 copper cofactors originate. Copper binding confers a net negative charge on the copper ligand and causes a decrease in its fluorescence. Using these properties, it was estimated that the majority of apo ligand complex is located within the cytoplasm while the majority of anionic CuL complex is found in mitochondria. The current model of mitochondrial copper homeostasis is that apo ligand binds copper in the cytosol and is then recruited to the mitochondrial matrix. This copper must be redistributed to the IMS for assembly into mitochondrial cuproenzymes (18).

Because the mitochondrial IM is impermeable to ions, transport proteins must exist to shuttle mitochondrial copper into and out of the matrix. We recently identified the first mitochondrial copper importer, Pic2, in *Saccharomyces cerevisiae* (Ch. 2). Pic2 is a member of

the mitochondrial carrier family (MCF). MCF proteins transport metabolites into and out of the mitochondrial matrix (19). Deletion of *PIC2* caused copper-dependent respiratory growth defects that were exacerbated by expression of matrix-targeted copper competitors. Mitochondria from these mutants had decreased total copper and a decreased capacity for copper uptake based on direct measurements of copper. However, the question remained as to whether Pic2 or mediates import of the CuL complex or whether an alternative model is more appropriate, where unbound copper is imported before binding to the ligand within the matrix. Here, we provide evidence that the entire ligand complex is imported into mitochondria. Consistent with previous observations, we saw expansion of the ligand pool upon addition of copper to the growth medium. In *pic2*Δ mutants, this expansion was attenuated. Purified Pic2 protein showed a direct physical interaction as measured by fluorescence anisotropy (FA). We also used the fluorescence of the CuL to measure its uptake observing its depletion from solution. Mitochondria from *pic2*Δ cells showed decreased ligand uptake relative to those from wild-type cells. Based on these data, we conclude that Pic2 mediates import of the entire CuL complex.

Materials and Methods

Yeast strains, culture conditions, and standard methods

Yeast strains used in this study were BY4741 (*MATa, leu2*Δ*, met15*Δ*, ura3*Δ*, his3*Δ) parental and the isogenic kanMX4- containing mutant, purchased from Invitrogen. All cultures were grown in YP (1% yeast extract, 2% peptone) medium or synthetic defined medium with 1-2% glucose added as a carbon source. Copper sulfate was added the medium when increased copper conditions were required. All cells were grown in a shaking incubator at 30 °C.

Expression of recombinant proteins

The *PIC2, MRS3,* and *MIR1* ORFs were cloned into pHis parallel 1. The fidelity of each construct was verified by dideoxynucleotide sequencing prior to use. BL21 (DE3) cells were transformed with appropriate vectors and protein expression was induced for 3 hours. Inclusion bodies were isolated in a manner similar to that described by Palmieri (20). Briefly, cells were resuspended in 1X potassium phosphate buffer (140 mM NaCl, 2.7 mM KCl, 8.3 mM K_2HPO_4, 1.8 mM KH_2PO_4), pH 7.5 and disrupted by sonication. Insoluble material was collected by centrifugation at 18,500 *g*. Insoluble material was resuspended in 1X potassium phosphate buffer, pH 7.5, and loaded onto a stepwise 40%, 53%, 70% sucrose (in 1X potassium phosphate buffer) gradient. Samples were centrifuged at 18,500 *g* for one hour and inclusion bodies were isolated as the band at the interface between the 53% and 70% layers. Presence of the protein of interest was confirmed by immunoblot. To load protein into liposomes, inclusion bodies were sonicated with egg yolk phospholipids and dialyzed for six hours in 1X potassium phosphate buffer.

Isolation of the copper ligand

Mitochondria were prepared and the soluble contents were fractionated as described previously (16). Whole cells or isolated mitochondria were treated with 100% methanol to extract soluble contents, which were dried and resuspended in water. Initial fractionations of CuL were prepared by pouring matrix contents over DE52 resin (Whatman). This was washed with 10 mM ammonium acetate at pH 8.0 and eluted with two bed volumes of 1 M ammonium acetate, pH 8.0. These samples were loaded onto a Sonoma C18 column using a Shimadzu UFLC. One mL fractions were collected across an 30 minute 20%- 100% methanol gradient. Final fractions were analyzed for copper by ICP-OES (PerkinElmer Life Sciences 9300-DV) and fluorescence

(PerkinElmer Life Sciences LS55 spectrofluorimeter or Shimadzu RF-2A prominence fluorescence detector). Excitation and emission scans of copper-containing fractions were conducted using 5 nm slit widths and used an excitation maximum of 220 nm and an emission maximum of 360 nm or an excitation maximum of 320 nm with an emission maximum of 400 nm.

Quantification of the CuL

The CuL was prepared from 5 mL overnight cultures as previously described (Ch 2). Ligand samples were treated with 1% KCN and loaded onto a Sonoma C18 column using a Shimadzu UFLC and detected by fluorescence using an excitation maximum of 320 nm and emission maximum of 400 nm with a Shimadzu RF-2A Prominence fluorescence detector. The area of the copper-responsive ligand peak was calculated and normalized to number of cells as measured by OD_{600}.

Ligand uptake assay

Isolated, intact mitochondria were incubated with CuL complex for variable time points and removed from solution by centrifugation. Resultant supernatants were separated on silica-coated TLC plates (GE Healthcare) using a mixture of 90% ethanol and 10% acetic acid. TLC plates were analyzed for fluorescence on an ImageQuant LAS 4000 (GE Healthcare) using the Cy2 detection filter (EX 492, EM 510). Bands were quantified using ImageJ.

Fluorescence anisotropy

CuL was diluted to give a fluorescence intensity of ~30 AU using an excitation maximum of 320 nm and emission maximum of 400 nm (slit widths were set to 5 nm). Inclusion bodies and MCF protein incorporated into liposomes were added in 1-5 μL increments. Anisotropy was measured using a PerkinElmer Life Sciences LS55 spectrofluorimeter.

Miscellaneous methods

Western blots were performed using mouse anti-His (Invitrogen) followed by Cy3-conjugated goat anti-mouse (GenScript). Blots were observed using an ImageQuant LAS 4000 (GE Healthcare). Egg yolk phospholipids were prepared using the Folch method of extraction in methanol and chloroform (21).

Results

CuL pool expansion is attenuated in a *pic2*Δ mutant

It has been shown previously that the CuL has a fluorescent emission at 360 nm when excited at 220 nm (17). This fluorescence was shown to decrease in response to copper and increase upon removal of copper with the strong chelator KCN. However, upon analysis by HPLC, multiple compounds in the anion exchange elution were found to exhibit similar fluorescence (not shown). At least one component of the copper containing fractions also had a fluorescent emission at 400 nm upon excitation at 320 nm (Figure 3.1A). This fluorescence was quenched in a concentration-dependent manner upon addition of Cu-acetonitrile (Figure 3.1B). Addition of cyanide to remove copper from the ligand caused an increase in the total fluorescence (Figure 3.1 C).

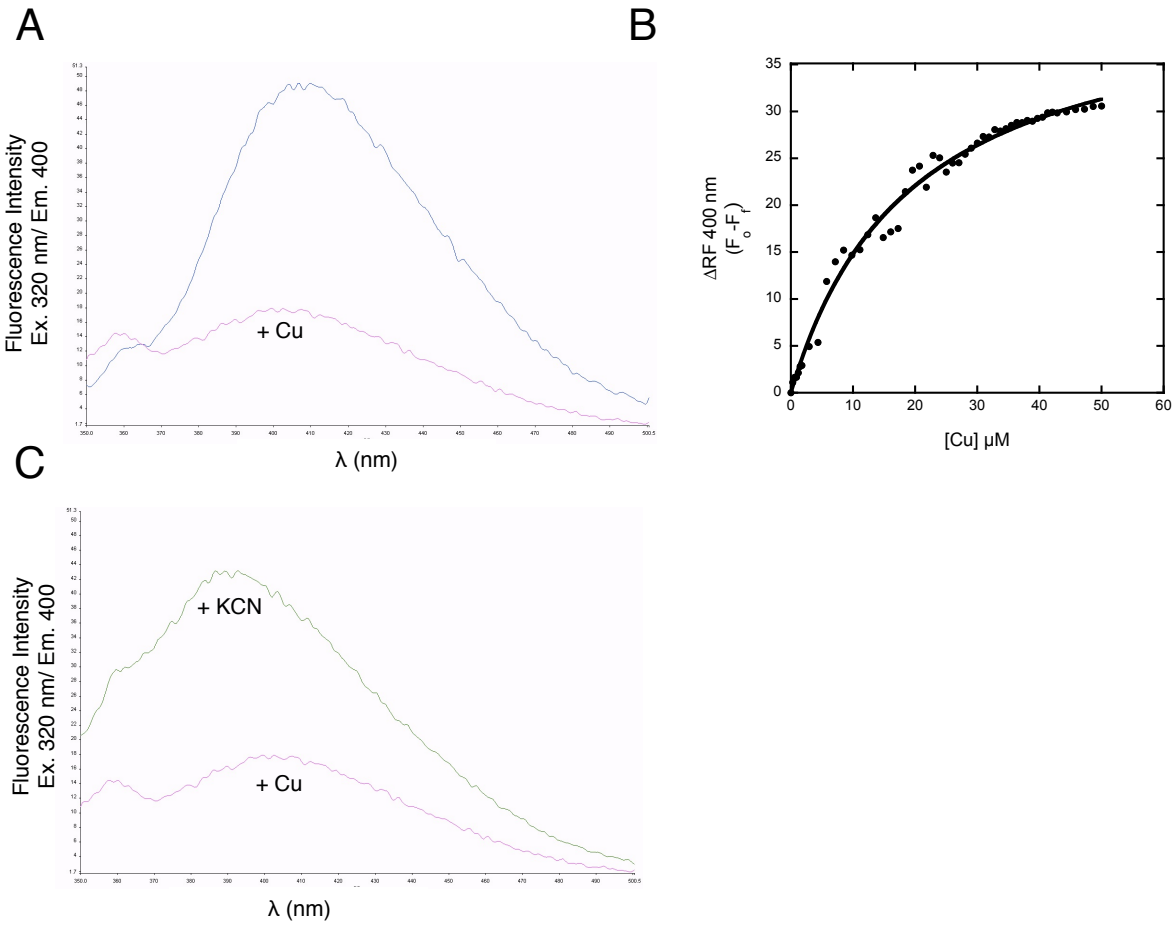

Figure 3.1: Unique fluorescence of the CuL complex

A) Fluorescent emission of purified CuL with an excitation maximum of 320 nm. B) Quenching of the 320/400 fluorescence signal with addition of Cu-acetonitrile. C) Addition of 1% KCN to remove copper led to a net increase in total fluorescence.

This fluorescence characteristic was used to quantify anionic CuL from yeast cells grown in synthetic medium with variable amounts of added copper. Ligand was isolated from cells by anion exchange chromatography. To observe maximum fluorescence, copper was removed from CuL by treatment with 1% KCN, which allowed for quantification of resultant peaks. These samples were then analyzed by HPLC and ligand detected using its 320 nm/400 nm emission characteristics (Figure 3.2 A). Growth in exogenous copper led to a concentration-dependent increase in the CuL pool (Figure 3.2 B). Expansion of the CuL pool peaked when cells were grown in 750 μM $CuSO_4$ and increased only slightly when cells were grown in 1mM $CuSO_4$. Growth in 750 μM $CuSO_4$ was used to compare CuL pools in wild-type and *pic2*Δ cells. Expansion occurred in both wild-type and mutants, but the *pic2*Δ cells expanded this pool only to about 60% of the wild-type levels (Figure 3.2 C). This result is consistent with previous observations that mitochondrial copper expansion is attenuated in *pic2*Δ (Ch. 2). Therefore, the involvement of Pic2 in matrix copper expansion is likely by import of the copper bound to the CuL complex.

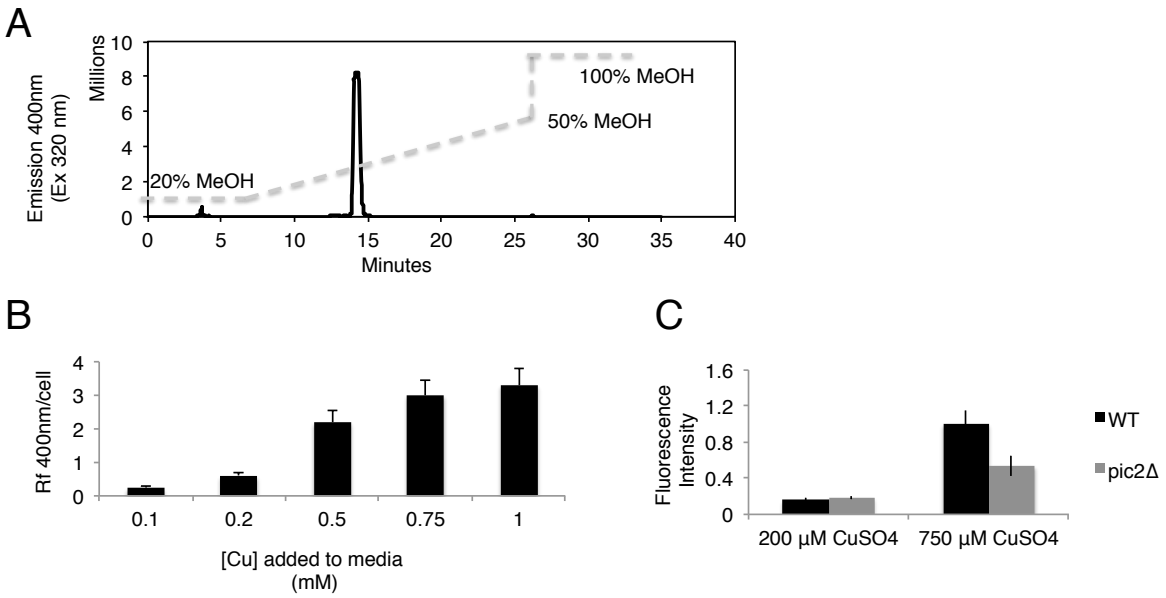

Figure 3.2: CuL quantification in wild-type and *pic2*Δ yeast

A) Reverse phase purification of anionic, copper-containing soluble matrix contents. Cells were grown overnight in SC (glucose) medium. Soluble contents were separated by anion exchange chromatography and resultant elutions were treated with cyanide to enhance fluorescence. CuL was observed as the fluorescent peak (Ex320Em400) that eluted at 14 minutes (~35% methanol). B) CuL was prepared as in (A) from wild-type cells grown in SC medium with increasing $CuSO_4$. The area of the 14 minute ligand peak was quantified. Shown are the average intensities from three separate experiments. Error bars represent standard deviation. C) Ligand was quantified from wild-type and *pic2*Δ cells grown in 200 µM or 750 µM $CuSO_4$. Shown is the average of three separate experiments. Error bars represent standard deviation.

CuL is transported into mitochondria

In previous experiments, mitochondria from wild-type and *pic2*Δ cells were assayed for uptake measured as an increase in copper after being incubated the CuL complex (Ch. 2). We sought to measure uptake of the ligand itself using its fluorescence as a means for detection. Dilutions of CuL in water were separated by TLC and observed using a Cy2 fluorescent filter (Figure 3.3 A). Resultant bands were quantified by ImageJ and showed concentration-dependent changes in intensity (Figure 3.3 B). To measure CuL uptake, we incubated intact mitochondria with the CuL and removed mitochondria by centrifugation at variable time points. The remaining ligand in solution was separated by TLC (Figure 3.3 C). Quantification of CuL bands revealed a time-dependent depletion of CuL from solution, indicating import of the CuL complex into mitochondria (Figure 3.3 D). Mitochondria isolated from *pic2*Δ cells took up around 50% less CuL than did those from wild-type cells, indicating that Pic2 participates in ligand import (Figure 3.3 D).

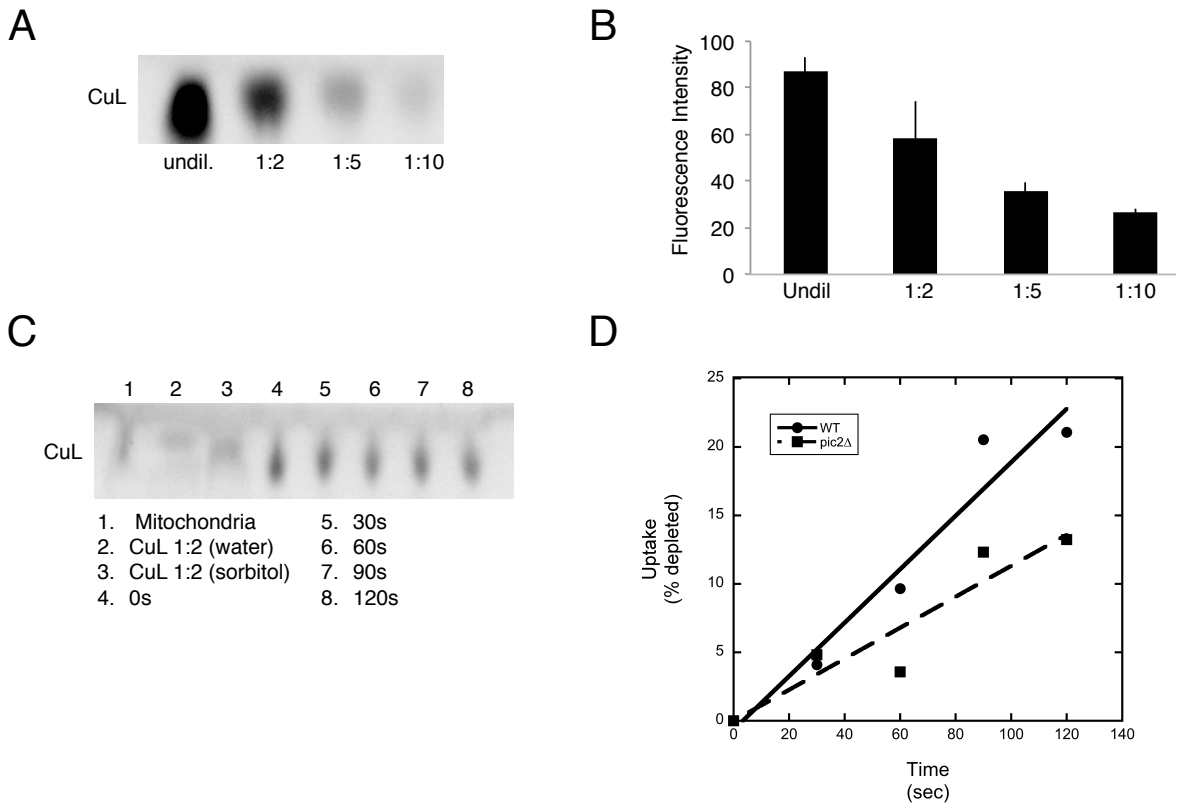

Figure 3.3: Mitochondria import the CuL complex

A) Purified CuL diluted in water, separated by TLC, and observed using a Cy2 fluorescence filter. Shown is one representative image from three separate experiments. B) Quantification of CuL dilutions from three separate experiments. Error bars represent standard deviation. C) Representative TLC from uptake assay using wild-type mitochondria. D) Isolated mitochondria from parental or *pic2*Δ cells assayed for *in vitro* uptake of the CuL as depletion of the fluorescent ligand from solution.

Pic2 interacts with the CuL

The ligand quantification and uptake assays implicate Pic2 in the expansion of the mitochondrial ligand pool and in import of the CuL complex. To determine a direct interaction between Pic2 and the CuL complex, we used fluorescence anisotropy to assay Pic2-ligand binding. Pic2 and the related MCF protein Mrs3 were expressed in *E. coli* and purified as inclusion bodies. These were assayed for CuL binding by fluorescence anisotropy using the 320 nm/400 nm fluorescence characteristics of the CuL. Pic2 showed enhanced stabilization of the CuL, indicating binding, while Mrs3 showed less interaction (Figure 3.4 A). Membranes from *E. coli* cells transformed with an empty vector showed no interaction with the CuL (not shown). Though it has been shown that proteins purified as inclusion bodies can be properly folded, we repeated this assay with Pic2 reconstituted into liposomes. This time another closely related MCF protein was used for comparison. Again, Pic2 enhanced the anisotropy of the CuL while Mir1 did not (Figure 3.4 B). These results suggest that Pic2 binds to the CuL complex and has a higher affinity for the CuL than both Mrs3 and Mir1.

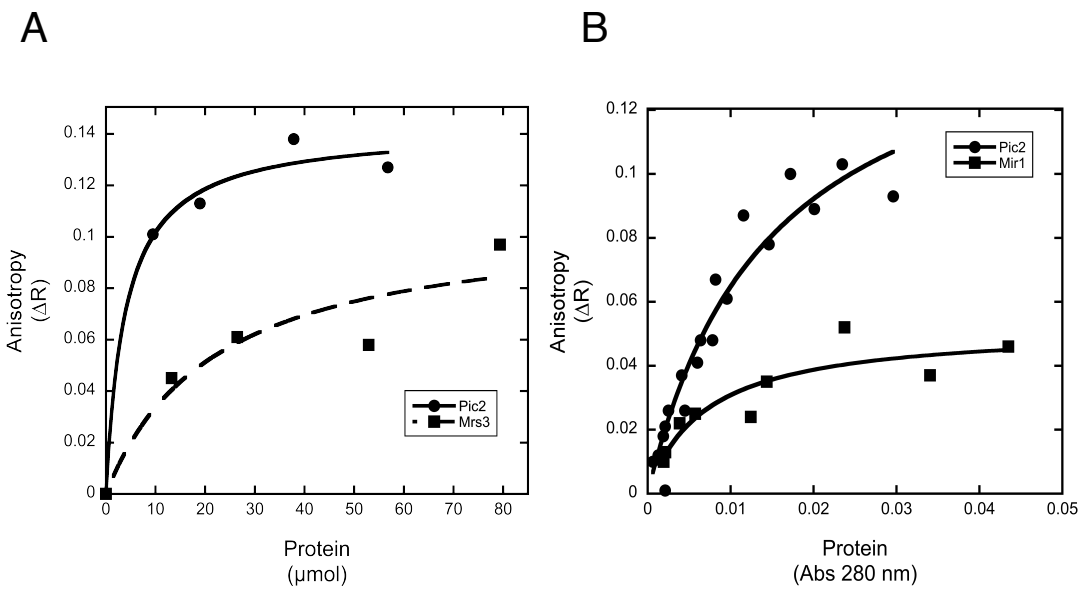

Figure 3.4: CuL binds the copper carrier Pic2

A) Change in anisotropy (Ex 320 Em 400) with addition of Pic2 and Mrs3 as inclusion bodies. B) Change in anisotropy with addition of Pic2 and Mir1 in liposomes.

Discussion

The majority of the apo-ligand complex exists within the cytosol while most of the anionic CuL is found within the matrix (17). Therefore, the current model of mitochondrial copper homeostasis states that apo-ligand binds copper in the cytosol and the CuL complex is imported into the mitochondrial matrix (18). The MCF protein Pic2 participates in mitochondrial copper import (Ch. 2). However, because Pic2 was capable of mediating uptake of copper salts when expressed in a heterologous system, the question remained as to whether Pic2 was responsible for uptake of the entire CuL complex or of copper alone (Ch. 2). The mitochondrial CuL pool expands in response to high copper conditions, but deletion of *PIC2* diminished this expansion, indicating that Pic2 participates in maintenance of the CuL pool. Mitochondria from *pic2*Δ cells had a defect in the uptake of CuL as measured by a depletion of the fluorescent complex from solution. The Pic2 protein enhanced stabilization of the CuL as measured by fluorescence anisotropy, indicating a direct physical interaction between carrier and substrate. Together these data show that the entire ligand complex is imported into the mitochondrial matrix by Pic2 and other complimentary transport systems.

This model of mitochondrial copper homeostasis is consistent with that observed in α-proteobacteria. Methanotrophic bacteria use secreted modified peptides called methanobactins to bind environmental copper (22). Methanobactin-bound copper is then imported back into the cytoplasm (23). This copper eventually is used by the membrane protein, particulate methane monooxygenase (pMMO), as disruption of Cu-methanobactin import disrupts copper insertion into pMMO (24). How this redistribution occurs is unknown. The bacterium *Rhodobacter capsulatus* has a major facilitator family protein in the cytoplasmic membrane that contributes to assembly of the *cbb3*-type oxidase. Deletion of this protein affects respiration without affecting

copper sensitivity or activity of the periplasmic multicopper oxidase CutO, indicating that there is a specific pathway by which copper enters the cytoplasm prior to redistribution to the periplasm to be used by the *cbb3* oxidase (25).

The endosymbiotic theory of eukaryotic origin states that a gram-negative α-proteobacterium was engulfed by another cell and became the mitochondrion in early eukaryotes (26). While the biosynthesis of iron-sulfur clusters is the sole essential function of modern mitochondria, initial stages of selection for retaining the endosymbiont could have depended on ATP production. Perhaps as a mechanism for ensuring its survival within cells, these endosymbionts may have maintained the original bacterial strategy for obtaining the copper that is critical for respiration. A small molecule ligand-mediated strategy for copper acquisition could have allowed the endosymbiotic bacterium to compete with other components of intracellular copper homeostasis for this cofactor. This hypothesis is supported by observations in cell lines from patients with mutations in the SCO proteins involved in CcO assembly (27). These patient cells have an overall copper deficiency while maintaining normal mitochondrial pools, suggesting that mitochondrial copper is sustained independently of total cellular copper status (28).

Several questions about mitochondrial copper homeostasis remain. Most pressing is the identity of the copper binding ligand complex. Fluorescence characteristics suggest the presence of a indole group. In fact, NMR spectra show an indole signature in the purified ligand complex (Cobine, unpublished). Tryptophan synthesis is a major pathway that contributes to the formation of the indole ring in yeast. Perhaps one or more intermediates from tryptophan biosynthesis or breakdown contributes to ligand biosynthesis. Mutations in certain genes involved in synthesis of either tryptophan or tryptophan precursors have been shown to increase copper sensitivity in *S.*

cerevisiae (29). This could be due to defects in CuL biosynthesis that result in aberrant copper handling within the cell. Whether mutations in these genes affects mitochondrial copper levels or the activity of mitochondrial copper enzymes remains to be tested. Tryptophan metabolism appears to crosstalk with copper homeostasis in higher eukaryotes as well. Gene expression analysis in hepatocytes from Wilson disease animal models in the early stages of copper accumulation have shown an up-regulation of tryptophan metabolic pathways (30). In later stages of the disease, mitochondria accumulate copper and show membrane damage (31). This may be an effect of the CuL-mediated import system being overwhelmed, leading to the presence of free copper. Finally, dietary tryptophan supplementation has been shown to decrease copper sensitivity in the carp species *Cyprinus carpio* (32). All of these data point to a role for tryptophan in copper detoxification and potentially in normal copper homeostasis.

The exact connections between tryptophan metabolism and copper homeostasis remain unknown and further experiments are needed to study the relevance of the CuL in connecting these pathways. We have shown that Pic2 contributes to copper import and that the species transported is likely the entire ligand complex. Therefore, deletion of the *PIC2* gene can be used to sensitize yeast strains to perturbed copper conditions. To further characterize the ligand and its synthesis, genetic strategies using *pic2*Δ mutants paired with tryptophan synthesis mutants can be used to elucidate the mitochondrial copper import pathway. The unique fluorescence characteristics and refined purification scheme described here can also be used to further characterize the structure of the CuL complex.

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Chapter 4: Characterization of Mrs3 and other Transporters

Abstract

Import of mitochondrial matrix copper is mediated by the mitochondrial carrier family (MCF) protein Pic2 in *Saccharomyces cerevisiae.* Deletion of this gene causes copper-dependent growth phenotypes as well as a depletion of mitochondrial copper and copper uptake. However, copper import is not completely reduced in these mutants, indicating the presence of alternative transport systems. Deletion of another MCF gene, *MRS3*, which encodes a component of the iron import machinery also caused a copper-dependent growth defect on non-fermentable carbon. Simultaneous deletion of *PIC2* and *MRS3* lead to severe respiratory growth defects and these strains failed to activate a heterologous copper-sensing enzyme. While mitochondrial copper was depleted in the double mutant, there was no observed import defect. Additionally, Mrs3 failed to mediate copper import when expressed in the bacterium *Lactococcus lactis*, though it did import iron in this system. Therefore, we conclude that *MRS3* acts as a genetic modifier of *PIC2*, imparting an indirect effect on mitochondrial copper import.

Introduction

Copper is an essential trace element used in various pathways including iron acquisition, respiration, and detoxification of reactive oxygen species (ROS). Its ability to cycle between oxidation states make copper an excellent cofactor for redox reactions but also renders it toxic.

Therefore cells must tightly control its import, localization, and storage. Cells use a combination of low-affinity and high-affinity transport systems to bring copper into the cytoplasm (1, 2). Once inside, protein and small molecule chaperones to sequester copper and deliver it to target enzymes. The chaperone Atx1 carries copper to the P-type ATPase Ccc2 in the trans-Golgi network for incorporation into the multicopper oxidase Fet3, which is required for high affinity iron uptake (3). Copper is delivered to the Cu,Zn superoxide dismutase (Sod1) by its chaperone Ccs1 in the cytosol and in the mitochondrial inter-membrane space (4, 5). A small molecule chelate, the copper ligand (CuL) is thought to be involved in recruiting copper to the mitochondrial matrix pool (6).

In mitochondria, in addition to Sod1, copper is used by cytochrome *c* oxidase, the final enzyme complex in the electron transport chain (7). This multi-subunit complex localizes to the inner membrane (IM) and cofactor insertion must be tightly coordinated with its assembly; a series of chaperone proteins add copper into the forming enzyme complex as the individual subunits are translated and inserted (8). The soluble IMS protein Cox17 delivers Cu to the IM proteins Sco1 and Cox11, which assemble the Cu_B site in the Cox1 subunit and the Cu_A site in the Cox2 subunit (9–11). While CcO is the major mitochondrial copper enzyme, the bulk of mitochondrial copper exist within the matrix, bound the copper ligand (CuL) (12). The CuL is transported into the mitochondrial matrix by the mitochondrial carrier family (MCF) protein Pic2 (Ch. 2, 3). It must then be redistributed to the IMS for assembly into mitochondrial cuproenzymes (13).

The mitochondrial carrier family proteins (MCF) are exclusive to eukaryotes and are involved in translocation of various TCA intermediates, nucleoside di- and triphosphates, and other substrates into and out of the mitochondrial matrix (14). Three pseudosymmetrical

transmembrane alpha-helical domains that form an opening across the membrane characterize conserved structure of these proteins (15). Conserved PX[DE]XX[KR] motifs within the transmembrane alpha-helices form salt bridges that close the channel (16). Substrate binding disrupts these salt bridges, opening the channel and allowing for translocation across the membrane (17).

Multiple MCF proteins are known to play a role in mitochondrial transition metal homeostasis. Mrs3 and Mrs4 are responsible for high-affinity iron transport across the IM (18). Deletion of both *MRS3* and *MRS4* causes a severe growth defect in yeast grown under iron-depleted conditions (19). Mutation of the metazoan homolog, mitoferrin, is embryonic lethal in zebrafish due to severe anemia, highlighting the importance of mitoferrin in mitochondrial iron import for heme synthesis in developing erythrocytes (20). Mtm1, originally identified due to a lack of Mn Sod2 activity, has been shown be required for correct iron handling to prevent mis-metallation of Sod2 with iron (21). It was later shown that iron accumulates as Fe (III) in *mtm1*Δ yeast, which suggests that Mtm1 contributes to Sod2 activity by some other mechanism (22). Ggc1 a GTP/GDP exchanger also is required for correct iron handling (23). Deletion of Ggc1 causes a defect in Fe-S and heme that can be reversed by normalization of the GTP levels by expression of a nucleoside diphosphate kinase in the mitochondrial matrix (24). Recently, another MCF protein, Rim2, was shown to mediate transport of nucleotide-bound iron across the inner membrane (25). These examples reveal that metabolites can indirectly modify metal-related phenotypes in yeast, highlighting the need for both phenotypic characterization and biochemical assessment.

Pic2 cannot be the only protein involved in mitochondrial copper import. Deletion of the *PIC2* gene causes a growth defect only under severely copper-limiting conditions. Mitochondria

from *pic2*Δ still have 40%-70% of wild-type copper levels and can still import copper, though to a lower capacity than wild-type mitochondria. Therefore, other transporters must exist. Here, we use a combination of growth phenotypes and biochemical assays to show that *MRS3* is a modifier of *PIC2* in the mitochondrial copper import pathway.

Materials and Methods

Yeast strains, culture conditions, and standard methods

Yeast strains used were BY4741 (*MATa, leu2*Δ*, met15*Δ*, ura3*Δ*, his3*Δ) and isogenic kanMX4 mutant from Open Biosystems (Huntsville, AL). Double mutants were constructed by homologous recombination of the *URA3MX* cassette at the *MRS3* locus. The Y8205 (*MATα, can1*Δ*::STE2pr-Sp_his5 lyp1*Δ *::STE3pr-LEU2 his3*Δ *leu2*Δ *ura3*Δ*)* strain was a kind gift from Scot Leary (University of Saskatchewan). Cultures were grown in 1% yeast extract, 2% peptone (YP) medium or in synthetic defined media with selective amino acids excluded using the appropriate carbon source. Bio101 yeast nitrogen base plus 0.1 mM ferrous sulfate was used to give copper deficient conditions. If required, further copper depletion was achieved by adding filter-sterilized extracellular copper chelator bathocuproine sulfonate (BCS), or silver was added as a mitochondrial copper competitor. Exogenous copper was provided by adding $CuSO_4$. Growth tests were performed at 30 °C with 1 in 10 serial dilutions of overnight pre-cultures grown in YP plus 1% glucose.

Vector constructs

IM-hSOD1 was constructed by inserting the hSod1 open reading frame in-frame with the sequence encoding the N-terminal 104 residues of Sco2 (YBR024W) that encode a targeting sequence and a transmembrane domain described previously (13). Constructs were verified by dideoxynucleotide sequencing prior to use.

Chromatographic fractionation of mitochondria

Preparation and fractionation of mitochondria was performed as previously described (12). Cells were extracted in 100% methanol. Dried methanol extracts were dried and resuspended in water. CuL was prepared using Whatman DE52 anion exchange resin, which were washed in 10 mM ammonium acetate (pH 8.0). Ligand fractions were eluted using two bed volumes of 1 M ammonium acetate, pH 8.0. These were dried and washed in water before being loaded onto a Sonoma C18 column and separated by a 0-100% methanol gradient for 30 minutes on a Shimadzu UFLC. One mL fractions were collected and copper was analyzed for fluorescence using an excitation maximum of 220 nm and emission maximum of 360 nm (PerkinElmer Life Sciences LS55 spectrofluorimeter) and copper by ICP-OES (PerkinElmer Life Sciences 9300-DV).

Expression of Mrs3 in *Lactococcus lactis*

L. lactis cells transformed with vector (pNZ8148) alone or pNZ8148 (MoBiTec) carrying the *MRS3* gene were grown overnight at 30˚C in M17 medium with 0.5% glucose and 10 µg/mL chloramphenicol. Cells were diluted into fresh medium at an OD_{600} of 0.1, grown to an OD_{600} of 0.4, and induced using 1 ng/mL nisin for five hours.

Copper uptake assay

Isolated mitochondria suspended in 0.6 M sorbitol were incubated with CuL for 30 second intervals and removed from solution by centrifugation. Uptake was measured by ICP-OES as an increase in copper over time. Copper uptake was assayed in *L. lactis* using a modified method where whole cells were resuspended in purified ligand, or copper salts in water. Cells were incubated for different time points at room temperature, removed by centrifugation, washed in

water, and total metals were measured by ICP-OES. Uptake was reported as the increase in copper over time.

Miscellaneous methods

The monoclonal mouse anti-human SOD1 was purchased from Santa Cruz Biosciences. Secondary antibodies used were the Cy3-linked goat anti mouse from GeneScript. Superoxide dismutase (SOD1) activity was measured using a xanthine oxidase-linked assay kit (Sigma Life Science) and absorbance was measured on a BioTek 96-well plate reader.

Results

Simultaneous deletion of *PIC2* and *MRS3* results in severe copper-related phenotypes

Pic2 was identified using a screen of single MCF gene deletions grown on rich medium with a non-fermentable carbon source in the presence of a cell-impermeable copper chelator, bathocuproinedisulfonic acid (BCS) and silver (Ch. 2). Under these conditions, the *mrs3Δ* mutants also had a severe growth defect (Figure 4.1 A). This defect was reversed when silver concentrations were decreased, and no defect was observed on glucose at the same concentration of silver (not shown). To test for synergistic phenotypes, we created a *pic2*Δ*mrs3*Δ double mutant. Double deletion of *PIC2* and *MRS3* resulted in poor growth on copper-depleted rich medium with a non-fermentable carbon source (Figure 4.1 B). Interestingly, deletion of the related gene *MRS4* in the *pic2*Δ background did not cause any further defect on non-fermentable carbon in the presence of both BCS and silver compared to *pic2*Δ alone (Figure 4.1 B). Double mutants also had a severe growth defect on synthetic medium with a non-fermentable carbon source, in the absence of chelators (Figure 4.1 C).

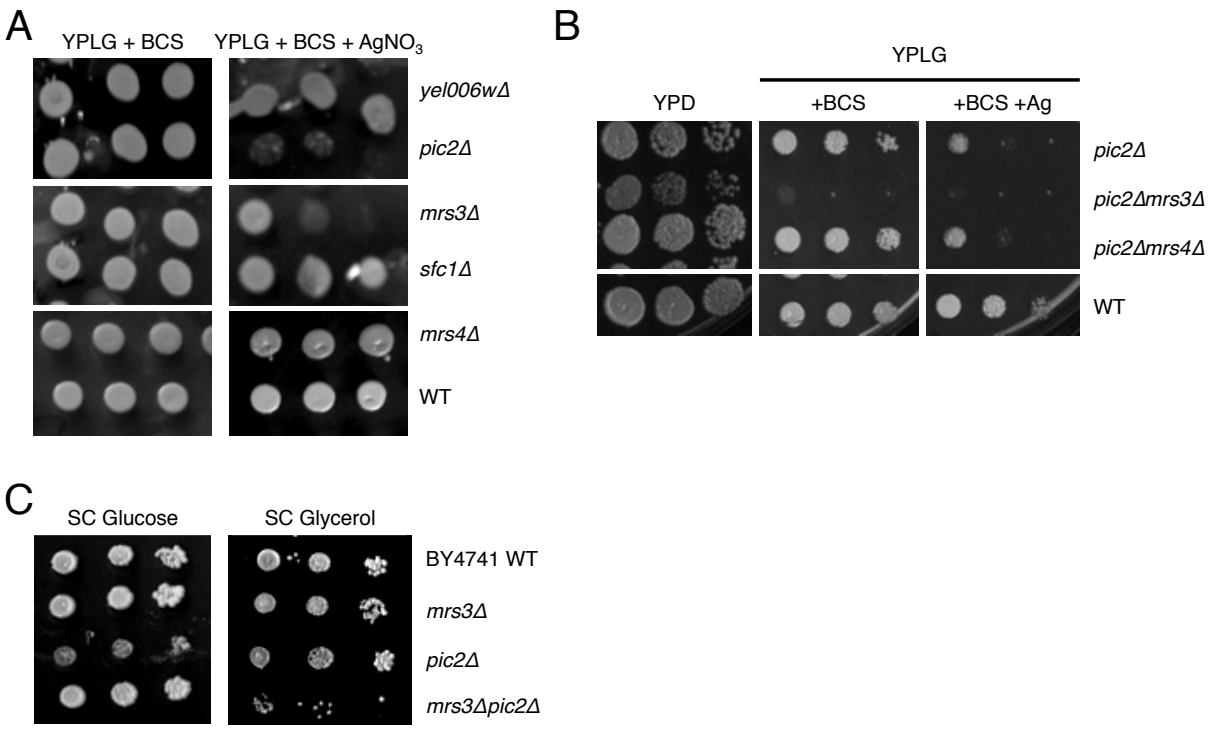

Figure 4.1: Copper related growth phenotypes in *pic2*Δ*mrs3*Δ

A) Serial dilutions of BY4741 and single MCF deletion strains grown on rich medium with a non-fermentable carbon source (lactate-glycerol; YPLG) in the presence of the cell-impermeable copper chelator BCS or in the presence of BCS and silver. B) Serial dilutions of BY4741 and *pic2*Δ, *pic2*Δ*mrs3*Δ, and *pic2*Δ*mrs4*Δ cells grown on rich medium with a fermentable (glucose, YPD) or a non-fermentable carbon source (lactate-glycerol; YPLG) in the presence of the cell-impermeable copper chelator BCS or in the presence of BCS and silver. C) Serial dilutions BY4741 and *mrs3*Δ, *pic2*Δ, and *pic2*Δ*mrs2*Δ cells grown on synthetic complete (SC) medium with either a fermentable (glucose) or non-fermentable (glycerol) carbon source.

Activation of heterologously expressed human Sod1

To test whether both *PIC2* and *MRS3* affect copper availability, these two genes were deleted in an copper-sensing yeast strain (see Ch. 2) (13). Deletion of the *CCS1* gene that encodes the copper chaperone for Sod1 renders yeast Sod1 inactive, as it cannot receive its cofactor (Figure 4.2 A) (5). Buildup of reactive oxygen in this background disrupts the iron-sulfur cluster in the enzyme homoaconitase and leads to a lysine auxotrophy (Figure 4.2 B) (26, 27). These *ccs1*Δ defects can be reversed by expressing an IM-tethered human Sod1 (IM-hSod1), which is capable of receiving copper in the absence of the Ccs1 chaperone (Figure 4.2 A, B). Activity of IM-hSod1 is dependent on available copper within the IMS and thus it can act as a sensor for mitochondrial copper availability. The copper used by IMS cuproenzymes originates from the matrix pool and so the *ccs1*Δ*::IM-hSod1* background can be used to probe availability of both matrix and IMS copper, though it cannot be used to differentiate between the two. Deletion of *PIC2* in this background caused a 40-50% decrease IM-hSod1 activity when these cells were grown in silver, but did not cause a lysine auxotrophy (Ch. 2).

The *ccs1*Δ*::IM-hSod1* strain was crossed with the *mrs3*Δ single mutant to generate the *mrs3*Δ *ccs1*Δ*::IM-hSod1* and the *pic2*Δ*mrs3*Δ double mutant to generate a *pic2*Δ*mrs3*Δ *ccs1*Δ*::IM-hSod1* triple mutant. While the *mrs3*Δ *ccs1*Δ*::IM-hSod1* strain showed no growth defect, *pic2*Δ*mrs3*Δ *ccs1*Δ*::IM-hSod1* mutant failed to grow on medium lacking lysine (Figure 4.2 C). As expected, the triple mutant completely lacked Sod1 activity, indicating a deficiency of available mitochondrial copper, while the *mrs3*Δ *ccs1*Δ*::IM-hSod1* double mutant had a mild deficiency of hSod1 activity (Figure 4.2 D). These results suggest that both *PIC2* and *MRS3* affect availability of copper to the IMS. However, it does not reveal whether this defect occurs during import or export.

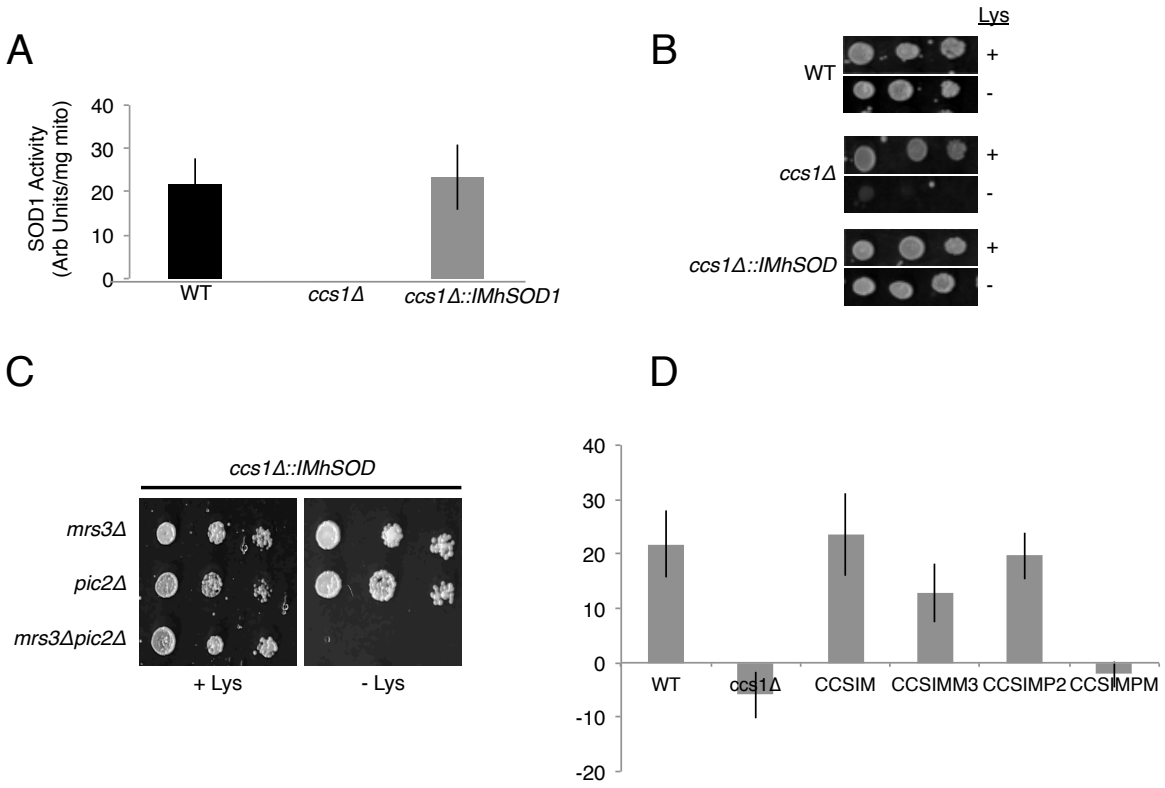

Figure 4.2: Deletion of *PIC2* and *MRS3* in a copper reporter strain

A) Activity of SOD1 in isolated mitochondria from Y8205, *ccs1*Δ, *ccs1*Δ*::IMhSOD1* cells grown in synthetic media with fermentable carbon source as measured by xanthine oxidase/tetrazolium salt assay (n=3) and normalized to total protein. B) Serial dilutions of BY4741 and *ccs1*Δ, *ccs1*Δ*::IMhSOD1* cells grown on synthetic medium under hyperoxic conditions with a fermentable carbon source (glucose) in the presence or absence of lysine. C) Serial dilutions of BY4741 and *ccs1*Δ, *ccs1*Δ*::IMhSOD1, mrs3*Δ*ccs1*Δ*::IMhSOD1, pic2*Δ*ccs1*Δ*::IMhSOD1,* and *pic2*Δ*mrs3*Δ*ccs1*Δ*::IMhSOD1* cells grown on synthetic medium under hyperoxic conditions with a fermentable carbon source (glucose) in the presence or absence of lysine. D) Activity of SOD1 in isolated mitochondria from Y8205, *ccs1*Δ, *ccs1*Δ*::IMhSOD1* (CCSIM), *mrs3*Δ*ccs1*Δ*::IMhSOD1* (CCSIMM3), *pic2*Δ*ccs1*Δ*::IMhSOD1* (CCSIMP2), and *pic2*Δ*mrs3*Δ*ccs1*Δ*::IMhSOD1* (CCSIMPM) cells grown in synthetic media with fermentable carbon source as measured by xanthine oxidase/tetrazolium salt assay (n=3) and normalized to total protein.

Biochemical characterization of *pic2*Δ*mrs3*Δ mitochondria

To look directly at mitochondrial copper, mitochondria were isolated from *pic2Δ*, *mrs3*Δ, and *pic2*Δ*mrs3*Δ cells grown in rich medium or synthetic medium with glucose as a carbon source. These were analyzed for total metals by ICP-OES and showed only a mild decrease in copper relative to mitochondria from wild-type cells (not shown). To exaggerate the copper defect, wild type, *pic2*Δ, and *mrs3*Δ, and *pic2*Δ*mrs3*Δ cells were grown in synthetic medium with 0.5 mM $CuSO_4$ supplemented. Both *pic2*Δ and *mrs3*Δ mitochondria accumulated copper to 0.4-fold and 0.25-fold of wild type mitochondria, respectively (Figure 4.3 A,B). The *mrs3*Δ mitochondria showed defects in a number of metals (Figure 4.3 B) while the *pic2*Δ mitochondria had copper-specific defects. The *pic2*Δ*mrs3*Δ double mutant accumulated only about 0.2-fold of wild-type copper while other metals remained at or greater than wild-type levels (Figure 4.3 C). It should be noted that, as previously observed (Ch. 2), mitochondria from all mutants expanded the copper pool when cells were grown in exogenous copper. However, single and double mutant strains did not expand this pool to the levels seen in the wild-type mitochondria.

Intact mitochondria from *pic2*Δ, *mrs3*Δ and wild-type cells were assayed for uptake of copper in the form of purified CuL. Copper was taken into *pic2*Δ mitochondria at a decreased initial rate at room temperature while uptake into *mrs3*Δ mitochondria resembled wild-type levels (Figure 4.4 A). Uptake into purified mitochondria from *pic2*Δ*mrs3*Δ cells resembled that observed in mitochondria from the *pic2*Δ single mutant (Figure 4.4 B). These data indicate that, while mitochondrial copper pools do not expand in *mrs3*Δ and *pic2*Δ*mrs3*Δ, this effect is not due to a direct defect in uptake.

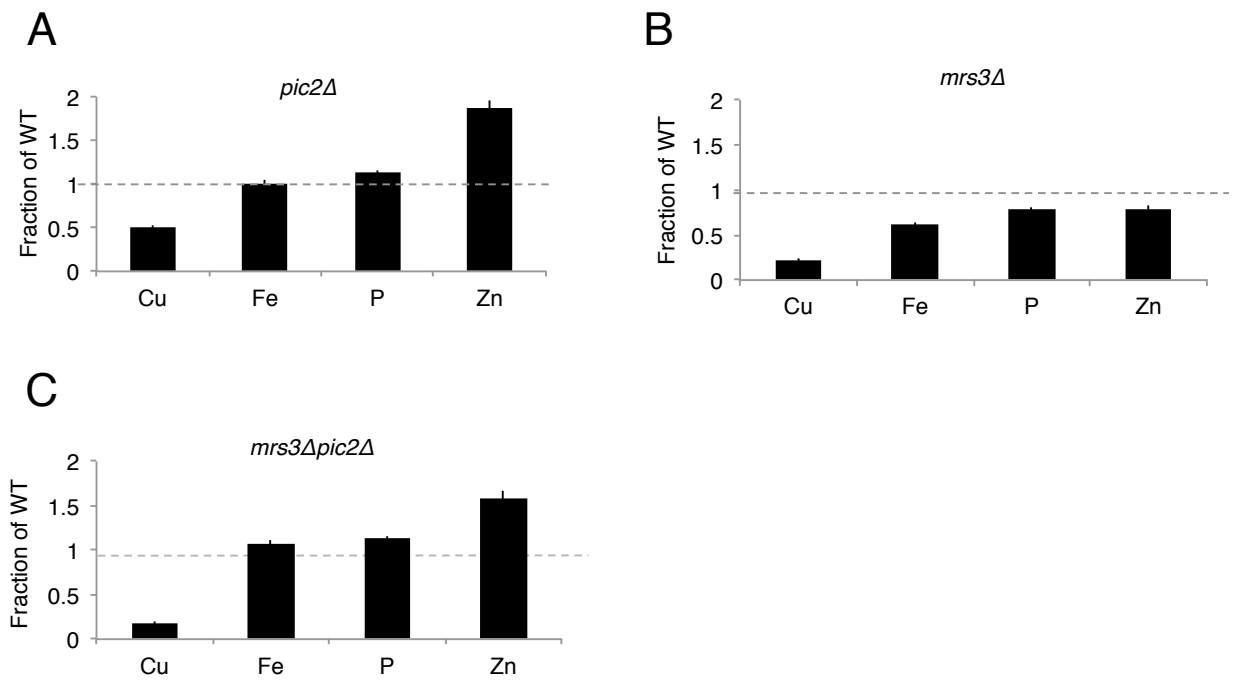

Figure 4.3: Total mitochondrial metals in *pic2*Δ, *mrs3*Δ, and *pic2*Δ*mrs3*Δ

Total amounts of Cu, Fe, P, and Zn in mitochondria from *pic2*Δ (A), *mrs3*Δ (B), and *pic2*Δ*mrs3*Δ (C) cells grown in synthetic medium with a fermentable carbon source (glucose) with added 0.5 mM $CuSO_4$. Metals were measured by ICP-OES and normalized on a per-sulfur basis before being calculated as a fraction of those found in wild-type cells.

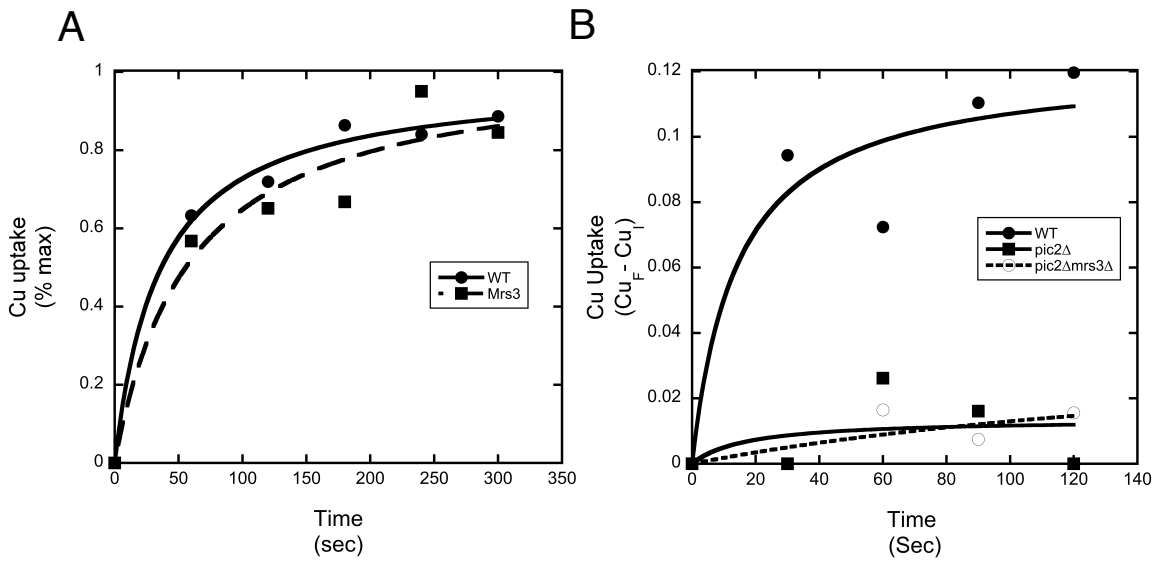

Figure 4.4: Mitochondrial copper uptake in WT, *pic2*Δ, *mrs3*Δ, and *pic2*Δ*mrs3*Δ

A) Isolated mitochondria from parental or *mrs3*Δ cells assayed for *in vitro* uptake of the CuL as measured by an increase in copper over time. Uptake is reported as the percentage of maximum uptake observed in wild-type mitochondria. B) Isolated mitochondria from parental, *pic2*Δ or *pic2*Δ*mrs3*Δ cells assayed for *in vitro* uptake of the CuL as measured by an increase in copper over time and reported as the final concentration less the initial concentration. Mitochondria were normalized for protein and copper was measured by ICP-OES and normalized on a per-sulfur basis.

Expression of Mrs3 in *Lactococcus lactis*

Although deletion of *MRS3* did not appear to cause any copper uptake defects, we sought to determine whether the Mrs3 protein could mediate copper uptake in the absence of other yeast proteins and metabolites. The *MRS3* gene was expressed in the gram positive bacterium *Lactococcus lactis*, which has been shown to express mitochondrial carrier family proteins in the plasma membrane (28). Uptake of CuL and $CuSO_4$ was assayed in whole cells containing *MRS3* or the empty vector. The *L. lactis* cells expressing Mrs3 failed to take up copper, given either as CuL or as $CuSO_4$ (Figure 4.5 A,B). However, these cells were capable of taking up $FeSO_4$, which is consistent with previous reports of Mrs3 as member of the high affinity inner membrane iron transport pathway (Figure 4.5 C). The ability of Mrs3 to mediate iron import suggests the presence of a functional protein. Therefore, the lack of copper transport is not likely due to defects in the heterologously-expressed protein.

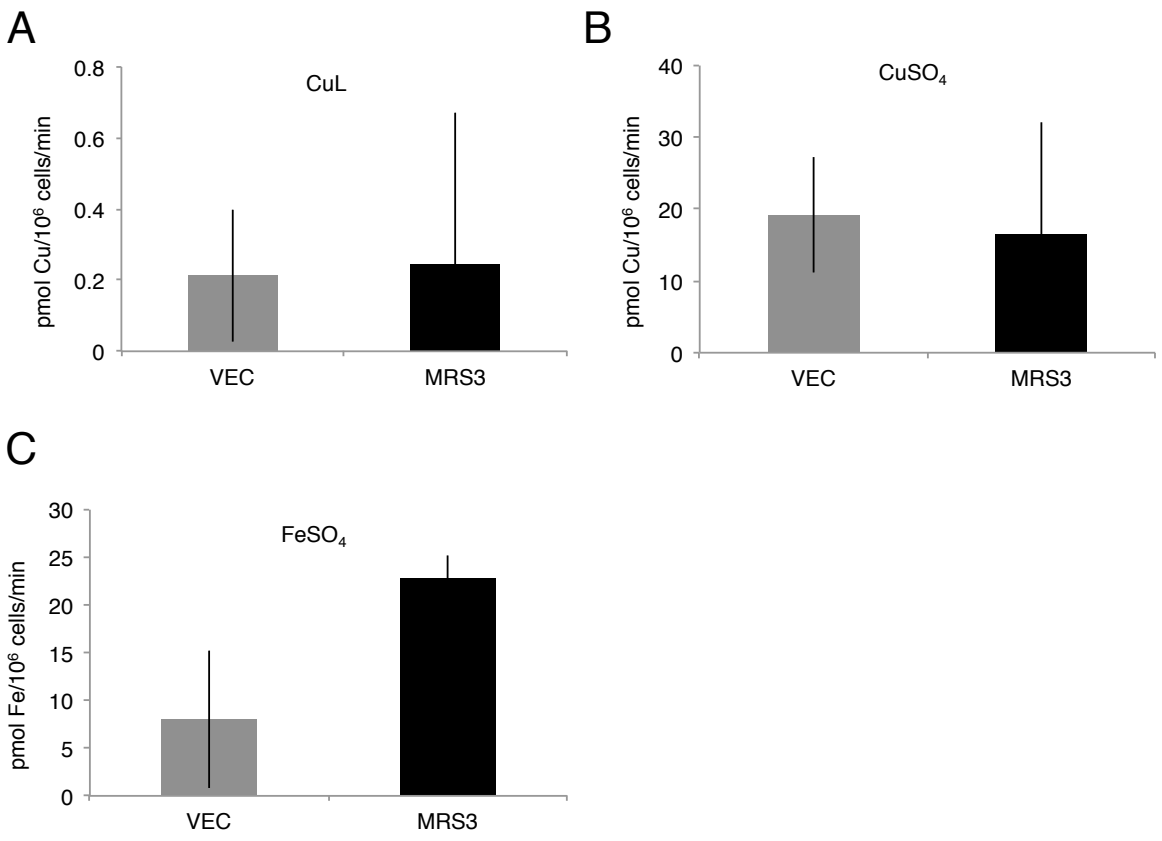

Figure 4.5: Expression of Mrs3 in *Lactococcus lactis*

A) Uptake of copper by intact cells transformed with an empty vector (VEC) or *MRS3* (MRS3) incubated at room temperature over time with 20 µM CuL (n=4). Rates of copper increase per minute were calculated on a per 10^6 cell basis. Error bars represent standard deviation. B) Uptake of copper by intact cells transformed with an empty vector (VEC) or *MRS3* (MRS3) incubated at room temperature over time with 20 µM $CuSO_4$ (n=4). Rates of copper increase per minute were calculated on a per 10^6 cell basis. Error bars represent standard deviation.. C) Uptake iron by intact cells transformed with an empty vector (VEC) or *MRS3* (MRS3) incubated at room temperature over time with 20 µM $FeSO_4$ (n=4). Rates of iron increase per minute were calculated on a per 10^6 cell basis. Error bars represent standard deviation.

Discussion

The data presented here suggest that *MRS3*, which encodes an inner-membrane iron transporter, acts as a genetic modifier of the gene encoding a mitochondrial copper importer, *PIC2*. The *pic2*Δ*mrs3*Δ double deletion shows severe copper-related growth defects and is unable to expand the mitochondrial pool. This reveals that the two genes do not have redundant functions in the pathway. The fact that deletion of *MRS3* and *PIC2* leads to a failure to activate an IM-tethered hSod indicates that *MRS3* has some in vivo function in mitochondrial copper homeostasis. These phenotypes, along with the defects mitochondrial copper expansion, suggest that Mrs3 may act as part of a secondary copper import pathway. However, mitochondria from *mrs3*Δ mutants have wild-type levels of copper uptake whereas the uptake in *pic2*Δ*mrs3*Δ mitochondria resemble that in the *pic2*Δ mutant. Interestingly, sub-mitochondrial particles from a *mrs3*Δ*mrs4*Δ double deletion have been shown to have defects in both $FeSO_4$ and $CuSO_4$ uptake (18). Here, we show that Mrs3 was able to mediate iron import when expressed in *L. lactis* but was unable to carry out copper import. The fact that these cells took up iron indicated that the protein was functional as expressed in this heterologous system. Based on these data, we conclude that Mrs3 does not play an active role in copper import but rather acts as an indirect modifier of the copper import pathway.

Both *PIC2* and *MRS3* have been shown to act as high copy suppressors of mutants of the mitochondrial K^+/H^+ exchanger (KHE) (29). The authors of this study postulated that *PIC2* may reverse defects found in the mitochondrial membrane potential of these mutants. Additionally, *PIC2* and *MRS3* overexpression reversed defects in mitochondrial morphology of the KHE mutants, which showed a higher ratio of fragmented to fused mitochondria relative to wild-type cells. Overexpression of *PIC2* mediated only partial return of the tubular network, but

overexpression of *MRS3* was able to completely reverse this defect. Perhaps deletion of *MRS3* in *pic2*Δ yeast leads to a morphological defect that affects copper or copper availability. Yeast *mrs3*Δ mutants have not been shown to have any defects in mitochondrial fusion but could have subtle defects that are exacerbated upon deletion of *PIC2*. Potentially even subtle morphological defects could be rescued by the presence of *MRS3*. How mitochondrial morphology affects the matrix copper pool remains unknown, but recent observations show a mitochondrial morphology defect in *coa1*Δ cells, which are also deficient for mitochondrial copper (Zhao and Cobine, unpublished). Other connections between copper and potassium transport have been observed. Addition of micromolar amounts of copper salts has been shown to activate potassium transport in rat liver mitochondria (30). Interestingly, the authors of this study also observed a decrease in mitochondrial membrane potential upon addition of $CuSO_4$. Perhaps there is some role for potassium in mitochondrial copper homeostasis and this is the mechanism by which *MRS3* affects *pic2*Δ mitochondria.

Because Mrs3 does function as an iron transporter, it is possible that some of the phenotypes observed here are related to defects in iron. However, under all growth conditions, iron was added to the medium to avoid any high affinity plasma membrane iron import defects caused by copper deficiency of Fet3. Moreover, mitochondria in the *pic2*Δ*mrs3*Δ double mutant maintain wild-type levels of iron. Therefore it is unlikely that observed copper defects are related to iron deficiency. Perhaps, as in the case of *mtm1*Δ, iron in these mitochondria may be insoluble or improperly incorporated into other proteins or metabolites. Further experiments are needed to describe overall mitochondrial function as well as the coordination of copper and transition metals in these mutants.

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Chapter 5: Concluding Remarks

Copper is an important redox cofactor that is involved in many different pathways including the electron transport chain, removal of reactive oxygen species, and iron homeostasis. Its ability to gain and accept electrons also renders copper toxic to cells. A series of protein chaperones are responsible for delivery of copper to most sites of utilization and the long-standing model was that all copper is bound to proteins within eukaryotic cells. The isolation of the mitochondrial CuL pool represented a paradigm shift from a solely protein-mediated delivery strategy to a strategy where cells also use proteins and small molecule metabolites to sequester and distribute copper.

While the identification of this pool was a critical step in the understanding of overall cellular copper handling, the mechanism by which it is maintained remained a black box in the understanding of mitochondrial copper homeostasis. For the first time, we have identified a protein involved in mitochondrial copper import in *Saccharomyces cerevisiae*. Identification of this carrier will be critical in elucidation of other components of the pathway (Figure 5.1). Yeast *pic2*Δ mutants can be used to sensitize other mutants by imparting a mitochondrial copper defect. This strategy can also be used to describe members of the mitochondrial copper pathway in humans, particularly to assay different disease states.

Most of the major copper enzymes in mitochondria have been identified but the number of proteins with the capability of binding copper continues to grow. While many of these

have been shown to bind copper in vitro, their relevance in vivo remains unknown. How cells regulate copper export from the matrix to the inter-membrane space is still a mystery, as is the mechanism by which cells control copper distribution between mitochondrial Sod1 and cytochrome c oxidase. However, the knowledge one importer component will be useful in answering these questions.

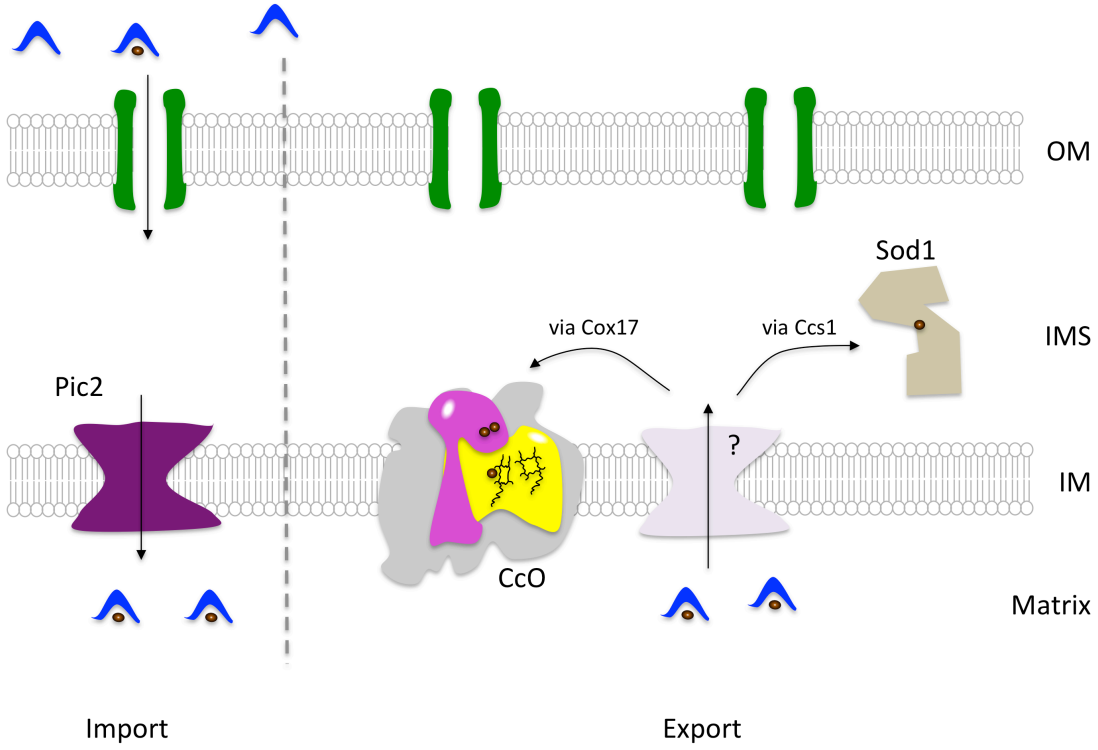

Figure 5.1 mitochondrial copper homeostasis

The biochemically characterized by unidentified CuL appears to found as a apo molecule in the cytoplasm were it presumably binds copper. The molecule that carries a net negative charge is transported into the mitochondria presumably via the voltage dependent anion channels (porins) into the IMS. It is then transported into the matrix by at least one mitochondrial carrier family protein (Pic2). Pic2 is capable of transporting both the intact complex and ionic copper. Once in the matrix it is stored until release to the IMS for Cox17 binding. The mechanism of transport and the transporter used are unknown. But biochemical depletion of the CuL complex in the matrix results in a loss of activity of CcO and Sod1 in the IMS.
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