FUNCTIONAL ANALYSES OF VARIANTS OF HUMAN SCO1, A MITOCHONDRIAL METALLOCHAPERONE

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ABSTRACT

Cytochrome *c* oxidase (COX) is a multimeric protein complex whose enzymatic activity contributes to the generation of an electrochemical potential required to synthesize adenosine triphosphate (ATP). Synthesis of Cytochrome *c* Oxidase 1 (SCO1) and SCO2 are two of the many accessory factors that are required to assemble individual structural subunits of COX into a functional holoenzyme complex. Mutations in either *SCO* gene cause severe, early onset forms of human disease.

SCO1 and *SCO2* are closely related paralogues localized to the inner mitochondrial membrane. Both proteins bind copper and exhibit a thiol disulphide oxidoreductase activity. Copper is bound by a highly conserved Cysteine x x x Cysteine motif and a histidine found within a thioredoxin fold, which is contained in the C-terminal half of the protein and projects into the mitochondrial intermembrane space. Mutations in either *SCO1* or *SCO2* affect their ability to deliver copper to COX II and metallate its Cu_A site, and also result in an increased rate of copper efflux from the cell. However, the relative importance of the ability to bind and transfer copper to SCO protein function remains poorly understood. Therefore, to investigate the significance of several cysteine residues and the conserved histidine to the copper-binding properties of SCO1, I functionally characterized a series of N- and C-terminal SCO1 mutant proteins by transducing them into control and patient fibroblasts, and quantifying their phenotypic effect on COX activity. I found that the two cysteines within the soluble, N-terminal matrix domain of SCO1 are not required for protein function. Overexpression of C-terminal SCO1 mutants only affected COX activity in *SCO1-2* patient fibroblasts. To further characterize the copper-binding properties of these C-terminal mutants, soluble forms of each SCO1 variant were expressed and purified from bacteria, and the amount of total bound copper and the relative abundance of Cu(I) and Cu(II) were quantified. Although these analyses suggested that one mutant, SCO1 C169H, binds significantly more Cu(I) than the wild-type protein, none of the SCO1 variants exhibited properties that furthered our understanding of the precise role of SCO1 in the biogenesis of the Cu_A site of COX II.

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"It takes courage to push yourself to places you have never been before… to test your limits… to break through barriers. And the day came when the risk to remain tight inside the bud was more painful than the risk it took to blossom."

–Anais Nin(1903-1977)

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LIST OF ABBREVIATIONS

Arh	Adrenodoxin reductase homolog
BCS	Bathocuproine disulfonic acid
Cmc	Cx_9C mitochondrial protein necessary for full assembly of cytochrome *c* oxidase
CoA	Coenzyme A
Coa1	COX assembly factor 1
CoQ	Coenzyme Q
COX	Cytochrome *c* oxidase
CPC motif	Cysteine-proline-cysteine motif
Crs	Copper resistant suppressor
CS	Citrate Synthase
DDM	n-Dodecyl-β-D-maltopyranoside
DMEM	Dulbecco's modified Eagle's medium
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Erv1	Essential for respiration and vegetative growth protein 1
$FADH_2$	Flavin adenine dinucleotide
Fet	Ferrous transport
Fre	Ferric reductase
H_2O_2	Hydrogen peroxide
HO•	Hydroxyl radical
Hot13	Helper of TIM of 13 kDa
Imp	Inner membrane peptidase
IMS	Mitochondrial intermembrane space
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LRPPRC	Leucine-rich PPR motif-containing protein
LSFC	Leigh syndrome, French-Canadian type

Mba	Multi-copy bypass of AFG3 (ATPase Family Gene)
Mdj	Mitochondrial DnaJ
Mge	Mitochondrial GrpE
MIA	The mitochondrial intermembrane space import and assembly machinery
Mss	Mitochondrial splicing suppressor
mtHsp70	Mitochondrial heat shock protein 70-kDa
NADH	Nicotinamide adenine dinucleotide
NMR	nuclear magnetic resonance
NTA	Nitrilotriacetic acid
Oxa1	Oxidase assembly mutant 1
PAM	Presequence translocase-associated motor
PBS	Phosphate buffered saline
Pet	PETite colonies
PIC	Protease inhibitor cocktail
PMSF	Phenylmethanesulfonyl fluoride
Pnt	PeNTamidine resistance
ROS	Reactive oxygen species
SCO	Synthesis of cytochrome *c* oxidase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Shy	SURF homolog of yeast
Smf	Suppressor of mitochondria import function
SOD	Cu, Zn-superoxide dismutase
TACO1	Translational activator of mitochondrial encoded COX I
TBS	Tris buffered saline
TIM	The translocase of the inner mitochondrial membrane
TNB	5-thio-2-nitrobenzoic acid
TOB	The translocase of outer membrane β-barrel protein
SAM	the sorting and assembly machinery
TOM	The translocase of the outer mitochondrial membrane
Yah	Yeast adrenodoxin homolog

1 INTRODUCTION

1.1 Mitochondria

Described as “cellular power plants”, mitochondria are organelles that are well known for their essential role in aerobic ATP production (McBride *et al.*, 2006). In fact, in most cell types, mitochondria supply the majority of ATP consumed by various energy-dependent processes. In addition, mitochondria also fulfill several other crucial roles in homeostatic pathways within the cell, including apoptosis, the regulation of cell division and growth, and the metabolism of essential micronutrients like iron and copper (Horn and Barrientos, 2008; McBride *et al.*, 2006; Pierrel *et al.*, 2007).

Mitochondria have several unique characteristics that presumably facilitate their ability to fulfill roles in a multitude of pathways. They are large, rod-shaped organelles consisting of two membranes (Henze and Martin, 2003) that divide the organelle into four distinct compartments; the outer membrane, the intermembrane space, the inner membrane and the matrix. Mitochondria are also semi-autonomous, in that they contain their own, multi-copy genome that encodes ribosomes and transfer RNAs required for the translation of its 13 messenger RNAs (Anderson *et al.*, 1981). However, because mitochondria consist of roughly 1000 proteins (Chacinska *et al.*, 2009; Pagliarini *et al.*, 2008; Perocchi *et al.*, 2006; Sickmann *et al.*, 2003), the overwhelming majority of which are encoded by nuclear DNA (Chacinska *et al.*, 2009), organelle biogenesis depends on the coordinated expression of both the mitochondrial and nuclear genomes. Finally, mitochondria are organized into a dynamic network or reticulum that undergoes constant fusion and fission, with movement of individual organelles along cytoskeletal tracts being important to maintaining functional organelles and cellular health (Liesa *et al.*, 2009).

1.1.1 Mitochondrial structure

Mitochondria are membrane-bound organelles that consist of an outer and inner membrane. These two phospholipid bilayers in turn define two distinct submitochondrial compartments; the intermembrane space (IMS) and matrix. The IMS is localized between the

outer and inner membranes, while the matrix is the aqueous phase contained by the inner membrane.

The mitochondrial outer membrane is semi-permeable. It contains two classes of channels that allow molecules to pass through the membrane from the cytosol into the IMS. Porin is a transmembrane channel protein that provides a means for the passive diffusion of ions and small molecules whose weight is less than 4,000-6,000 Daltons (Colombini, 1979; Freitag *et al.*, 1982; Sakaguchi *et al.*, 1992; Zalman *et al.*, 1980). The other channel is provided by the TOM (the translocase of the outer mitochondrial membrane) complex, which is composed of many proteins that collectively function as a translocon. Serving as a general point of entry into the organelle, the TOM complex recognizes proteins that carry mitochondrial targeting sequences and facilitates their translocation across the outer membrane (Neupert and Herrmann, 2007).

Unlike the outer membrane, the mitochondrial inner membrane is impermeable. Movement of proteins, molecules and even ions across the inner membrane therefore requires dedicated transporters, such as the TIM (translocase of the inner mitochondrial membrane) complex (Endo *et al.*, 2011; Neupert and Herrmann, 2007). The impermeable nature of the inner membrane is a necessary feature to establish the electrochemical gradient that is ultimately required for aerobic ATP production. ATP is produced in a process known as oxidative phosphorylation, by the concerted efforts of five multimeric enzyme complexes that are embedded in the inner membrane. The first four complexes form the respiratory chain, and generate an electrochemical gradient by pumping protons across the inner membrane in a series of electron transfer reactions. This gradient is then used by Complex V, ATP synthase to produce ATP (Figure 1.1). In cells where ATP demand is high, a higher concentration of these five complexes is accommodated by increasing the total surface area of the inner membrane through the formation of cristae.

Enclosed by the outer and inner membranes, the IMS of mitochondria is a more oxidizing environment than either the matrix or the cytosol (Herrmann and Hell, 2005; Hu *et al.*, 2008). It also tends to have a relatively low pH, which is at least in part a result of the proton gradient which is generated by the respiratory chain. Unlike the IMS, the mitochondrial matrix contains its own transcriptional and translational apparatus to support the expression of mtDNA-encoded proteins. The matrix also houses many enzymes which participate in the

citric acid cycle and the oxidation of fatty acids. In addition, it contains labile pools of iron, copper, zinc and manganese that are used to mature relevant mitochondrial targets, and that are important to the regulation of cellular metal ion homeostasis (Horn and Barrientos, 2008; Pierrel *et al.*, 2007).

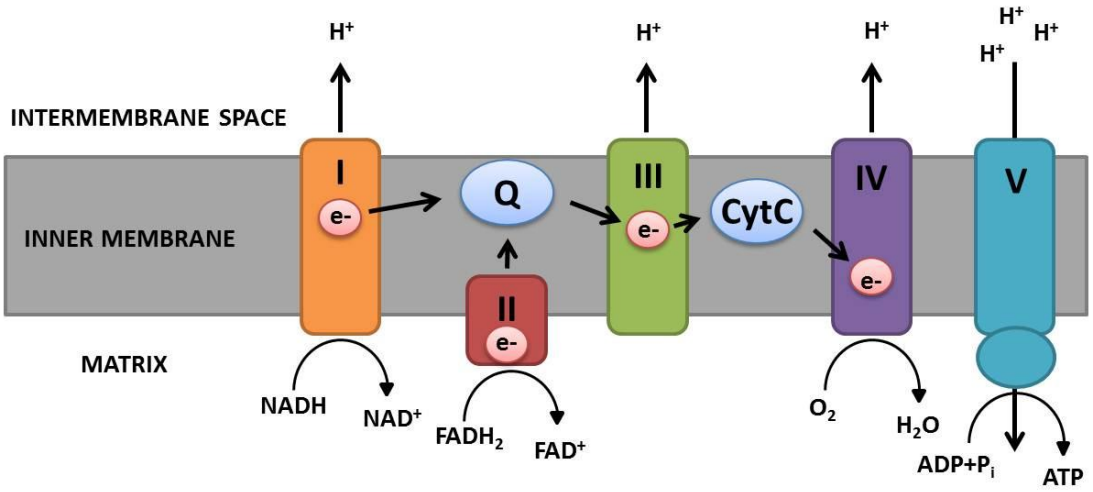

Figure 1.1 Schematic of oxidative phosphorylation. Oxidative phosphorylation is a process in which transfer of electrons from NADH (nicotinamide adenine dinucleotide) or succinate to O_2 is coupled to the synthesis of ATP. Complexes I and II shuttle electron from NADH and succinate, respectively, to Coenzyme Q (CoQ). Complex III subsequently mediates electron transfer from CoQ to cytochrome *c* (CytC). Finally, Complex IV completes electron transfer by utilizing electrons from cytochrome *c* to reduce molecular oxygen into water. Electron flow through Complexes I, III and IV is coupled with the pumping of protons from the matrix to the IMS, which contributes to the formation of proton gradient. Complex V utilizes this proton gradient to synthesize ATP. Both CoQ and CytC serve as electron shuttles that facilitate electron transfer between the complexes (Adapted from Ho *et al.*, 2012).

1.1.2 Mitochondrial biogenesis

1.1.2.1 Biogenesis of nuclear-encoded proteins

De novo synthesis of mitochondria requires coordinated expression of the nuclear and mitochondrial genomes. The overwhelming majority of mitochondrial proteins, including those required for mitochondrial translation, assembly of the respiratory chain and inner membrane transporters, are all nuclear-encoded (Becker *et al.*, 2012). These nuclear-encoded proteins are synthesized in the cytosol as precursors, and are targeted to the organelle by amino terminal signaling sequences. For most matrix and inner membrane proteins, the targeting signal is

cleaved upon import (Endo and Kohda, 2002; Roise and Schatz, 1988). However, there are many mitochondrial proteins that contain internal targeting signals that are recognized by the import machinery but that are not cleaved. Irrespective of the fate of the targeting signal upon import, mitochondrial proteins are recognized by specific subunits of the mitochondrial outer membrane translocon, or TOM complex, and transported across the outer membrane (Endo *et al.*, 2011). Proteins are then sorted and imported into their respective mitochondrial compartments via different sorting-specific import machineries.

1.1.2.1.1 TOM complex

The TOM complex is a translocon in the mitochondrial outer membrane (Figure 1.2) that facilitates the import of most mitochondrial proteins from the cytosol into the organelle (Chacinska *et al.*, 2009). It consists of 7 subunits, which can be grouped by function into receptors (Tom20 and Tom70) (Kiebler *et al.*, 1993; Saitoh *et al.*, 2007) and pore components (Tom40, Tom22, Tom5, Tom6 and Tom7) (Endo *et al.*, 2011). Tom40 is the central component of the TOM complex. It is a membrane-embedded protein with a β-barrel structure. Although Tom40 has been suggested to form two to three pores that serve as protein-conducting channels, the detailed mechanism of pore formation is still unclear (Becker *et al.*, 2005; Endo *et al.*, 2011; Herrmann *et al.*, 2012; Hill *et al.*, 1998; Kunkele *et al.*, 1998). Three small Tom proteins, Tom5, Tom6 and Tom7, are involved in the assembly, stability and dynamics of the TOM complex. Tom5 facilitates formation of the TOM complex (Dietmeier *et al.*, 1997). Tom6 and Tom7 function antagonistically, with Tom6 stabilizing the TOM complex and Tom7 promoting its dissociation (Model *et al.*, 2001; Wiedemann *et al.*, 2003). Tom22 is anchored in the outer membrane, and a cytosolically exposed N-terminal domain and an IMS-localized C-terminal domain provide specific binding sites for precursors on both sides of the outer membrane (van Wilpe *et al.*, 1999). The N-terminal domain of Tom22 along with receptors Tom20 and Tom70 collectively form a *cis*-binding site on the cytosolic side, and its C-terminal domain forms a *trans*-binding site in cooperation with Tom40 and Tom7 on the IMS side (van Wilpe *et al.*, 1999). Tom20 and Tom70 are major initial recognition sites for precursors. Both proteins are tethered to the outer membrane via their transmembrane segments, and expose hydrophilic receptor domains to the cytosol. Tom20 and Tom70 exhibit differences in their substrate

specificity. Tom20 preferentially recognizes precursor proteins with N-terminal cleavable presequences (Abe *et al.*, 2000; Neupert and Herrmann, 2007), while Tom70 preferentially recognizes precursor proteins containing internal targeting signals (Chan *et al.*, 2006; Neupert and Herrmann, 2007; Wu *et al.*, 2006; Wu and Sha, 2006). After precursor proteins are recognized by either Tom20 or Tom70, they are transferred to Tom22, and forwarded to the Tom40 channel via Tom5. Once precursor proteins cross the outer membrane through the Tom40 channel, they are recognized by the *trans*-binding site and directed to distinct mitochondrial subcompartments by different sorting machineries. The TIM23 (the presequence translocase of the inner mitochondrial membrane) complex interacts with proteins destined for the matrix and inner membrane, the TIM22 (the carrier translocase of the inner mitochondrial membrane) complex interacts with proteins targeted to the inner membrane, the TOB/SAM (the translocase of outer membrane β-barrel protein/the sorting and assembly machinery) complex recognizes proteins of the outer membrane, and the MIA (the mitochondrial intermembrane space import and assembly) pathway oxidizes the cysteines of several small soluble proteins to promote their retention within the IMS (Becker *et al.*, 2008; Chacinska *et al.*, 2009; Endo *et al.*, 2011; Koehler and Tienson, 2009; Paschen *et al.*, 2005). Because SCO1[1] is an inner mitochondrial membrane protein that interacts with several of these small, cysteine-rich IMS factors to fulfill its functions (Banci *et al.*, 2008a; Buchwald *et al.*, 1991; Leary *et al.*, 2007; Leary *et al.*, 2013b; Leary *et al.*, 2004; Leary *et al.*, 2009), I will focus on introducing the TIM23 and MIA sorting machineries (Figure 1.3).

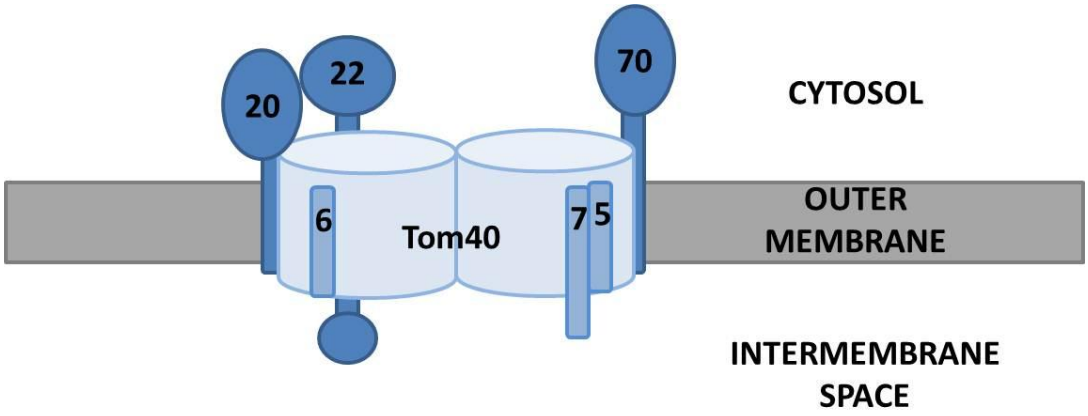

Figure 1.2 The composition of TOM complex. The TOM complex consists of 7 subunits; the receptor subunits Tom70 and Tom20, and the pore component Tom40, Tom22, Tom5, Tom6 and Tom7 (Adapted from Chacinska *et al.*, 2009).

[1] For the sake of consistency, the human nomenclature is used for SCO genes and proteins throughout this thesis.

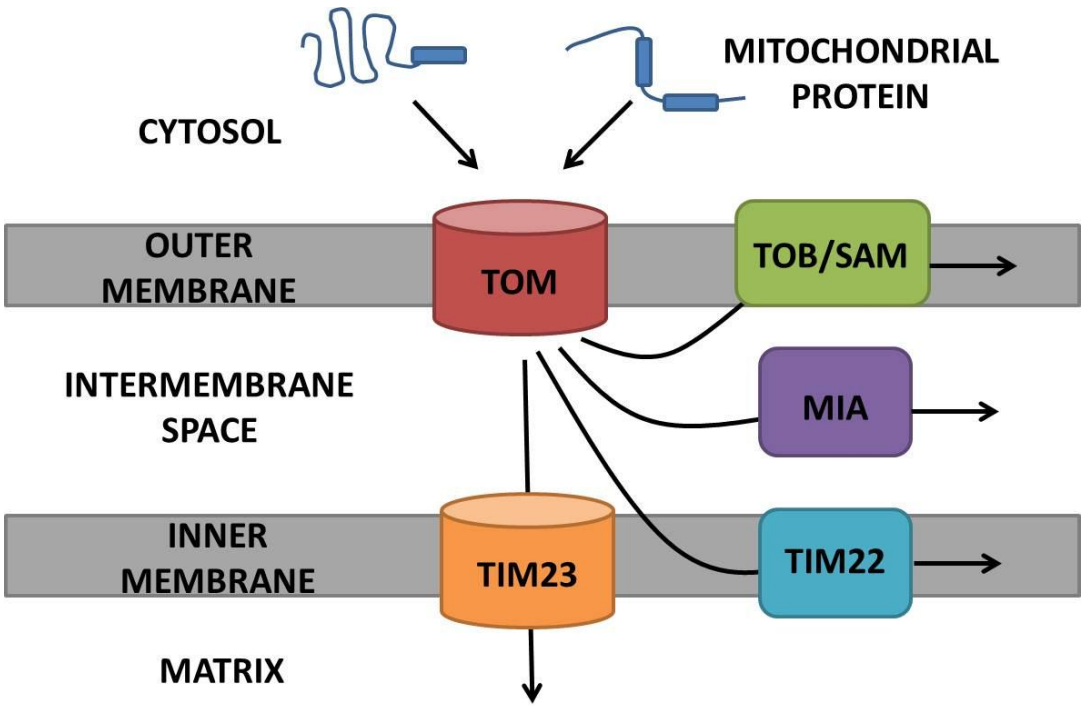

Figure 1.3 Sorting pathways of mitochondrial proteins. TOM complex is the main entry gate for proteins import into mitochondria. Mitochondrial proteins are subsequently directed to distinct mitochondrial subcompartments by different sorting machineries; the TOB/SAM complex to the outer membrane, the MIA pathway into the IMS, the TIM22 complex to the inner membrane, and the TIM23 complex to the matrix and inner membrane. (Adapted from Chacinska *et al.*, 2009)

1.1.2.1.2 TIM23 complex

The TIM23 complex is a translocon located in the inner membrane (Figure 1.4) that regulates the translocation or inner membrane insertion of precursor proteins containing cleavable N-terminal targeting sequences (Chacinska *et al.*, 2009; Endo *et al.*, 2011). The TIM23 complex can be divided into two major components; a protein-conducting channel which transports precursor proteins from the IMS to the matrix, and an import motor, known as PAM (presequence translocase-associated motor), that provides the energy source required for protein import (Herrmann *et al.*, 2012; Neupert and Herrmann, 2007). The protein-conducting channel of TIM23 consists of three crucial inner membrane subunits (Tim50, Tim17 and Tim23), as well as a dispensable subunit (Tim21). Tim50 functions as a receptor. It exposes its C-terminal domain to the IMS, and uses it to interact with the TOM complex and recognize incoming presequence-containing precursors (Chacinska *et al.*, 2005; Geissler *et al.*, 2002;

Mokranjac *et al.*, 2009; Tamura *et al.*, 2009; Yamamoto *et al.*, 2002). Tim23 consists of a membrane domain making up the translocation channel, and an IMS domain, which may in conjunction with Tim50 provide a recognition site for precursor proteins (Alder *et al.*, 2008; Truscott *et al.*, 2001). Tim17 has also been suggested to be part of the protein-conducting channel, and it also plays a key role in lateral sorting of preproteins (Chacinska *et al.*, 2005; Martinez-Caballero *et al.*, 2007). PAM is a multisubunit import motor, which is associated with the TIM23 complex at its matrix side. It consists of a central mtHsp70 (mitochondrial heat shock protein 70) subunit, and several subunits that regulate its activity (Mge1, Tim44, Pam18/Tim14 and Pam16/Tim16), as well as a non-essential subunit Pam17 (Neupert and Herrmann, 2007). mtHsp70 binds ATP and hydrolyzes it to drive the translocation of precursor proteins (Chacinska *et al.*, 2009). Mge1 (mitochondrial GrpE) is a nucleotide exchange factor that assists in the release of ADP from mtHsp70 (Schneider *et al.*, 1996). Tim44, Pam18/Tim14 and Pam16/Tim16 are co-chaperones of mtHsp70, directing and regulating the activity of mtHsp70 (D'Silva *et al.*, 2003; Kozany *et al.*, 2004; Mokranjac *et al.*, 2003; Truscott *et al.*, 2003). The protein-conducting channel and import motor of the TIM complex work cooperatively to translocate presequence-contain polypeptides through the inner membrane.

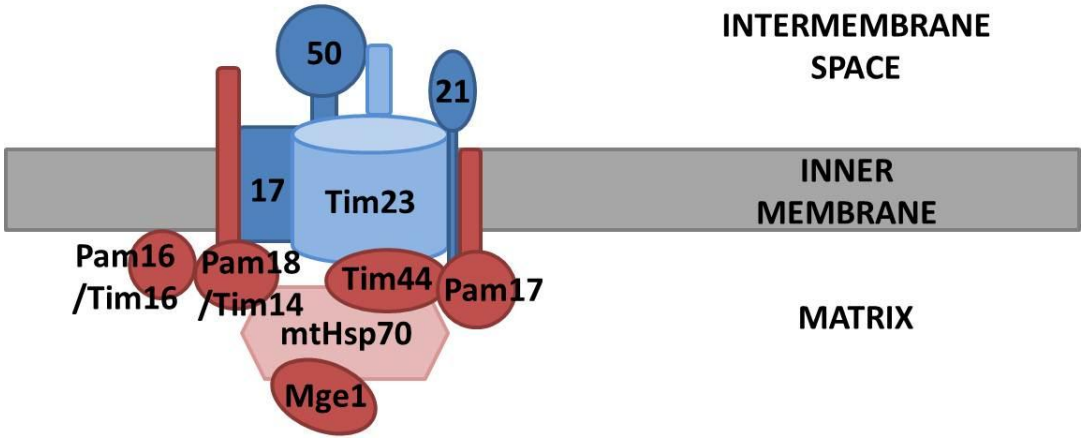

Figure 1.4 The composition of TIM23 complex. The TIM23 complex can be divided into two major components; a protein-conducting channel (blue), and an import motor, known as PAM (red). The protein-conducting channel of TIM23 consists of three crucial subunits (Tim50, Tim17 and Tim23), as well as a dispensable subunit (Tim21). PAM consists of a central subunit mtHsp70 (mitochondrial heat shock protein 70), and several regulatory subunits of mtHsp70 (Mge1, Tim44, Pam18/Tim14 and Pam16/Tim16), as well as a non-essential subunit Pam17 (Adapted from Chacinska *et al.*, 2009).

1.1.2.1.3 MIA pathway

The MIA pathway functions as a disulfide relay system, and is involved in the import of a specific subclass of mitochondrial proteins into the IMS (Endo *et al.*, 2011) (Figure 1.5). MIA recognizes mitochondrial proteins that contain characteristic twin Cx_3C or Cx_9C motifs, and oxidizes the cysteines within these motifs to form intramolecular disulfide bonds (Becker *et al.*, 2012; Herrmann *et al.*, 2012). The formation of intramolecular disulfide bonds changes the structure of IMS proteins from a linear, disordered polypeptide to one that has a helical hairpin fold, a structural transition that prevents retrotranslocation to the cytosol (Allen *et al.*, 2003; Lutz *et al.*, 2003).

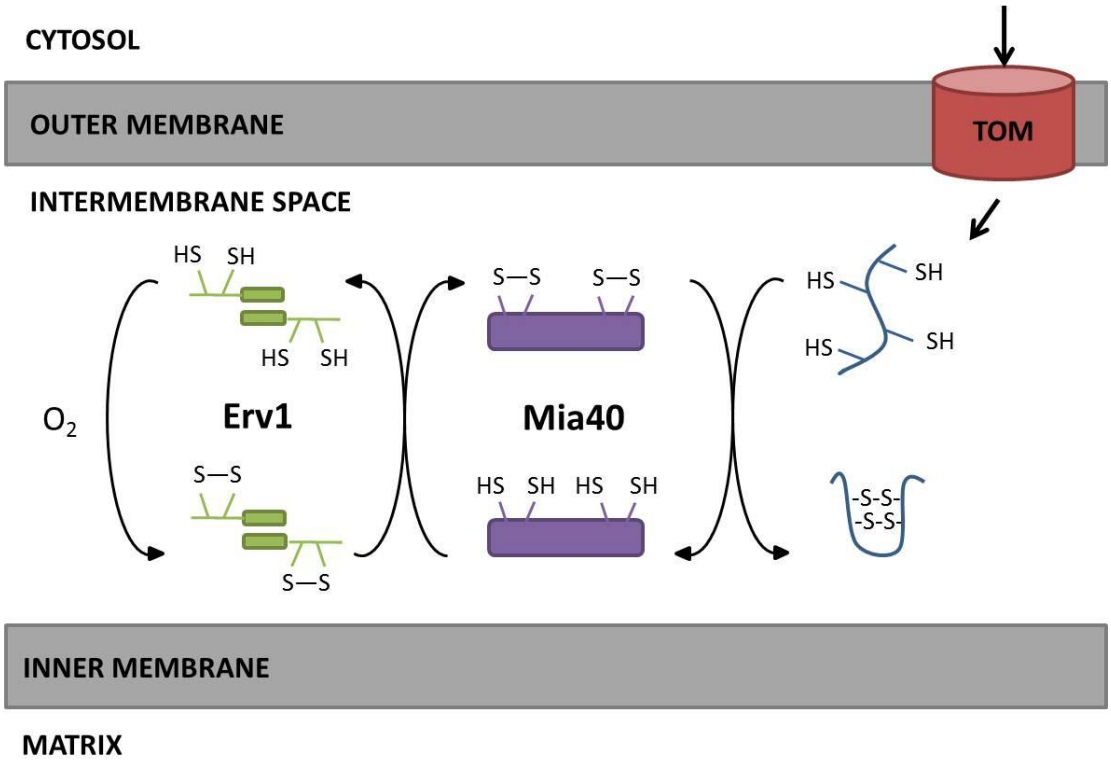

Figure 1.5 Model of the MIA pathway. The MIA pathway is a disulfide relay system which oxidatively traps cysteine-rich proteins imported from the cytosol into the IMS. It is composed of an oxidoreductase Mia40/Tim40 and a FAD-dependent sulfhydryl oxidase Erv1. Mia40/Tim40 oxidizes the twin Cx_3C or Cx_9C motifs of newly imported precursor proteins which promotes the formation of intramolecular disulfide bonds and alters their conformation, trapping them in the IMS. During this process, Mia40 becomes reduced and requires the activity of Erv1 to prepare it for the next round of disulfide bonding. Reduced and oxidized thiol groups are indicated by SH and S-S, respectively (Adapted from Allen *et al.*, 2008).

MIA is made up of two core components; Mia40/Tim40 and Erv1 (essential for respiration and vegetative growth protein 1) (Chacinska *et al.*, 2009; Chacinska *et al.*, 2004; Mesecke *et al.*, 2005; Naoe *et al.*, 2004). Mia40/Tim40 is an oxidoreductase (Herrmann *et al.*, 2012) that contains six conserved cysteines which form three intramolecular disulfide bonds. The first disulfide bond is formed by cysteines within a CPC motif (cysteine-proline-cysteine), which is critical for Mia40 function because this motif is redox active (Endo *et al.*, 2011; Grumbt *et al.*, 2007; Milenkovic *et al.*, 2007). Mia40/Tim40 in yeast is tethered to the inner membrane via a transmembrane domain, while Mia40/Tim40 in animals lacks the transmembrane domain and is therefore a soluble IMS protein (Hofmann *et al.*, 2005; Naoe *et al.*, 2004; Terziyska *et al.*, 2005). Mia40/Tim40 recognizes and oxidizes the twin Cx_3C or Cx_9C motifs of incoming IMS precursors, which results in its reduction (Figure 1.5). Erv1 is a FAD-dependent sulfhydryl oxidase that carries out the oxidation of reduced Mia40/Tim40, preparing it for the next round of disulfide bonding (Mesecke *et al.*, 2005; Rissler *et al.*, 2005). Hot13 (helper of Tim of 13 kDa) may be an additional component of MIA, as it has been suggested to promote the Erv1-dependent reoxidation of Mia40 (Curran *et al.*, 2004; Mesecke *et al.*, 2008).

1.1.2.2 Biogenesis of mitochondrially-encoded proteins

Compared to the number of nuclear-encoded mitochondrial proteins, the number of mitochondrial translation products is very small (~1%), and the mitochondrial genome in mammals only encodes a total of 13 polypeptides (Anderson *et al.*, 1981). Although few in number, these mitochondrially-encoded proteins are critical to organelle function, because they are all core catalytic subunits of a multimeric enzyme required for oxidative phosphorylation; ND1-ND6 and ND4L of Complex I, cytochrome b of Complex III, COX I-III of Complex IV and, ATP6 and ATP9 of Complex V (Ott and Herrmann, 2010). The transcription of mRNAs encoding these subunits and their subsequent translation relies on protein machinery unique from that localized in the cytosol. The mitochondrial transcriptional apparatus is composed of a single RNA polymerase, two transcription factors and a termination factor, while the translation machinery consists of mt-ribosomes, mt-tRNA, translational activators and other regulatory factors (Bonawitz *et al.*, 2006; Herrmann *et al.*, 2013; Scarpulla, 2008; Scarpulla *et al.*, 2012).

Following their translation in the matrix, newly synthesized mitochondrial proteins must be inserted into the inner membrane, and Oxa1 (Oxidase assembly mutant 1) plays a prominent role in this process (Herrmann *et al.*, 2012). Embedded in the inner membrane (Bonnefoy *et al.*, 1994), Oxa1 has a C-terminal tail that contains a long, positively charged domain localized to the matrix which is believed to bind the ribosome tightly. It has been shown that Oxa1 engages with newly synthesized polypeptides, mediating their co-translational insertion into the lipid bilayer (Hell *et al.*, 2001). Oxa1 also serves as a general insertion site for nuclear-encoded proteins, exporting them from the mitochondrial matrix into the inner membrane following cleavage of their presequence (Stuart, 2002). The function of Oxa1 in the context of protein insertion into the lipid bilayer is supported by an additional protein, Mba1 (multi-copy bypass of AFG3 (ATPase Family Gene)) (Ott *et al.*, 2006; Preuss *et al.*, 2001). Mba1 also serves as a ribosome receptor to recruit ribosomes to the inner membrane (Ott *et al.*, 2006), and helps Oxa1 to align the ribosome exit tunnel with the site of protein insertion (Ott *et al.*, 2006).

1.1.3 Mitochondrial dysfunction and quality control

Environmental changes, nutrient depletion, aging, DNA mutation and even normal mitochondrial function may result in irreversible oxidative damage to the organelle that impairs its integrity and in turn leads to mitochondrial dysfunction (Kubli and Gustafsson, 2012; Schapira, 2012; Youle and van der Bliek, 2012). Cells have therefore evolved several quality control systems to monitor mitochondria, and maintain those that are healthy and clear those that are irreversibly damaged. These quality control systems are contained within the organelle, as well as within the cytosol. Cytosolic quality control systems recruit proteins to damaged mitochondria that target the entire organelle for destruction via a specialized form of autophagy known as mitophagy (Michel *et al.*, 2012; Youle and Narendra, 2011). Mitochondrial fusion and fission (Chan, 2012) both play an important role in the maintaining a healthy organellar population within the cell. Fusion allows for cross-complementation of defective organelles, primarily because the healthy organelle contains a sufficient number of copies of unmutated mtDNA (Youle and van der Bliek, 2012). Mitochondrial fission allows for selective removal of irreversibly damaged components of the reticulum and their degradation by mitophagy (Klionsky, 2007; Kubli and Gustafsson, 2012; Michel *et al.*, 2012).

1.1.4 Mitochondrial disease

Mitochondrial diseases are some of the most common genetic disorders in humans (Schon *et al.*, 2012). More than 150 different mitochondrial diseases have been identified, and at least 1 in 5,000 individuals are affected by one of these disorders (Vafai and Mootha, 2012). Mitochondrial disorders can result from mutations in either mitochondrial or nuclear DNA. Pathogenic mutations in mtDNA include point mutations and rearrangements, such as deletions, duplications or inversions, all of which are caused by replication errors and are heritable (Schapira, 2012). Mutations in nuclear-encoded genes that are essential for mtDNA expression and the biogenesis of the complexes of oxidative phosphorylation also contribute significantly to the number and diversity of mitochondrial diseases.

The majority of mitochondrial diseases arise from defects in oxidative phosphorylation (Vafai and Mootha, 2012). With the exception of Complex II which is entirely nuclear-encoded, all complexes of oxidative phosphorylation have a dual genetic origin; most of the core subunits of these complexes are contained within the mitochondrial genome, while the remaining, peripheral subunits are encoded in the nuclear genome. The assembly of individual structural subunits into a functional, multimeric protein complex requires numerous nuclear-encoded accessory proteins. These accessory proteins, termed assembly factors, function broadly to promote the expression of both mitochondrially- and nuclear-encoded structural subunits, the insertion of these subunits into the inner membrane, and the synthesis, delivery and incorporation of several prosthetic groups during holoenzyme assembly that are ultimately required for catalytic competence (Ghezzi and Zeviani, 2012). Therefore, mutations in genes encoding structural subunits of oxidative phosphorylation complexes, or in those that support their assembly or stability, severely impair ATP synthesis. To date, more than 200 pathogenic mutations in mtDNA and over 100 pathogenic mutations in nuclear-encoded mitochondrial genes have been described in humans (Vafai and Mootha, 2012). A long-standing goal of the field therefore is to understand the molecular function of genes which, when mutated, cause mitochondrial dysfunction associated with both early (e.g. Leigh's syndrome) and late (e.g. Alzheimer's disease) onset forms of disease (Schapira, 2012) for which effective therapies are currently lacking (Pfeffer *et al.*, 2012; Vafai and Mootha, 2012)

1.2 Cytochrome *c* oxidase

COX[2], also known as Complex IV, is a multimeric protein complex embedded in the inner mitochondrial membrane in eukaryotes. It catalyzes the last in a series of electron transfer reactions within the respiratory chain. It uses electrons from reduced cytochrome *c* to convert oxygen into water, while simultaneously pumping a proton across the inner membrane to the IMS. As such, it contributes to the establishment of a chemiosmotic potential that is harnessed by Complex V for ATP synthesis (Figure 1.6) (DiMauro *et al.*, 2012).

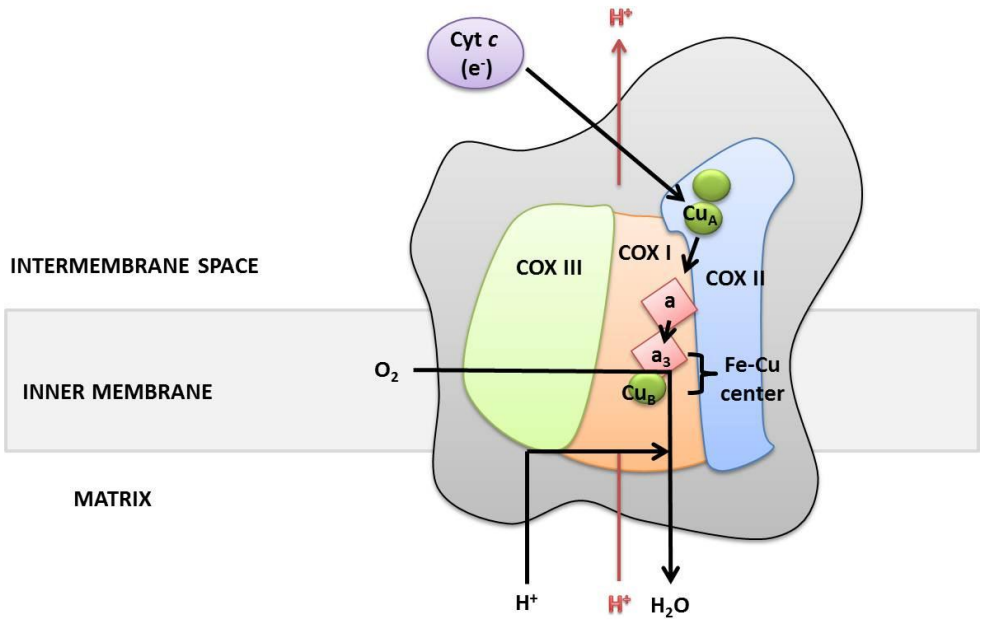

Figure 1.6 Function of core subunits of COX complex. The mitochondrially-encoded subunits I, II and III are essential to electron flow. The schematic of COX is shown here, with COX I (orange), COX II (blue) and COX III (green). COX I harbors two heme moieties, designated *a* and a_3 (red), and a copper ion, Cu_B (green sphere). COX II contains two Cu ions (green spheres) in a binuclear center, Cu_A. Electron transfer through COX starts with reduced cytochrome *c* delivering electrons to the Cu_A site. From the Cu_A site, electrons are transported through heme *a* to the Fe-Cu center. There, oxygen is reduced to water by the electrons, with consumption of protons from the matrix. At the same time, protons are pumped across the inner membrane from the matrix into the IMS (Adapted from Lehninger, A. *et al.*, 2004).

1.2.1 Structure of COX

[2] For the sake of consistency, the human nomenclature for COX genes and proteins is used throughout this thesis

COX is a multimeric protein complex composed of 11 or 13 structural subunits in yeast and humans, respectively (Fontanesi *et al.*, 2008). It requires several distinct metal co-factors for its catalytic activity; two heme moieties, two copper groups, and a magnesium and zinc ion (Tsukihara *et al.*, 1995). Mitochondrially-encoded COX I, II and III are the three largest structural subunits, and form the catalytic core of the holoenzyme (Carr and Winge, 2003; Poyton and McEwen, 1996). COX I contains two heme moieties, designated *a* and a_3, and a mononuclear Cu_B site (Khalimonchuk and Rodel, 2005). COX II contains a binuclear Cu_A site (Arnold, 2012). These four metal co-factors play an essential role in electron transfer (Figure 1.6). The Cu_A site receives electrons from cytochrome *c*, and transfers them to heme *a*. The heme a_3 and Cu_B constitute a binuclear Fe-Cu center which transport electrons from heme *a* to O_2 (Yoshikawa *et al.*, 2012). The catalytic core is surrounded by the remaining, nuclear-encoded structural subunits. They are thought to stabilize the holoenzyme and provide sites for the allosteric regulation of its activity (Fontanesi *et al.*, 2006; Stiburek and Zeman, 2010). A subset of these nuclear-encoded subunits (COX VI , COX V, COX VI and COX VII in mammals, COX VI and COX VII in yeast) have isoforms (Khalimonchuk and Rodel, 2005) that are expressed in a tissue-specific manner or at particular stages of development.

1.2.2 COX assembly

The biogenesis of COX is a complex, sequential process. It requires a myriad of steps, including the coordinate expression of both the mitochondrially- and nuclear-encoded subunits, protein translocation into the organelle, and the synthesis, delivery and incorporation of metal co-factors into relevant structural subunits at the appropriate stage of assembly (Cobine *et al.*, 2006c; Stiburek and Zeman, 2010). Studies of respiratory-deficient yeast mutants have identified more than 30 nuclear-encoded accessory proteins that assist with one or more of these aspects of holoenzyme assembly, and most of these proteins are not contained in the mature COX complex itself (Fontanesi *et al.*, 2008). The elegant genetic studies in yeast have been invaluable to the identification of human homologues of these accessory proteins (Barrientos *et al.*, 2002).

1.2.2.1 Synthesis and membrane insertion of mitochondrially-encoded COX subunits

COX I-III are highly hydrophobic, and are synthesized on matrix-localized ribosomes that are associated with the inner membrane to protect newly synthesized proteins from aggregating (Green-Willms *et al.*, 2001; Jia *et al.*, 2003; Szyrach *et al.*, 2003). The expression of these core catalytic subunits is tightly regulated. Several translational activators (such as Mss51, Pet111 and Pet122) have been found in yeast (Brown *et al.*, 1994; Manthey and McEwen, 1995; Mulero and Fox, 1993), and these factors regulate the translation of COX I-III via binding to the 5' untranslated region of their mRNAs (Naithani *et al.*, 2003; Sanchirico *et al.*, 1998). In mammals, only two mitochondrial translational activators have been identified to date. TACO1 is a translational activator of mitochondrially-encoded COX I and has a yeast orthologue, YGR021w, with about 72% of similarity (Weraarpachai *et al.*, 2009). LRPPRC (Leucine-rich PPR motif-containing protein) is also predicted to be the human homolog of the yeast translational activator Pet309 (PETite colonies), but its exact role is ill defined. The synthesis of COX I in yeast requires the assistance of two translational activators Mss51 (mitochondrial splicing suppressor) and Pet309 (Figure 1.7). In addition to a role in the translation of *COX I* mRNA, Mss51 has been reported to interact with newly synthesized COX I, and result in a transient Mss51-COX I complex (Barrientos *et al.*, 2004; Perez-Martinez *et al.*, 2003). Such an interaction has been proposed to act as a negative feedback loop, by preventing Mss51 from promoting the translation of additional *COX I* mRNA. Moreover, it could serve to recruit other assembly factors including Cox14, Cox25, Ssc1 and Mdj1 (mitochondrial DnaJ) (Barrientos *et al.*, 2004; Fontanesi *et al.*, 2011; Fontanesi *et al.*, 2010; Mick *et al.*, 2010). These assembly factors may work cooperatively to promote the stabilization and proper folding of nascent COX I (Soto *et al.*, 2012; Westermann *et al.*, 1996). The translation of *COX II* mRNA is mediated by the membrane bound translational activator Pet111 (Mulero and Fox, 1993; Poutre and Fox, 1987). Pet111 is specific for *COX II* mRNA, and its low abundance within the inner membrane limits synthesis of the COX II protein (Mulero and Fox, 1993; Poutre and Fox, 1987). COX III synthesis requires the translational activators Pet54, Pet122 and Pet494 (Costanzo *et al.*, 1986; Kloeckener-Gruissem *et al.*, 1988), which form a complex to promote translation of *COX III* mRNA (Brown *et al.*, 1994; Costanzo and Fox, 1988, 1995; Kaspar *et al.*, 2008).

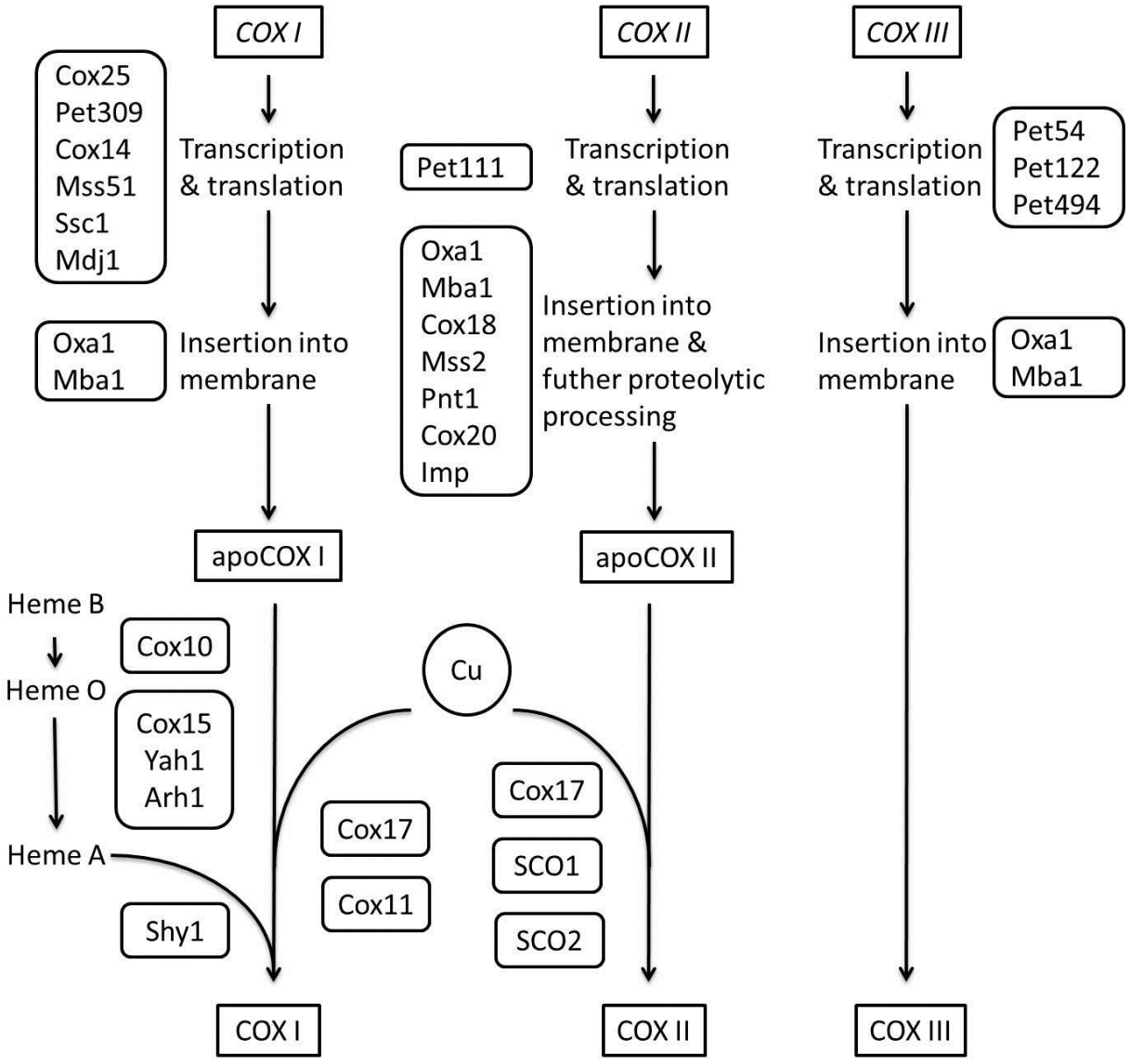

Figure 1.7 The biosynthesis of COX I-III in yeast. COX subunits are in rectangles, and COX assembly factors are in rounded rectangles (Adapted from Zee and Glerum, 2006).

Following their translation, newly synthesized COX I-III require additional accessory proteins for their proper insertion into the inner membrane. The insertion pathway of COX II in particular has been thoroughly investigated. COX II is an integral membrane protein with two transmembrane domains. Its two termini protrude into the IMS, resulting in an N-out and C-out topology (Soto *et al.*, 2012). Among the three core subunits of COX, COX II is the only one that is proteolytically processed during its maturation. Prior to cleavage, the precursor depends on the Oxa1 machinery to insert its first transmembrane domain and export its N-terminal tail across the inner membrane (He and Fox, 1997; Hell *et al.*, 1998). Anchoring of the second, C-

terminal transmembrane domain within the inner membrane requires the cooperative action of Cox18, Mss2 and Pnt1 (PeNTamidine resistance) (Broadley *et al.*, 2001; Fiumera *et al.*, 2007; Saracco and Fox, 2002; Souza *et al.*, 2000). After its insertion into the inner membrane is complete, COX II associates with another chaperone, Cox20 (Hell *et al.*, 2000), which presents the precursor to the inner membrane peptidase (Imp) complex. The Imp complex then cleaves a portion of the soluble, N-terminal region of COX II, yielding the mature form of protein (Gakh *et al.*, 2002; Jan *et al.*, 2000). The mechanisms responsible for inserting COX I and COX III into the inner membrane remain poorly understood (Khalimonchuk and Rodel, 2005). It has been shown that incorporation of COX I and COX III into the inner membrane relies in part on the activity of Oxa1 (Hell *et al.*, 2001; Herrmann and Neupert, 2003; Stuart, 2002). Mitochondrial import and subsequent membrane integration of the nuclear-encoded COX polypeptides occurs after their translation on cytoplasmic ribosomes, and is mediated by the TOM and TIM23 machineries (Mokranjac and Neupert, 2010).

1.2.2.2 Incorporation of metal prosthetic groups

The incorporation of two heme moieties and two copper groups is essential for the maturation and correct folding of COX I and COX II. It appears that metal insertion occurs prior to full assembly of the holoenzyme (Cobine *et al.*, 2006c). However, when and how COX I and COX II are metallated is not yet fully understood.

COX is the only mitochondrial enzyme that requires heme *a* moieties for its activity. Hemylation of COX I requires two steps; biosynthesis of heme *a* moieties and their subsequent delivery and insertion into the COX I protein. Heme A is synthesized from its ancestral heme B form primarily by Cox10 (Glerum and Tzagoloff, 1994) and Cox15 (Barros *et al.*, 2001; Barros and Tzagoloff, 2002). Cox10 is a farnesyl-transferase, and it catalyzes the first reaction of heme A biosynthesis, converting heme B to a heme O intermediate (Tzagoloff *et al.*, 1993). The subsequent oxidation of heme O to heme A is performed in two separate monooxygenase steps, which rely on the activity of Cox15, and in all likelihood the ferredoxin Yah1 (yeast adrenodoxin homolog) and the putative ferredoxin reductase Arh1 (Adrenodoxin reductase homolog) (Barros *et al.*, 2001; Barros *et al.*, 2002; Brown *et al.*, 2002). Heme A incorporation into COX I relies on the activity of Shy1, the yeast homolog of human SURF1 (Smith *et al.*,

2005). However, little is known about the mechanism of heme a insertion, and whether Shy1 and SURF1 fulfill the same functions.

COX and Cu, Zn-superoxide dismutase (SOD1) are the only two copper metalloenzymes contained within mitochondria. It is believed that the copper found in these two enzymes comes from a labile copper pool housed in the matrix (Cobine *et al.*, 2006a). Metallation of these two enzymes therefore depends on specific copper transport pathways that facilitate the movement of copper across the inner membrane. At present, however, it is unclear how copper is delivered to mitochondria, and how it is trafficked within the organelle.

Cox17 is the first metallochaperone implicated in copper delivery to COX (Glerum *et al.*, 1996a). This small hydrophilic protein exists in both the cytoplasm and the IMS (Beers e*t al.*, 1997). It contains a twin Cx_9C motif and a cysteine-cysteine motif at its N-terminus that is essential for Cu(I) binding (Arnesano *et al.*, 2005; Banci *et al.*, 2008b; Palumaa *et al.*, 2004). Cox17 functions as Cu(I) donor for Cox11 (Carr *et al.*, 2002), SCO1 (Glerum *et al.*, 1996b) and SCO2 (Banci *et al.*, 2008a; Banci *et al.*, 2007b; Horng *et al.*, 2004; Papadopoulou *et al.*, 1999), which in turn deliver copper to COX I or COX II (Horng *et al.*, 2004). How Cox17 itself is metallated remains an open question. In addition to Cox17, the IMS contains a number of other proteins with twin Cx_9C motifs, many of which are critical for COX assembly and may regulate copper trafficking within the IMS (Horn and Barrientos, 2008; Longen *et al.*, 2009). These twin Cx_9C motif-containing proteins include Cox19 (Rigby *et al.*, 2007), Cox23 (Barros *et al.*, 2004), Pet191 (McEwen *et al.*, 1993), Cmc1 (Cx9C mitochondrial protein necessary for full assembly of cytochrome *c* oxidase) (Horn *et al.*, 2008) and Cmc2 (Horn *et al.*, 2010). All of these yeast proteins have human orthologues, and their import and retention within the IMS relies on the TOM complex and the MIA pathway (Fraga and Ventura, 2012; Herrmann and Riemer, 2012; Soto *et al.*, 2012). Other than Cox17, however, the molecular function of these twin Cx_9C motif-containing proteins remains poorly understood.

The Cu_B site of COX I consists of three histidines for the coordination of the copper ion (Yoshikawa *et al.*, 2012). The copper metallochaperone of COX I, Cox11 is an integral inner membrane protein with a single transmembrane helix (Hiser *et al.*, 2000; Tzagoloff *et al.*, 1990). Its C-terminal tail contains a copper-binding domain that projects into the IMS (Carr *et al.*, 2002). A role for Cox11 in the metallation of COX I was inferred by the observation that the Cu_B site was absent when the COX complex was purified from a strain of *Rhodobacter*

sphaeroides in which the Cox11 gene had been deleted (Hiser *et al.*, 2000). Additional evidence from a study in yeast showed that mutation of the copper-coordinating amino acids in Cox11 resulted in an isolated COX deficiency (Carr *et al.*, 2002). The mechanism by which Cox11 delivers copper to the Cu_B site of COX I will require further investigation.

The Cu_A center in COX II is formed by two copper ions present as a [Cu^{2+}/Cu^{1+}] complex (Lappalainen *et al.*, 1993; Malmstrom and Aasa, 1993). These two copper ions are coordinated by two cysteines, two histidines, a methionine and a glutamate (Tsukihara *et al.*, 1995). The two copper-binding cysteines are within a CxxxC motif that faces the IMS (Iwata *et al.*, 1995; Tsukihara *et al.*, 1995; Yoshikawa *et al.*, 2012). Further details concerned with COX II metallation will be provided in Section 1.3.

At present, it is not known how the magnesium and zinc ions are delivered and inserted into COX, or if their presence has a direct effect on enzyme function (Cobine *et al.*, 2006c; Fontanesi *et al.*, 2006).

1.2.2.3 The process of COX assembly

COX assembly is best described as an ordered process in which structural subunits are sequentially added to COX I (Figure 1.8) (Fontanesi *et al.*, 2008). Three distinct assembly intermediates (S1-S3) are formed prior to the biogenesis of the mature holoenzyme (S4) (Cobine *et al.*, 2006c; Fontanesi *et al.*, 2006), and the basic steps by which COX is assembled appear to be conserved between yeast and mammals (Fontanesi *et al.*, 2008). In mammals, the first step of COX assembly is the insertion of COX I into the inner membrane (S1). The second step involves the incorporation of COX IV and COX V_a to form S2. Addition of the bulk of the remaining subunits allows for COX assembly to proceed to the S3 stage, with the subsequent incorporation of COX VI_a and COX $VII_{a/b}$ producing the mature holoenzyme (S4) (Fontanesi *et al.*, 2008; Nijtmans *et al.*, 1998; Williams *et al.*, 2004).

1.2.3 Isolated COX deficiency

An isolated COX deficiency is one of the most common causes of respiratory chain defects in humans, and pathogenic mutations have been described in genes encoding both structural subunits and accessory proteins critical for holoenzyme assembly (Fernandez-Vizarra

et al., 2009; Ghezzi and Zeviani, 2012). Pathogenic mutations have been reported for all three mitochondrially-encoded COX subunits and these are known to affect COX assembly and stability (Bruno *et al.*, 1999; Campos *et al.*, 2001; Tiranti *et al.*, 2000). Patients with mutations in COX I, II or III genes exhibit remarkably different, tissue-specific clinical phenotypes, even among patients with mutations in the same gene (Shoubridge, 2001).

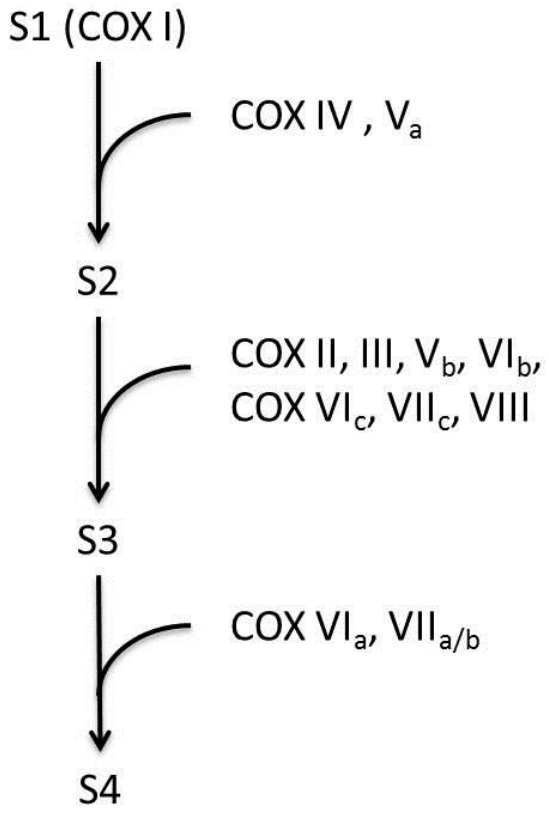

Figure 1.8 Schematic representation of COX assembly. S1- S3 represent the intermediate steps in the assembly process, and S4 refers to the fully assembled holoenzyme complex (Adapted from Shoubridge, 2001).

Defects in 3 nuclear genes encoding COX structural subunits have been characterized to date; COX IV, COX VI_b and COX VII_b. COX IV is located at the matrix-site of the holoenzyme (Shteyer *et al.*, 2009). It is thought to be critical for early steps of COX complex biogenesis (Li *et al.*, 2006). Patients with mutations in COX IV present with exocrine pancreatic insufficiency, dyserythropoietic anemia and calvarial hyperostosis (Shteyer *et al.*, 2009). COX VI_b is a hydrophilic extramembrane protein facing the IMS that is proposed to

facilitate the connection of two monomers of COX to yield the dimeric form of the enzyme that is found *in vivo* (Tsukihara *et al.*, 1996; Yoshikawa *et al.*, 1998). Moreover, COX VI_b may be involved in the interaction between the holoenzyme and cytochrome *c* (Huttemann *et al.*, 2003; Sampson and Alleyne, 2001). Pathogenic mutations in COX VI_b cause mitochondrial encephalomyopathy associated with an isolated COX defect (Massa *et al.*, 2008). The relevance of COX VII_b to COX function is still poorly understood. However, it must be indispensable for enzyme function, given that mutations in this gene cause microphthalmia with linear skin lesions, an X-linked dominant disorder that is lethal in males (Indrieri *et al.*, 2012).

The most frequent cause of COX deficiency is attributable to mutations in genes encoding accessory proteins, which severely impair various steps of holoenzyme biogenesis. To date, variable clinical phenotypes associated with pathogenic mutations in *TACO1* (Weraarpachai *et al.*, 2009), *SURF1* (Tiranti *et al.*, 1998; Zhu *et al.*, 1998b), *SCO1* (Valnot *et al.*, 2000a), *SCO2* (Papadopoulou *et al.*, 1999), *COX10* (Valnot *et al.*, 2000c), *COX15* (Antonicka *et al.*, 2003b), *LRPPRC* (Mootha *et al.*, 2003), *FAM36A/COX20* (Szklarczyk *et al.*, 2013), *C12orf62* (Weraarpachai *et al.*, 2012) and *COA5/PET191* (Huigsloot *et al.*, 2011) have been described. COX10 and COX15 are required for the biogenesis of heme A critical for the maturation of the heme *a* and a_3 sites contained within COX I. Mutations in *COX10* lead to a loss in total heme A content, and cause a range of clinical conditions including hypertrophic cardiomyopathy, Leigh syndrome and renal tubulopathy (Antonicka *et al.*, 2003a; Coenen *et al.*, 2004; Valnot *et al.*, 2000b). Mutations in *COX15* are associated with an isolated COX deficiency and an early onset, fatal hypertrophic cardiomyopathy, as well as a reduction in the levels of heme A (Antonicka *et al.*, 2003b). While SURF1 is also proposed to be involved in the biogenesis of the heme a_3 site of COX I, its precise function is poorly understood (Fernandez-Vizarra *et al.*, 2009; Ghezzi and Zeviani, 2012). Over 40 distinct mutations have been described in *SURF1*, all of which are associated with Leigh syndrome and an isolated COX deficiency (Pecina *et al.*, 2004; Pequignot *et al.*, 2001; Tiranti *et al.*, 1998; Zhu *et al.*, 1998a). With few exceptions, *SURF1* mutations result in a lack of detectable SURF1 protein, which leads to the accumulation of the S1 and S2 COX assembly intermediates and a reduction in the amount of the fully assembled holoenzyme (Stiburek *et al.*, 2005; Tiranti *et al.*, 1999; Williams *et al.*, 2004).

1.3 SCO1 and SCO2

SCO1 and SCO2 are involved in the metallation of the Cu_A site of COX II, which is perhaps the most thoroughly characterized aspect of COX assembly. SCO1 and SCO2 were first identified in a suppressor screen that used a yeast *Cox17* null mutant (Glerum *et al.*, 1996b). While both SCO1 and SCO2 suppressed the respiratory deficient phenotype of a yeast *Cox17* null strain (Glerum *et al.*, 1996b), subsequent deletion of each gene revealed that only SCO1 is required for growth on a non-fermentable carbon source. While SCO2 overexpression was unable to rescue a *SCO1* null mutant, it was able to functionally complement the glycerol growth defect in a yeast strain expressing a SCO1 point mutant (Glerum *et al.*, 1996b). This led to the suggestion that while only SCO1 is essential for COX assembly in yeast, the functions of SCO1 and SCO2 partially overlap (Glerum *et al.*, 1996b). To date, however, a function for yeast SCO2 has yet to be identified (Khalimonchuk and Rodel, 2005).

Unlike yeast, human SCO1 and SCO2 are essential and mutations in either gene result in a severe, isolated COX deficiency that is associated with early onset, tissue-specific forms of disease with fatal clinical outcomes. Thus far, three *SCO1* pedigrees have been identified. Patients with a homozygous *G132S* mutation presented with a fatal hypertrophic cardiomyopathy, while those that carried a *P174L* missense mutation on one allele and a nonsense mutation on the second allele ultimately died from neonatal liver failure (Stiburek *et al.*, 2009; Valnot *et al.*, 2000a). The third *SCO1* pedigree was only recently characterized (Leary *et al.*, 2013a). The patient carried a *M294V* missense mutation on one allele and a premature stop codon on the second allele, and presented with a fatal encephalopathy. Mutations in *SCO2* are relatively more common. Nearly all of the roughly 30 pedigrees identified thus far carry at least one allele with an *E140K* missense mutation (Mobley *et al.*, 2009; Sambuughin *et al.*, 2013). Although patients may be *E140K* heterozygous or homozygous, all of them ultimately succumb from a fatal hypertrophic cardiomyopathy (Jaksch *et al.*, 2001a; Leary *et al.*, 2006). The distinct forms of disease are not caused by tissue-specific expression of these two genes, since both *SCO1* and *SCO2* are ubiquitously expressed and display a similar expression pattern across human tissues (Papadopoulou *et al.*, 1999). However, a molecular genetic explanation as to how mutations in these two housekeeping genes produce such strikingly different clinical phenotypes is not currently understood (Khalimonchuk and Winge, 2008; Stiburek and Zeman, 2010).

Human SCO1 and SCO2 are closely related paralogues (i.e. divergence from gene duplication event). SCO1 is a 301 aa protein that consists of a mitochondrial targeting sequence at its N-terminus, a matrix domain, a single transmembrane helix and a C-terminal domain that protrudes into the IMS (Banci *et al.*, 2006; Williams *et al.*, 2005). SCO2 is marginally smaller than SCO1, a difference that is reflected mostly in the size of its soluble, matrix-localized N-terminal domain (Williams *et al.*, 2005). Both SCO1 and SCO2 are homodimers *in vivo* (Leary *et al.*, 2004), and they share a great degree of sequence identity in their C-terminal domains, which are globular and contain four α-helices and nine β-strands that collectively form a highly conserved thioredoxin fold that contains a copper-binding site (Figure 1.9) (Banci *et al.*, 2006; Banci *et al.*, 2007a). The copper-binding site consists of two cysteines present in a CxxxC motif and a histidine, all of which are spatially close to each other in the quaternary structure of the proteins (Banci *et al.*, 2006; Banci *et al.*, 2007a). This site binds Cu(I), and the solution structures of the apo- and Cu(I)-loaded conformers of human SCO1 are similar, with the exception of loop 8 which shows significant rearrangements depending on the metallation state of the protein. The structural dynamics of loop 8 imply it may be a region of the protein critical to interactions between SCO1 and its partners (Banci *et al.*, 2006). SCO1 and SCO2 are also able to bind Cu(II). The solution structure of the Ni(II) derivative of human SCO1 has been solved, and indicates that divalent metal ions like Cu(II) can also be coordinated by the same histidine residue and the two cysteine residues of CxxxC motif used to bind Cu(I), with the help of a donor ligand from an undetermined Asp residue (Banci *et al.*, 2006). In addition to their ability to bind copper, it appears that human SCO1 and SCO2 also have a thiol disulphide oxidoreductase activity (Leary *et al.*, 2009; Williams *et al.*, 2005).

While SCO proteins clearly require the ability to bind copper for their function, their relative roles in COX assembly are poorly understood. Several lines of evidence argue that human SCO1 and SCO2 function as metallochaperones and are responsible for copper delivery to COX II during the biogenesis of the Cu_A site. The function of SCO1 and SCO2 is abrogated by replacing any of the copper-coordinating amino acids (cysteine, cysteine and histidine) with alanines, arguing that copper binding is essential to their biological activity (Horng *et al.*, 2005). Supplementation of the culture media with exogenous copper fully also rescued the COX deficiency of *SCO2* patient cells and it partially rescued that of *SCO1* patient cells (Jaksch *et al.*,

2001b; Leary *et al.*, 2004; Salviati *et al.*, 2002). However, it is not yet clear whether both SCO proteins transfer copper to COX II during the maturation of its Cu_A site. Since the Cu_A site is composed of a Cu(I) and a Cu(II) ion, it is possible that SCO1 and SCO2 each transfer one atom of copper during the metallation reaction. Alternatively, one or both SCO proteins may instead catalyze metallation of the Cu_A site by fulfilling a critical redox function in the absence of copper transfer. Consistent with this idea, overexpression of wild-type SCO2 in *SCO2* patient fibroblasts enriched for the presence of oxidized cysteines within the CxxxC motif of SCO1 (Leary *et al.*, 2009). The observation that fibroblasts derived from both *SCO* backgrounds accumulate the same S2 assembly intermediate implies that SCO1 and SCO2 each function at the same stage of COX complex biogenesis. These functions appear to be unique, because overexpression of wild-type SCO1 in *SCO2* patient fibroblasts, or vice versa, further exacerbates the COX deficiency in the relevant patient background (Leary *et al.*, 2004). Accordingly, it has been shown that SCO2 is critical for normal rates of COX II synthesis (Leary *et al.*, 2009), while SCO1 is required for the stability of newly synthesized COX II (Cobine *et al.*, 2006).

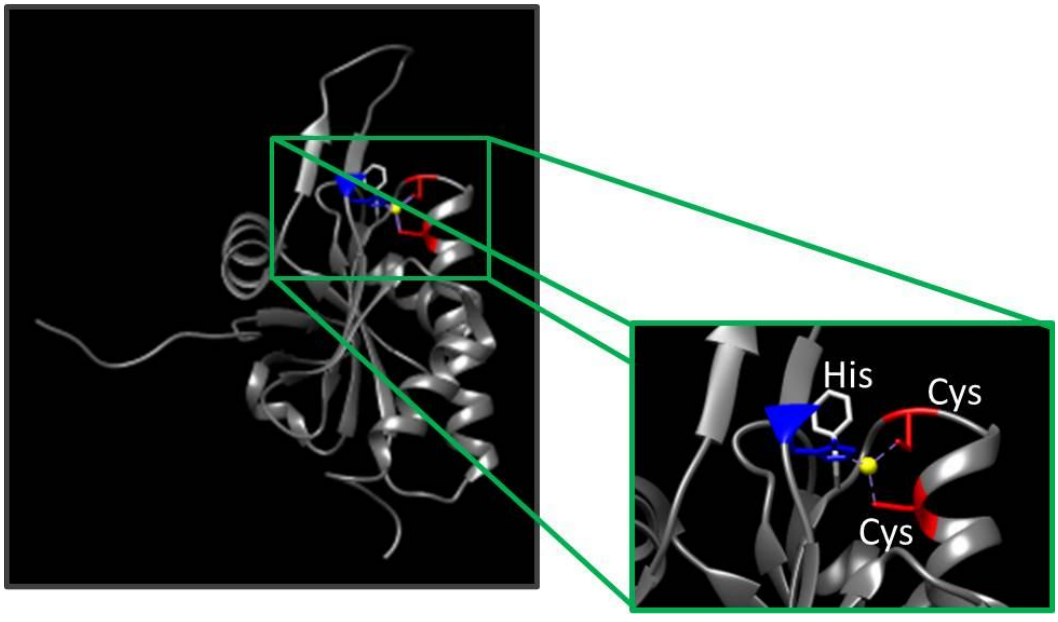

Figure 1.9 Solution structure of copper-loaded human SCO1. The average structures of the lowest energy ensemble of human SCO1 with bound Cu(I) is shown. The copper binding amino acids cysteine (Cys169, Cys173) and His260 are depicted in red and blue, respectively. The Cu(I) ion is shown in yellow (Adapted from Banci *et al.*, 2006).

Understanding the relative roles of SCO1 and SCO2 is complicated by the fact that both proteins have additional, novel functions in the regulation of cellular copper homeostasis. *SCO1* and *SCO2* patient fibroblasts are both COX and copper deficient (Leary *et al.*, 2007). Even though the overexpression of a wild-type *SCO1* cDNA in *SCO1* patient fibroblasts completely suppressed the COX deficiency, it did not rescue the total cellular copper deficiency (Leary *et al.*, 2007). In contrast overexpression of a wild-type *SCO2* cDNA in *SCO1* patient fibroblasts partially rescued the copper deficiency, while exacerbating the COX deficiency (Leary *et al.*, 2007). These data argue that the copper deficiency phenotype can be completely dissociated from the defects in COX assembly, and further indicate that it can be suppressed by overexpressing SCO2, but not SCO1. Subsequent pulse-chase experiments with ^{64}Cu revealed that *SCO1* and *SCO2* patient cells are unable to retain ^{64}Cu, and exhibit elevated rates of copper efflux rather than defects in high affinity uptake (Leary *et al.*, 2007). To explain these observations, a model was proposed in which SCO2 alters the redox state of the cysteine of the CxxxC motif of SCO1, leading to activation of the cellular copper efflux signaling pathway (Briere and Tzagoloff, 2007; Leary *et al.*, 2007). A recent study confirms and further develops this model by characterizing a SCO1-dependent mitochondrial redox signal that requires COX19 to regulate ATP7A-mediated copper efflux from the cell (Leary *et al.*, 2013b). However, the detailed mechanisms describing how the redox and/or metallation state of SCO1 affects cellular copper homeostasis have yet to be elucidated.

1.4 Hypothesis and objectives

SCO1 and SCO2 function in the biogenesis of the binuclear Cu_A site of COX II (Lode *et al.*, 2000). Mutations in either *SCO1* or *SCO2* produce a severe, isolated COX deficiency, and result in distinct, tissue-specific clinical phenotypes (Papadopoulou *et al.*, 1999; Valnot *et al.*, 2000a). Because *SCO* patient fibroblasts accumulate the S2 assembly intermediate, and the overexpression of either SCO in the reciprocal patient background exacerbates the COX deficiency, it is thought that both SCO1 and SCO2 fulfill unique functions at the same stage of COX assembly (Leary *et al.*, 2004). The function of SCO proteins requires that they be able to bind copper (Horng *et al.*, 2005; Nittis *et al.*, 2001). Both SCO1 and SCO2 can bind Cu(I) and Cu(II) within a highly conserved thioredoxin fold contained within their C-terminus that

protrudes into the IMS (Beers *et al.*, 2002; Horng *et al.*, 2005; Jaksch *et al.*, 2001b; Nittis *et al.*, 2001). This copper-binding region consists of two cysteine residues within a CxxxC motif and a highly conserved histidine (Balatri *et al.*, 2003; Banci *et al.*, 2007a; Nittis *et al.*, 2001). Protein function is abrogated by alanine substitutions of any of these three residues (Horng *et al.*, 2005; Nittis *et al.*, 2001). Although significant progress has been made in characterizing the function of SCO proteins in yeast and humans, their precise molecular functions remain unclear. In fact, our current understanding of the respective roles of SCO1 and SCO2 in the maturation of the Cu_A site of COX II is limited. In particular, it remains unclear which SCO protein delivers copper to COX II, and how their redox states affect COX assembly. Understanding the relative importance of the metallation state of SCO proteins to their roles as COX assembly factors may also provide insight into how copper-loading of SCO1 relates to the generation of a mitochondrial redox dependent signal that regulates the rate of copper efflux from the cell (Leary *et al.*, 2007; Leary *et al.*, 2013b).

Hypothesis:

SCO1 transfers copper to the Cu_A site of COX II during COX assembly, and the metallation state of SCO1 *in vivo* acts as a rheostat that contributes to the regulation of cellular copper homeostasis. Therefore, overexpression of a SCO1 mutant that binds copper with greater affinity than the wild-type protein and that is unable to transfer the metal ion to an interacting partner would be predicted to exacerbate the COX and cellular copper deficiencies in *SCO* patient fibroblasts.

Objectives:

1. Functionally characterize a series of SCO1 point mutants that had been rationally designed to potentiate or abolish copper-binding. Methionine, cysteine and histidine are common copper-binding amino acids (Giri *et al.*, 2004), while serine is structurally similar to cysteine but is incapable of coordinating copper. Therefore, the two cysteines of the CxxxC motif (Cys169, Cys173) and the conserved histidine (His260) of SCO1 were substituted with various combinations of these four amino acids. These SCO1 mutants were then functionally characterized by quantifying the phenotypic effect of their overexpression on residual COX activity in control, *SCO1* and *SCO2* patient fibroblasts.

2. Quantify the copper-binding properties of this panel of SCO1 point mutants *in vitro*, using histidine-tagged, soluble truncates expressed in and purified from *E. coli* (Horng *et al.*, 2005).

2 MATERIALS AND METHODS

2.1 Reagents

Names of reagents and suppliers are listed in Table 2.1. Addresses for each supplier are subsequently listed in Table 2.2.

Table 2.1 List of reagents and suppliers

General Reagent	**Supplier**
Bathocuproine disulfonic acid (BCS)	Sigma-Aldrich
Bradford reagent	Bio-Rad
Chloroquine	Sigma-Aldrich
Dithiothreitol (DTT)	Bioshop
Ethylenediaminetetraacetic acid (EDTA)	Bioshop
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Bioshop
K_2HPO_4	Bioshop
KH_2PO_4	Bioshop
n-Dodecyl-β-D-maltopyranoside (DDM)	Bioshop
Oxaloacetic acid	Sigma-Aldrich
Phenylmethanesulfonyl fluoride (PMSF)	Bioshop
Phosphate buffered saline (PBS)	Bioshop
Potassium Phosphate	Bioshop
Triton X-100	Bioshop
Cell Culture Reagent	**Supplier**
Antibiotic-antimycotic	Lonza
Dulbecco's modified Eagle's medium (DMEM)	Corning cellgro
Fetal bovine serum	Sigma-Aldrich
Hygromycin B	Calbiochem-Millipore
Puromycin	Sigma-Aldrich
Bacteria Culture Reagent	**Supplier**
Ampicillin	Bioshop
Kanamycin	Bioshop

Sodium chloride	Bioshop
Tryptone	Bioshop
Yeast Extract	Becton Dickinson
Commercial kit	**Supplier**
E.Z.N.A. Plasmid Mini Kit	Omega Bio-Tek
Gel Extraction Kit	QiaQuick
jetPRIME^{TM} DNA Transfection Reagent	Polyplus Battery
Kapa HiFi PCR Kit	Kapa Biosystems
Ni-NTA Agarose	Qiagen
PCR Purification Kit	Qiagen

Table 2.2 Name and addresses of suppliers

Becton Dickinson	Mississauga, Ontario, Canada
Bio-Rad	Mississauga, Ontario, Canada
Bioshop	Burlington, Ontario, Canada
Calbiochem-Millipore	Billerica, MA, USA
Corning cellgro	Manassa, VA, USA
Kapa Biosystems	Woburn, MA, USA
Lonza	Basel, Switzerland
Omega Bio-Tek	Norcross, GA, USA
Polyplus Battery	Berkeley, CA, USA
Qiagen	Toronto, Ontario, Canada
Sigma-Aldrich	Oakville, Ontario, Canada

2.2 Mammalian cells and growth media

Primary skin fibroblasts were derived from controls, and *SCO1* (*SCO1-1*, T146X/P174L (Valnot *et al.*, 2000a); *SCO1-2*, V93X/M294V (Leary *et al.*, 2013a) and *SCO2* (R90X/E140K) patients (Leary *et al.*, 2013b), and immortalized by stable transduction with the *E7* gene of

human papillomavirus and the catalytic subunit of human telomerase (Lochmuller *et al.*, 1999). Immortalized fibroblasts were stably transduced with individual *SCO1* cDNAs using retrovirus that was produced by the Phoenix amphotropic packaging cell line (kind gift of Dr. G. Nolan, Stanford University). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Corning cellgro) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1x antibiotic-antimycotic (Life technologies) at 37°C in an atmosphere of 5% CO_2. Cell lines were selected and maintained by their co-culture in media supplemented with 100 mUnits/mL of hygromycin B (Calbiochem) or 2 μg/mL of puromycin (Sigma) to ensure stable overexpression of a given construct.

2.3 Plasmids, bacterial strains and growth media

For *in vivo* experiments, a series of *SCO1* point mutants was generated by site-directed mutagenesis. The first generation retroviral expression vector pLXSH (Miller *et al.*, 1993) containing a wild-type *SCO1* cDNA was used as the PCR template. I generated all of the mutants listed below (Figures 2.1 & 2.2), except for the *SCO1* N-terminal point (C45A, C61A and C45AC61A) and truncation (Δ17, Δ37 and Δ57) mutants (generated by Ms. Shelley Stewart). Plasmid DNA for transduction was amplified using the competent *E. coli* strains DH5α or XL-1 Blue.

For *in vitro* experiments, soluble *SCO1* point mutants lacking the N-terminal 333 bp (Figure 2.3) were amplified by PCR using the appropriate pLXSH expression vector as a template, and cloned into the pHis parallel vector 2 which contains a hexahistidine tag 5' of the start methionine that allows for subsequent protein purification (Sheffield *et al.*, 1999). Plasmid DNA was transformed into the competent *E. coli* strain BL21 (DE3) which carries the T7 polymerase under the control of an IPTG-inducible lacUV5 promoter.

All *SCO1* constructs generated in this study were submitted to the Plant Biotechnology Institute (Saskatoon, SK) for Sanger sequencing to confirm the introduction of the desired mutation(s) and the fidelity of the remainder of the sequence.

2 YT media (16 g/L tryptone, 10 g/L yeast extract, 5 g/L sodium chloride, pH 7.4) containing the appropriate antibiotic (either 100 μg/mL ampicillin or 50 μg/mL of kanamycin)

were utilized for bacterial culture. All bacterial strains were grown at 37°C at 225 rpm in a shaking incubator.

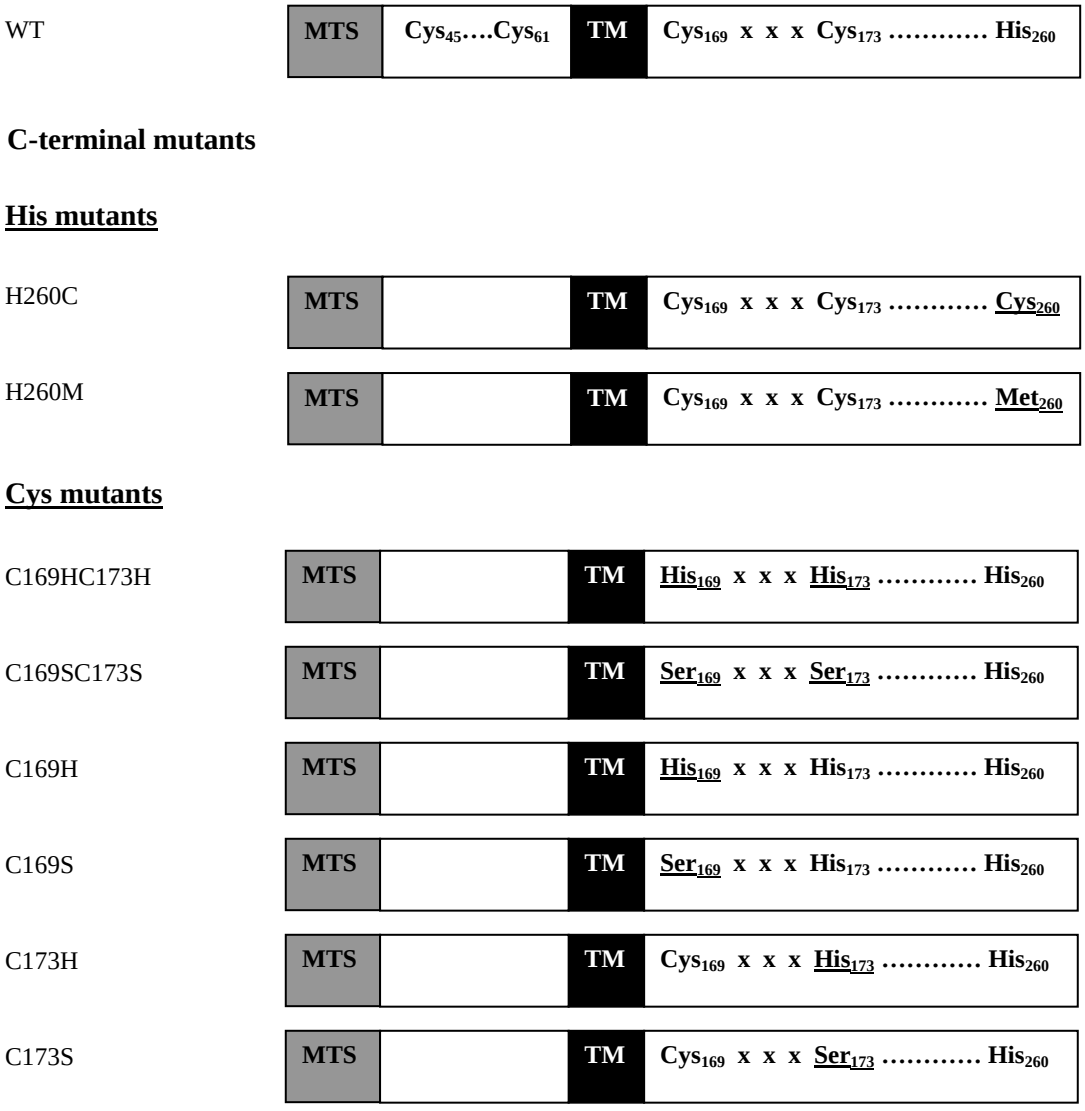

Figure 2.1 Schematic of C-terminal SCO1 point mutants. Cys169, Cys173 and His260 were substituted via site-directed mutagenesis with amino acids expected to abolish (serine) or alter (cysteine, histidine, methionine) the copper-binding properties of SCO1. The substituted amino acids are underlined. MTS, mitochondrial targeting sequence; TM, transmembrane helix.

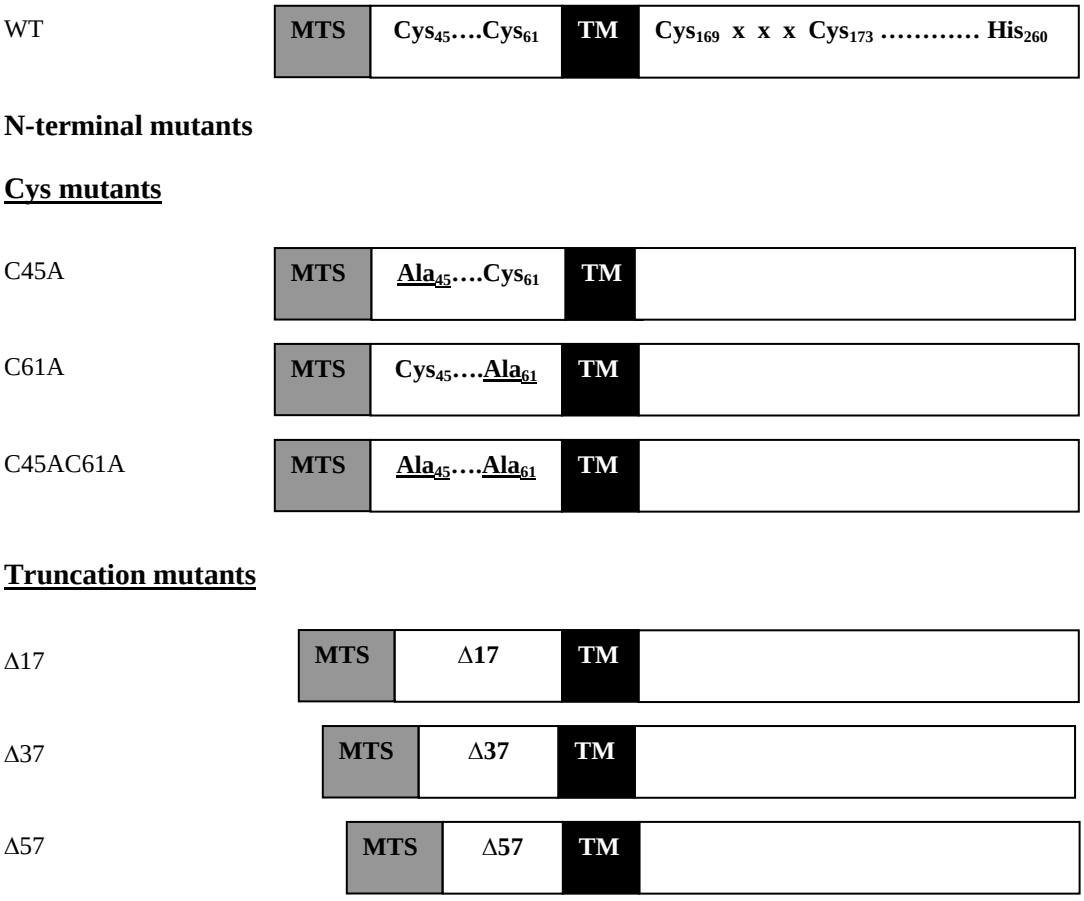

Figure 2.2 Schematic of N-terminal SCO1 mutants. Two sets of SCO1 N-terminal mutants were used in the study. One set was comprised of cysteine to alanine mutants, and included C45A, C61A and C45AC61A substitutions. The other set was comprised of truncation mutants and included Δ17, Δ37 and Δ57. Substituted amino acids are underlined. MTS, mitochondrial targeting sequence; TM, transmembrane helix.

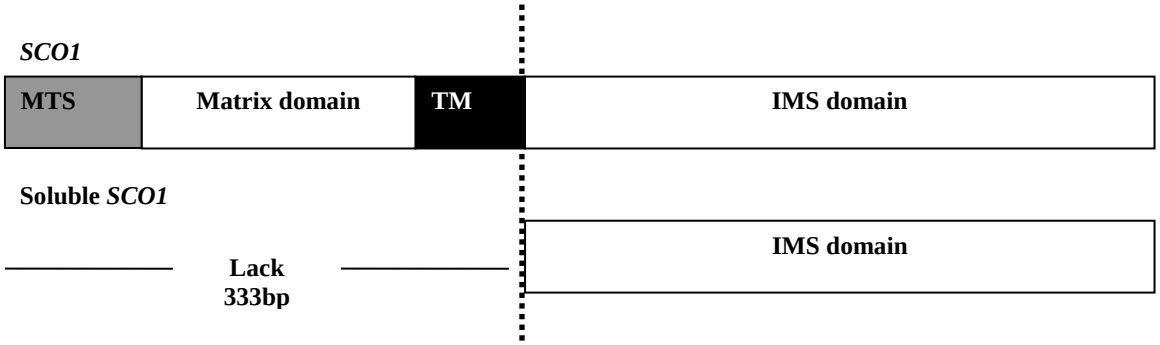

Figure 2.3 Schematic of soluble SCO1 variants. Soluble *SCO1* variants lacking the mitochondrial targeting sequence (MTS), matrix domain and the transmembrane helix (TM) were fused in frame with an N-terminal 6x histidine tag to allow for their purification (Horng *et al.*, 2005).

2.4 DNA methods

2.4.1 Site-directed mutagenesis

Site-directed mutagenesis was used to generate all of the *SCO1* point mutants (Figure 2.2). Primers were designed to adhere to the properties recommended by the manufacturer (Stratagene), and synthesized by Sigma-Aldrich (Table 2.3). All point mutations were introduced using the Kapa HiFi PCR Kit (KAPABIOSYSTEMS). PCR amplifications were performed in 25 μL reactions that contained 1x buffer (supplemented with 2.0 mM Mg^{2+}), 0.3 mM dNTPs, 0.3 μM of both the forward and reverse primers, 5-10 ng of template DNA, and 0.5 Units KAPA HiFi DNA Polymerase. The cycling conditions were as follows; initial denaturation at 95°C for 2 minutes, followed by 16 cycles at 98°C for 20 seconds, 52-75°C for 1 minute, 72°C for 8 minutes, with a final extension step at 72°C for 7 minutes. To remove methylated, parental DNA, the resultant PCR products were treated with 10 Units DpnI (BioLabs) in 1x buffer 4 (NEB) at 37°C for 2 hours, and then used for transformation (refer to section 2.4.3).

Table 2.3 List of primers

Mutant name	Primer name	Primer sequence	Annealing temperature
H260C	H260C-F	GAAGACTACATAGTGGATTGCACAATAATAATGTACTTGATTGGACC	75 °C
	H260C-R	GGTCCAATCAAGTACATTATTATTGTGCAATCCACTATGTAGTCTTC	
H260M	H260M-F	GAAGACTACATAGTGGATATGACAATAATAATGTACTTGATTGGACC	55 °C
	H260M-R	GGTCCAATCAAGTACATTATTATTGTCATATCCACTATGTAGTCTTC	
C169H	C169H-F	GGCTTCACTCATCACCCTGATGTCTGTCCAG	52 °C
	C169H-R	CTGGACAGACATCAGGGTGATGAGTGAAGCC	
C169S	C169S-F	GGCTTCACTCATTCCCCTGATGTCTGTCCAG	55 °C
	C169S-R	CTGGACAGACATCAGGGGAATGAGTGAAGCC	
C173H	C173H-F	CACTCATTGCCCTGATGTCCATCCAGAAGAACTAG	55 °C
	C173H-R	CTAGTTCTTCTGGATGGACATCAGGGCAATGAGTG	
C173S	C173S-F	CACTCATTGCCCTGATGTCTCTCCAGAAGAACTAG	55 °C
	C173S-R	CTAGTTCTTCTGGAGAGACATCAGGGCAATGAGTG	
C169HC173H	C169HC173H-F	GGCTTCACTCATCACCCTGATGTCCATCCAGAAGAACTAG	55 °C
	C169HC173H-R	CTAGTTCTTCTGGATGGACATCAGGGTGATGAGTGAAGCC	
C169SC173S	C169SC173S-F	GGCTTCACTCATTCCCCTGATGTCTCTCCAGAAGAACTAG	55 °C
	C169SC173S-R	CTAGTTCTTCTGGAGAGACATCAGGGGAATGAGTGAAGCC	
Soluble SCO	SCO His-F	AGATCTGGATCCATGAAGCACGTCAAGAAAGA (*BamH*I)	72 °C
	SCO His-R	AGATCTGTCGACCTAGCTCTTTTTTCTGTATG (*Sal*I)	

2.4.2 Subcloning

Soluble *SCO1* variants (lacking the first 333 bp) were amplified by PCR from the appropriate pLXSH parental plasmid using pHis forward and reverse primers (Table 2.3) in 40 µL reactions that contained 1x Phusion HF buffer (supplemented with 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 0.5 µM of both the forward and reverse primers, 4 ng template DNA, and 0.8 Unit High-Fidelity DNA polymerase (Thermo Scientific). The cycling conditions were as follows; initial denaturation at 98°C for 10 seconds, followed by 30 cycles at 54°C for 30 seconds, 72°C for 45 seconds, with a final extension step at 72°C for 7 minutes. Successful amplification of each construct was confirmed by electrophoresing 1-3 µL of the PCR product on a 1% agarose gel. The remainder of the PCR product was purified using a spin column, according to the manufacturer's instructions (QiaQuick).

The pHis-parallel 2 vector was prepared from bacterial culture for subsequent subcloning (refer to section 2.4.4). Digestion of the purified PCR product and of the pHis parallel 2 vector with *BamH*I (Thermo Scientific) and *Sal*I (NEB) then allowed for conventional cloning via ligation. The double digestion was performed in a 50 µL reaction with 1x buffer 3 (BioLabs) at 37°C for 1 hour and 50 minutes. *BamH*I was added to the reaction in the last 50 minutes to avoid its star activity. Large amounts of purified insert and linearized vector proved to be necessary for successful ligation. All digestion products were electrophoresed and the desired DNA fragments were excised from the agarose gel. Each gel slice was incubated with QG buffer (QIAquick) at 50°C for 10 minutes until it was completely dissolved, followed by its purification using a spin column (QIAquick) according to the manufacturer's specifications. To increase ligation efficiency, purified DNA concentration was quantified by electrophoresis using a DNA Mass ladder (NEB). Ligations were performed in 20 µL reactions with 1x T4 DNA ligase reaction buffer (BioLabs) and 400 Units T4 DNA ligase (BioLabs) at 19°C overnight, using a 1:4 molar ratio of vector to insert. The ligation product was then used for transformation (refer to section 2.4.3).

2.4.3 Transformation

XL1-blue, DH5α and XL10-gold competent cells were used for transformation in the study. 1-5 µL of ligation reaction was added to tubes containing competent cells (2 µL for

XL10-Gold). The tubes were gently swirled and placed on ice for 30 minutes. Next, the tubes were heat-pulsed in a 42°C water bath for 45 seconds (30 seconds for XL10-Gold), and incubated on ice for 2 minutes. 500 µL of 2YT (NZY^+ for XL10-Gold) was added to the tubes and cells were allowed to recover at 37°C for 1 hour with shaking at 225 rpm prior to plating. After an overnight incubation at 37°C on 2YT plates containing the appropriate antibiotic, positive clones containing the desired plasmid were screened first by colony PCR. Plasmid from potential positives was then isolated, double digested and sent for sequencing.

2.4.4 Plasmid isolation and DNA quantification

Plasmid DNA was isolated from bacterial cultures using the E.Z.N.A. plasmid mini kit as described by the manufacturer. The concentration of plasmid DNA was determined spectrophotometrically using a SpectraMAX 190 (Molecular Devices). Plasmid DNA was diluted 1:20 with double-distilled H_2O, and its concentration quantified by measuring the absorbance at a wavelength of 260 nm. Relative purity of plasmid DNA was evaluated by calculating the A_{260}/A_{280} ratio. To confirm its integrity prior to use in transfections or transformations, plasmid DNA was subjected to digestion with the appropriate restriction enzymes and DNA was visualized by electrophoresis.

2.5 Retroviral transduction

Retroviral transduction was utilized to overexpress *SCO1* variants in human fibroblasts, as the low transfection efficiency of this cell type is well established. Retrovirus was packaged with the Phoenix Amphotropic, helper-free production system (Swift *et al.*, 2001), and used to stably transduce recipient cells as follows;

Day 1, Transfection of Phoenix cells: Phoenix cells were transfected at 60% to 80% confluency. Media was replaced with fresh media containing 25 µM chloroquine, to prevent lysosomal degradation of plasmid DNA. Typically, 100 mm plates were used, with 5 µg of plasmid DNA and 5 µg of carrier DNA (Sigma-Aldrich) being added to 500 µL jetPRIME™ (Polyplus) buffer, followed by the addition of 20 µL of jetPRIME™ reagent. The transfection mix was vortexed for 10 seconds, incubated for 10 minutes at room temperature, and added to the plate in a drop wise manner. Phoenix cells were then placed in the incubator for 24 hours.

Day 2, Detoxification of Phoenix cells: 24 hours after transfection, media was replaced with fresh media to avoid the toxic side effects of continued exposure to chloroquine, and to obtain a high viral titer. Human fibroblasts were split such that they would be 40-60% confluent at the time of transduction.

Day 3, Transduction of human fibroblasts: 48 hours after transfection, the retroviral-containing media was harvested and filtered through a 0.45 μm syringe filter into sterile 15 mL tubes to remove whole cells and cell debris. Polybrene was added to a final concentration of 5 μg/mL, to neutralize the charge repulsion between retrovirus and sialic acid on the cell surface and therefore increase the transduction efficiency. 1.5-4 mL of the retroviral filtrate was then added to human fibroblasts proliferating in 60 or 100 mm plates. Recipient cells were returned to the incubator for 2 hours, after which fresh media containing 5 μg/mL polybrene was added and the fibroblasts were left overnight.

Days 4 & 5, Removal of retroviral-containing media and selection for stable overexpressing lines: The day after transduction, media was replaced. Hygromycin was then added the following day to a final concentration of 100 mUnits/mL to select for a stably transduced bulk culture.

2.6 Protein analysis

2.6.1 Sample preparation

Fibroblasts overexpressing SCO1 variants were harvested in 1 mL of ice-cold PBS via scraping or by trypsinization and subsequent neutralization. Cells were centrifuged at 14,000 *x g* for 1 minute at 4°C. The resultant cell pellets were resuspended in 50 to 200 μL of extraction buffer (50 mM triethanolamine, 1.0 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4), homogenized with a hand held, motorized pestle and used immediately for kinetic and endpoint assays.

2.6.2 Citrate Synthase (CS) activity assay

CS is a nuclear-encoded enzyme that localizes to the mitochondrial matrix that is often used as a marker of mitochondrial content. The activity of CS was quantified by monitoring the

rate of breakdown of acetyl Coenzyme A to Coenzyme A (CoA-SH), a byproduct that then reacts with Ellman's reagent (DTNB). Specifically, homogenate was mixed with or without 10 μL of oxaloacetic acid (OAA) and 150 μL assay buffer (100 mM Tris (pH 7.4), 0.3 mM acetyl CoA, 200 mM Ellman's reagent and 10% triton X-100), and changes in absorbance at 412 nm were monitored at 30°C using a spectrophotometer (SpectraMAX 190, Molecular Devices). CS activity was calculated using an extinction coefficient 25.9 $M^{-1}cm^{-1}$ using the following formula; CS activity = (A_{412} [+OAA] - A_{412} [-OAA]) × 25.9 $M^{-1}cm^{-1}$ / (volume of sample × protein concentration of sample)

2.6.3 COX activity assay

COX activity was measured by quantifying the rate of oxidation of reduced cytochrome *c*. Specifically, homogenate was mixed with assay buffer (50 mM potassium phosphate (pH 7.0), 0.2 mM cytochrome *c*, 2 mM dodecylmaltoside), and changes in absorbance at 550 nm were monitored spectrophotometrically at 30°C. COX activity was calculated using an extinction coefficient 12.4 $M^{-1}cm^{-1}$ using the following formula; COX activity= A_{550} × 12.4 $M^{-1}cm^{-1}$ / (volume of sample × protein concentration of sample)

COX activity was normalized to CS activity, and expressed as a percentage of either parental, untransduced cells or matched, control fibroblasts. A one-way analysis of variance (ANOVA) followed by a Tukey's HSD post-hoc test was then used to determine if SCO1 variants had a significant effect on COX activity in control and patient cells.

2.6.4 Protein concentration

The Bradford assay (Bradford, 1976) was used to quantify the protein concentration of all samples. A standard curve (0-8 ng) was set up using 1 mg/mL BSA (Bioshop) in a 96-well plate. Standards and samples were diluted with 250 μL of 1x Bradford reagent (Bio-Rad) and the absorbance at 595 nm was measured spectrophotometrically. SOFTmax PRO 4.0 software (Life Sciences Edition) was then used to calculate the protein concentration of samples, based on the accompanying BSA standard curve.

2.7 Recombinant protein expression and purification

2.7.1 Protein expression trials

Protein expression conditions were optimized for truncated, soluble SCO1 point mutants, to obtain large amount of Cu-loaded recombinant proteins for subsequent *in vitro* characterization. Two experimental variables were examined; the $CuSO_4$ concentration (0.5 mM or 1 mM) and isopropyl β-D-1-thiogalactopyranoside (IPTG) concentration (0.5 mM or 1 mM). Since nearly no copper or Cu-bound proteins exist in the cytoplasm of *E. coli*, and inclusion of copper in the culture medium slows down bacterial growth, the $CuSO_4$ was added to the culture upon IPTG induction.

2.7.2 Expression of histidine-tagged proteins and harvesting cells

pHis-parallel 2 plasmids containing soluble *SCO1* variants were transformed into BL21 (DE3) expression cells by electroporation, and plated on 2YT agar plates containing 100 μg/mL ampicillin. A single colony was isolated for each soluble *SCO1* variant, and grown in 5 mL LB starter culture (containing 100 μg/mL ampicillin) at 37°C for 8 hours, at 225 rpm. These starter cultures were then used to inoculate 50 mL of fresh media, and grown overnight at 37°C while shaking at 225 rpm. The overnight cultures were then inoculated into 1 L media in 2 L flasks to ensure proper aeration of the culture. These cultures were grown to an OD_{600} of 0.6-0.8 whereupon 1 mM IPTG and 0.5 mM $CuSO_4$ were added to induce protein expression and provide a source of copper for metallation. Following another 2-3 hours of growth, cells were harvested by centrifugation at 2,000 *x g* for 10 minutes and the cell pellets were stored at -80°C prior to their analysis.

2.7.3 Lysis of cells and protein purification

The first step for cell lysis was to resuspend the cell pellet in 40 mL of lysis/wash buffer (1x phosphate buffered saline (PBS), 10 mM imidazole, 0.2 mM dithiothreitol (DTT), pH7.4). 40 mL of lysis buffer containing 62.5 μM $CuSO_4$ was added to the pellet, which was resuspended by vortexing. The purpose of adding exogenous $CuSO_4$ to the lysate was to ensure full copper-loading of SCO1 variants. Cell suspensions were sonicated on ice for three 30

second pulses, with roughly an 8 minute delay between pulses. The resultant lysate was clarified at 16,000 *x g* for 30 minutes at 4°C to separate the soluble fraction from unbroken cells and cell debris.

The clarified lysates were gently loaded onto home-made Ni-NTA (Qiagen and Novagen) columns with a 3 mL bed volume. Soluble SCO1 variants bound to Ni-NTA were washed with 50 mL of lysis/wash buffer to remove unbound or non-specifically bound protein. Soluble SCO1 mutant proteins were then eluted with 3 x 5 mL of elution buffer (1x PBS, 200 mM imidazole, 0.2 mM DTT, pH 7.4). The Ni-NTA columns were then washed with another 50 mL of lysis/wash buffer to prepare the column for subsequent round of protein purification.

Elution fractions 1 and 2 contained most of purified soluble SCO1, and were therefore pooled and concentrated by centrifugation at 2,000 *x g* for 40 minutes at 4°C.

2.8 Characterization of the copper-binding affinity of SCO1 variants

2.8.1 Total bound copper and Cu(II)

To characterize the Cu(II)-binding affinity of soluble SCO1 variants, purified proteins were dialyzed overnight at 4°C in PBS alone or in PBS containing 1 mM EDTA. All dialyzed samples were scanned using an optical emission spectrometer (Perkin Elmer Optima 7300 DV) and a UV-Vis spectrophotometer (Shimadzu UV-2450) to quantify their total copper content and Cu(II) content, respectively. The Cu(II) content of the samples was measured by monitoring the absorbance at 360 nm using an extinction coefficient of 3100 cm^{-1} M^{-1} (Horng *et al.*, 2005). Total copper content of a sample was quantified by dividing the mM concentration of Cu by the mM protein concentration, while the Cu(II) content was calculated using the following formula; Cu(II) content (M) = A_{360} / (3100 cm^{-1} M^{-1} × 1 cm)

2.8.2 Cu(I)

A bathocuproine disulfonic acid (BCS) assay was used to quantify the Cu(I)-binding affinity of soluble SCO1 variants dialyzed overnight in PBS containing 1mM EDTA. BCS is a Cu(I) chelator that forms a Cu-$(BCS)_2$ complex, which has a maximal absorbance at 483 nm. 100 μL of each purified soluble SCO1 variant was added to a well of a 96-well plate. 100 μL

ddH_2O was also added to the plate, and served as a blank. 100 mM BCS was added to each well, and the A_{483} was measured spectrophotometrically at 0, 30, and 60 minutes and 12 hours. The amount of Cu(I) bound by BCS was calculated using a previously published extinction coefficient 12,250 cm^{-1} M^{-1} (Horng *et al.*, 2005) and the following formula; Cu(I) content of BCS (M) = A_{483} / (12,250 cm^{-1} M^{-1} × 1 cm)

2.9 Protein visualization techniques

2.9.1 Western blotting

For *in vivo* experiments, Western blotting was used to verify that SCO1 variants were overexpressed in transduced human fibroblasts. Cells were washed with 4 mL ice-cold PBS, and harvested by scraping in 1 mL ice-cold 1x PBS. Cells were centrifuged at 14,000 *x g* for 1 minute at 4°C. The cell pellets were kept and resuspended in 1x PBS buffer containing 1x protease inhibitor cocktail (PIC) (Roche) and 0.5 mM phenylmethanesulfonyl fluoride (PMSF). Total protein content of each sample was quantified by Bradford, and the total volume was adjusted to a final protein concentration of roughly 4 μg/uL in PIC/PMSF/PBS containing 1.5% of the non-ionic detergent n-Dodecyl-β-D-maltopyranoside (DDM). Samples were vortexed every 5 minutes during a 30 minute incubation on ice. Samples were then centrifuged at 14,000 *x g* for 10 minutes at 4°C, the protein concentration of the soluble fraction was quantified by Bradford to ensure it was no greater than 4 μg/μL. More concentrated samples were diluted with PIC/PMSF/PBS mix containing 1.5% DDM, and re-extracted as described above.

A 2x sample loading buffer (Bio-Rad) containing 200 mM dithiothreitol (DTT) was added to each sample, which was then incubated at 95°C for 5 minutes. 20 μg of total protein was loaded per lane, and fractionated with a 15% SDS-PAGE gel (sodium dodecyl sulfate polyacrylamide gel electrophoresis) as follows; 90 V for 30 minutes, 110 V for 60 minutes, 130 V for 30 minutes and 150 V for 30 to 60 minutes. Once the dye front reached the bottom of the gel, electrophoresis was discontinued and the gel was transferred to a nitrocellulose membrane under semi-dry conditions. To assess the efficiency of transfer, nitrocellulose was washed with deionized water and stained with ponceau S for 1 minute. The membrane was then incubated in blocking buffer (5% BSA dissolved in Tris buffered saline (TBS) containing 0.1% tween 20) for 24 hours at 4°C, and then incubated overnight at 4°C with an unpurified polyclonal SCO1

antibody (1:200, (Leary *et al.*, 2004)). To remove unbound antibody, the membrane was washed 6 times for 30 minutes with blocking buffer. The membrane was then incubated in goat anti-rabbit secondary antibody (1:5000, Bio-Rad) for 1 hour at room temperature, followed by washing 6 times for 30 minutes with blocking buffer. The membrane was rinsed with regular TBS-T, and then visualized by enhanced chemiluminescence (Cell Signaling). The membrane was subsequently reblocked without stripping and incubated with primary antibodies raised against SDH70, a subunit of Complex II of the mitochondrial respiratory chain, and actin, both of which served as internal loading controls.

2.9.2 Polyacrylamide gel electrophoresis

For *in vitro* experiments, SDS-PAGE was used to track SCO1 during the purification process and verify the relative purity of soluble SCO1 variants in the eluate fractions. Samples from purified soluble SCO1 variants or from each step of the purification process were mixed with an equal volume of 2x sample loading buffer containing 200 mM dithiothreitol (DTT). Samples were then boiled for 5 minutes and loaded onto a 12% SDS-PAGE gel. The gel was run at 90 V for 30 minutes until the samples entered the resolving phase, whereupon the voltage was increased to 120 V. When the dye front reached the bottom of the gel, electrophoresis was discontinued and protein was visualized by staining the gel with Coomassie Brilliant Blue R250 for 1 hour at room temperature. The gel was then incubated in destaining solution (50% methanol and 10% acetic acid) with gentle agitation until the unbound dye was removed from the gel and the proteins were clearly visible.

3 RESULTS

3.1 Characterization of the phenotypic effect of overexpressing N-terminal SCO1 mutants in control and patient fibroblasts on COX activity

To investigate the significance of the N-terminal domain of SCO1 to its function as a COX assembly factor, I overexpressed three cysteine to alanine point mutants (Cys45, Cys61 and Cys45Cys61) and three truncation mutants (Δ17, Δ37 and Δ57) lacking the indicated amino acids within the N-terminus of the protein in control and *SCO* patient fibroblasts. Wild-type SCO1 was included in these analyses as a positive control. If SCO1 interacts with an inner membrane Cu(I) transporter that moves the metal ion from the matrix to the IMS, it is reasonable to predict that the matrix-localized cysteines of SCO1 may be involved since cysteine is a common, copper-binding amino acid (Giri *et al.*, 2004). Overexpression of all three SCO1 cysteine to alanine variants functionally complemented the COX deficiency in *SCO1-1* patient backgrounds, and acted as a dominant-negative in *SCO2* patient backgrounds, similar to wild-type SCO1 (Figure 3.1). These data argue that Cys45 and Cys61 are not essential for SCO1 function. Overexpression of the N-terminal truncation mutants of SCO1 showed that Δ17 and Δ37 also rescued the COX deficiency in *SCO1-1* patient fibroblasts, while residual COX activity was unaltered by overexpression of Δ57 (data not shown). However, Western blot analysis of these truncated variants revealed multiple SCO1-specific immunoreactive bands (Figure 3.2) whose molecular weight could not be reconciled with the *in silico* prediction of a cleavable mitochondrial targeting sequence of ~40 residues at the N-terminus of the protein. These data suggest that our truncation mutants perturb elements within the N-terminus of SCO1 that are critical to its mitochondrial targeting and subsequent processing of the precursor protein.

3.2 Characterization of the phenotypic effect of overexpressing C-terminal SCO1 mutants in control and patient fibroblasts on COX activity

To address whether the copper contained within COX II originates from SCO1, a series of C-terminal *SCO1* variants with point mutations in copper-coordinating amino acids were generated. As Cys169, Cys173, and His260 are all required for the coordination of Cu(I) and Cu(II) (Balatri *et al.*, 2003), we substituted these three amino acids with the known copper-

binding amino acids cysteine, methionine or histidine (Giri *et al.*, 2004) or with serine, which is incapable of copper-binding. Eight C-terminal *SCO1* variants were generated, and included two histidine mutants (H260C, H260M), and six cysteine mutants (C169HC173H, C169SC173S, C169H, C173H, C169S and C173S). We hypothesized that these point mutations would either abolish or enhance the ability of SCO1 to bind copper. Variants with cysteine to serine substitutions, along with wild-type *SCO1* and *SCO1* H260A and *SCO1* C169AC173A (Horng *et al.*, 2005), were included in these analyses as internal controls.

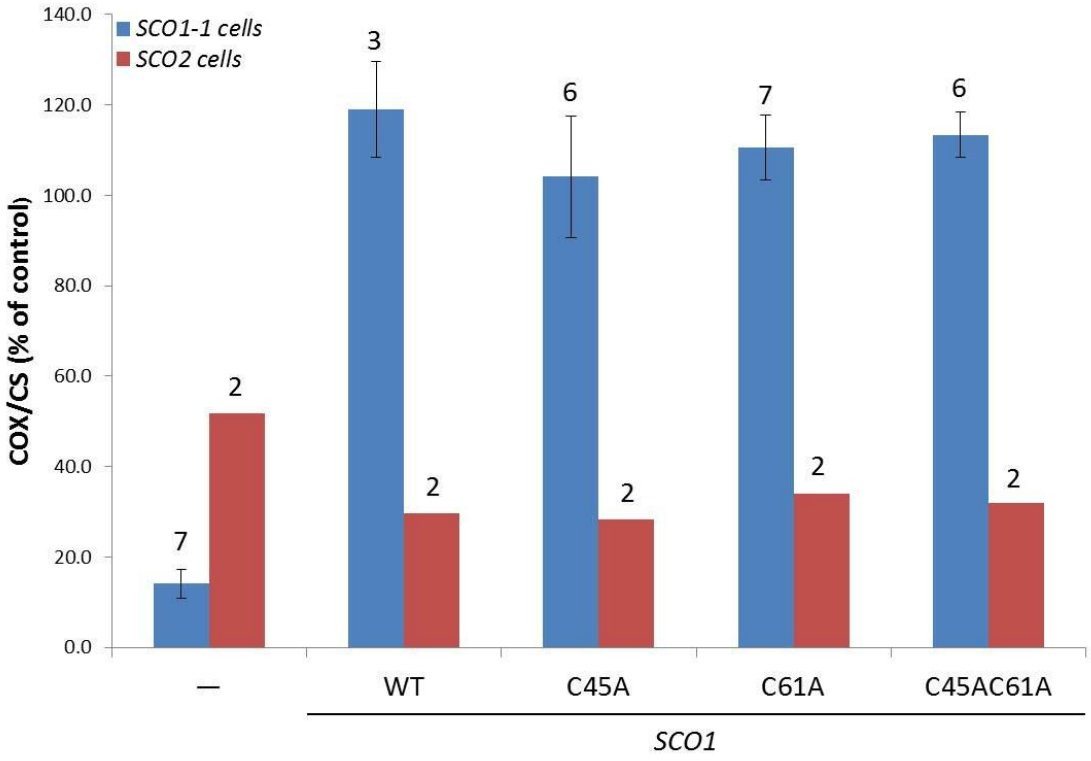

Figure 3.1 COX/CS in SCO1-1 and SCO2 patient fibroblasts alone (-) or overexpressing WT and N-terminal cysteine to alanine point mutants of SCO1. Control, *SCO1-1* and *SCO2* patient fibroblasts were transduced with retroviral vectors containing cDNAs encoding the following SCO1 mutants: *C45A*, *C61A* and *C45AC61A SCO1*. Wild-type SCO1 was included as a positive control in these analyses. The activity of COX was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and reported as a percentage of COX/CS in control cells. The number of replicates for each experimental group is indicated above the mean +/- the standard error.

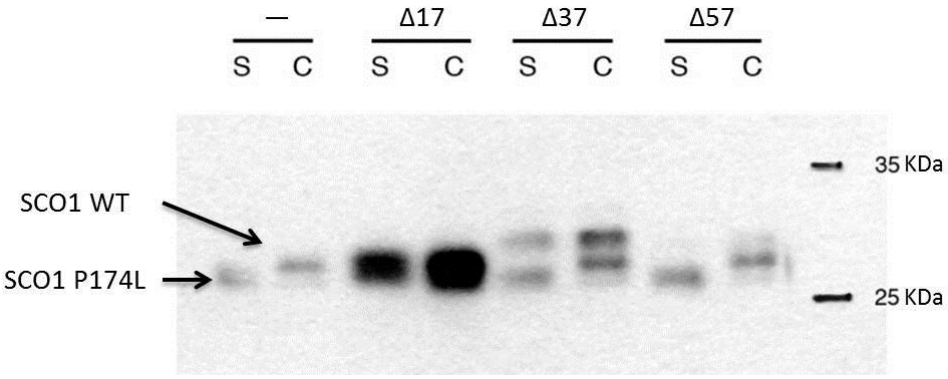

Figure 3.2 Western blot analysis of SCO1 expression in control and SCO1-1 patient fibroblast alone (-) or overexpressing SCO1 N-terminal deletion mutants. Protein extracts were prepared from control (C) and *SCO1-1* patient fibroblasts (S) alone (-) or those stably expressing one of 3 N-terminal deletion mutants of SCO1 (Δ17, Δ37 and Δ57), and separated using a 15% SDS-PAGE gel. The resultant membrane was then immunoblotted with SCO1 antiserum. Arrows indicate the bonafide wild-type (WT) and mutant (P174L) SCO1 proteins. The relevant molecular weight markers are shown on the right hand side.

Initial attempts to generate stably transduced cell lines that expressed SCO1 variants were severely constrained by very low transduction efficiencies. To evaluate whether this was caused by the fact we were using a first generation retroviral expression vector (pLXSH) (Miller *et al.*, 1993), all *SCO1* variants were subcloned into a third generation retroviral vector (pMys-puromycin) (Kitamura *et al.*, 2003). Although a significant improvement in the transduction efficiency was observed with the pMys-puromycin expression vector, the expression levels of SCO1 variants were inconsistent and very low, and the data from downstream biochemical analyses were highly variable (data not shown). Therefore, only data generated using the pLXSH retroviral expression vectors is presented herein.

Control human fibroblasts used in my M.Sc. thesis studies contain two wild-type alleles of both *SCO1* and *SCO2*. As shown in Figure 3.3A, COX activity was expressed as a function of citrate synthase (CS) activity for each data point (i.e. COX/CS), and normalized by expressing it as a percentage of the median control value in untransduced cells. Although the abundance of all SCO1 variants in stably transduced cells was significantly higher than that of endogenous SCO1 in control cells alone (-) (Figure 3.3B), COX activity was unchanged.

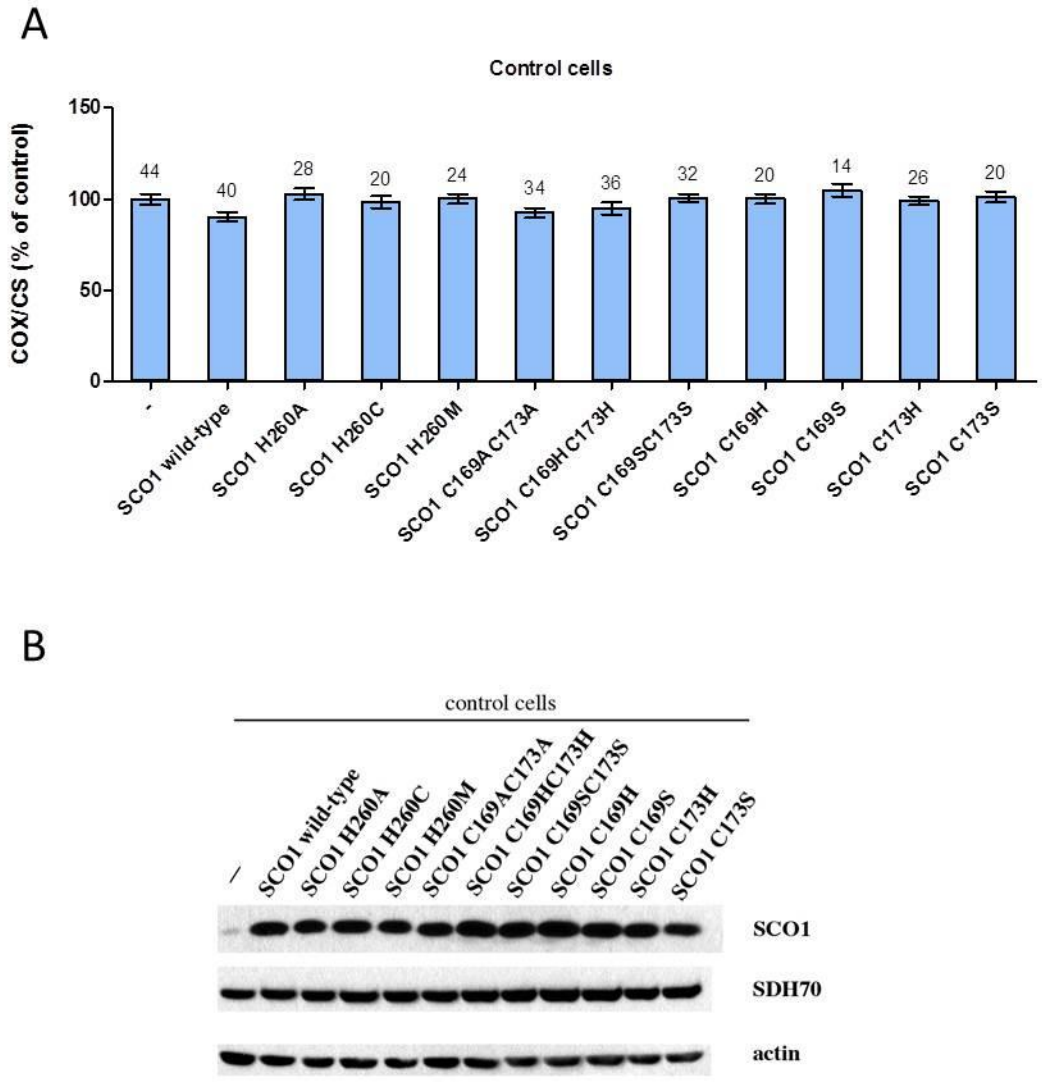

Figure 3.3 Phenotypic effect of overexpressing SCO1 variants on COX activity in control fibroblasts. **A.** Control fibroblasts stably expressing a given SCO1 variant were generated via their retroviral transduction and subsequent drug selection. The COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in baseline cells (-) which had not been transduced with retrovirus. The number of replicates for each experimental group is indicated above the mean +/- the standard error. **B.** Western blot analysis of the steady-state levels of SCO1 in control fibroblasts alone (-) and in those overexpressing a given SCO1 variant. SDH70, a subunit of Complex II of the mitochondrial respiratory chain, and actin served as internal loading controls.

SCO1-1 and *SCO1-2* patient fibroblasts utilized in this study are from different pedigrees and harbor different point mutations (Leary *et al.*, 2013a; Valnot *et al.*, 2000a). The P174L variant expressed in *SCO1-1* patient fibroblasts significantly impairs the ability of SCO1 to interact with COX17, and produces a very severe COX deficiency because the mutant protein cannot be copper loaded (Banci *et al.*, 2007b; Cobine *et al.*, 2006b). In contrast, expression of the M294V variant in *SCO1-2* cells is associated with a milder COX deficiency, even though the steady-state levels of the mutant protein are negligible (Leary *et al.*, 2013a). While the COX deficiency in both *SCO1* backgrounds was rescued by overexpressing a wild-type *SCO1* cDNA, expression of C-terminal *SCO1* variant cDNAs failed to functionally complement the biochemical defect (Figures 3.4, 3.5). Overexpression of SCO1 C169SC173S further reduced residual COX activity in *SCO1-1* patient fibroblasts (Figure 3.4), although the effect did not reach statistical significance (P=0.0609). In contrast, COX activity was significantly reduced to an equivalent degree in *SCO1-2* patient fibroblasts upon overexpression of all SCO1 variants (Figure 3.5). These data collectively suggest that all variants have the same effect on SCO1 function.

SCO2 patient fibroblasts utilized in this study are compound heterozygotes, carrying an E140K missense mutation on one allele and a nonsense mutation (R93X) on the other allele (Papadopoulou *et al.*, 1999). *SCO2* patient cells also have a severe, isolated COX deficiency (Papadopoulou *et al.*, 1999), which is attributable to very low expression levels of the mutant protein (Leary *et al.*, 2004). Similar to control and *SCO1-1* cells, overexpression of C-terminal SCO1 variants did not have any effect on residual COX activity in *SCO2* patient cells (Figure 3.6).

To confirm that the effect on COX activity only depends on overexpression of SCO1 variants, and was not affected by the endogenously expressed mutant SCO protein in patient cells, we conducted complementary analyses in control, *SCO1-1* and *SCO2* patient cells in which *SCO1* expression had been stably knocked down using an shRNA that targets the 3'UTR of the *SCO1* mRNA (Leary *et al.*, 2007). Identical results were obtained (Figure 3.7 – 3.10), with COX activity remaining unchanged upon overexpression of SCO1 variants in control (Figure 3.7), *SCO1-1* (Figure 3.8) and *SCO2* cells (Figure 3.10), or being significantly reduced in *SCO1-2* patient cells (Figure 3.9).

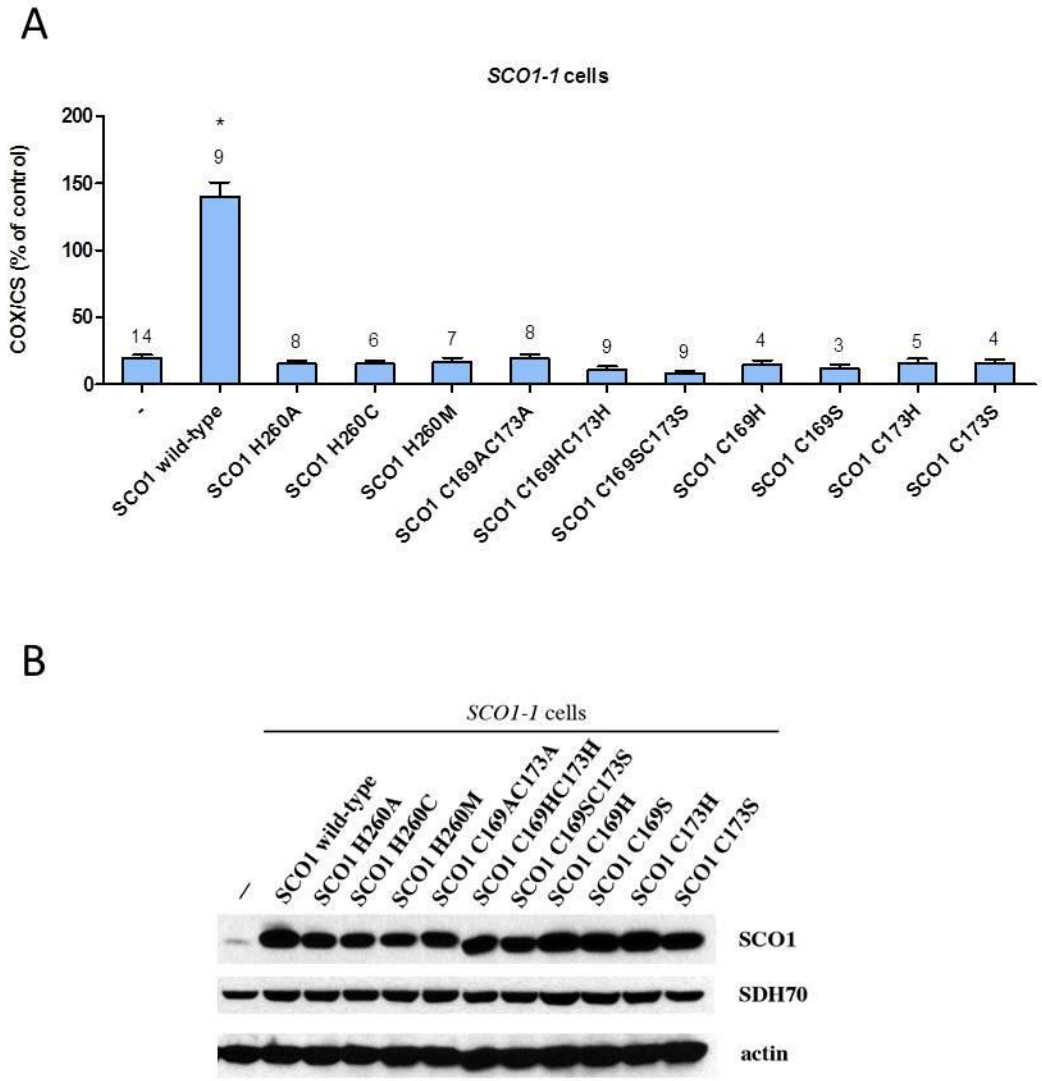

Figure 3.4 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO1-1 patient fibroblasts. **A.** *SCO1-1* patient fibroblasts stably expressing a given SCO1 variant were generated via their retroviral transduction and subsequent drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in matched control cells. The number of replicates for each experimental group is indicated above the mean +/- the standard error. Statistical analysis identified a significant difference in COX activity between untransduced patient fibroblasts and those overexpressing wild-type SCO1 (*, $p<0.0001$). **B.** Western blot analysis of the steady-state levels of SCO1 in *SCO1-1* patient fibroblasts alone (-) and in those overexpressing a given SCO1 variant. SDH70, a subunit of Complex II of the mitochondrial respiratory chain, and actin served as internal loading controls.

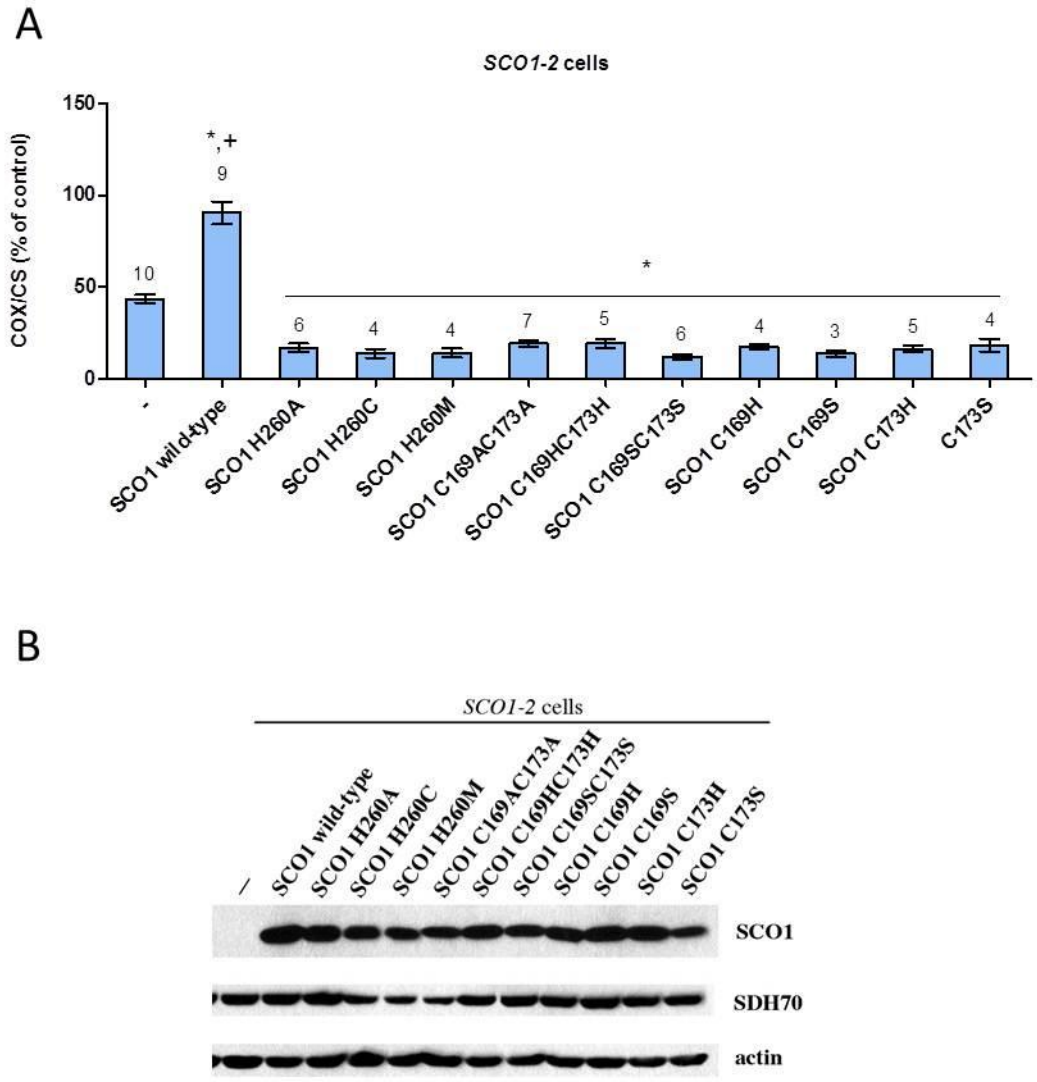

Figure 3.5 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO1-2 patient fibroblasts. **A.** *SCO1-2* patient fibroblasts stably expressing a given SCO1 variant were generated via their retroviral transduction and subsequent drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in matched control cells. The number of replicates for each experimental group is indicated above the mean +/- the standard error. Statistical analysis identified a significant difference in COX activity between untransduced patient fibroblasts and those overexpressing each SCO1 variant (*, $p<0.001$). COX activity was also significantly different in patient fibroblasts overexpressing wild-type SCO1 when compared to those overexpressing all other SCO1 variants (+, $p<0.001$). **B.** Western blot analysis of the steady-state levels of SCO1 in *SCO1-2* patient fibroblasts alone (-) and in those overexpressing a given SCO1 variant. SDH70, a subunit of Complex II of the mitochondrial respiratory chain, and actin served as internal loading controls.

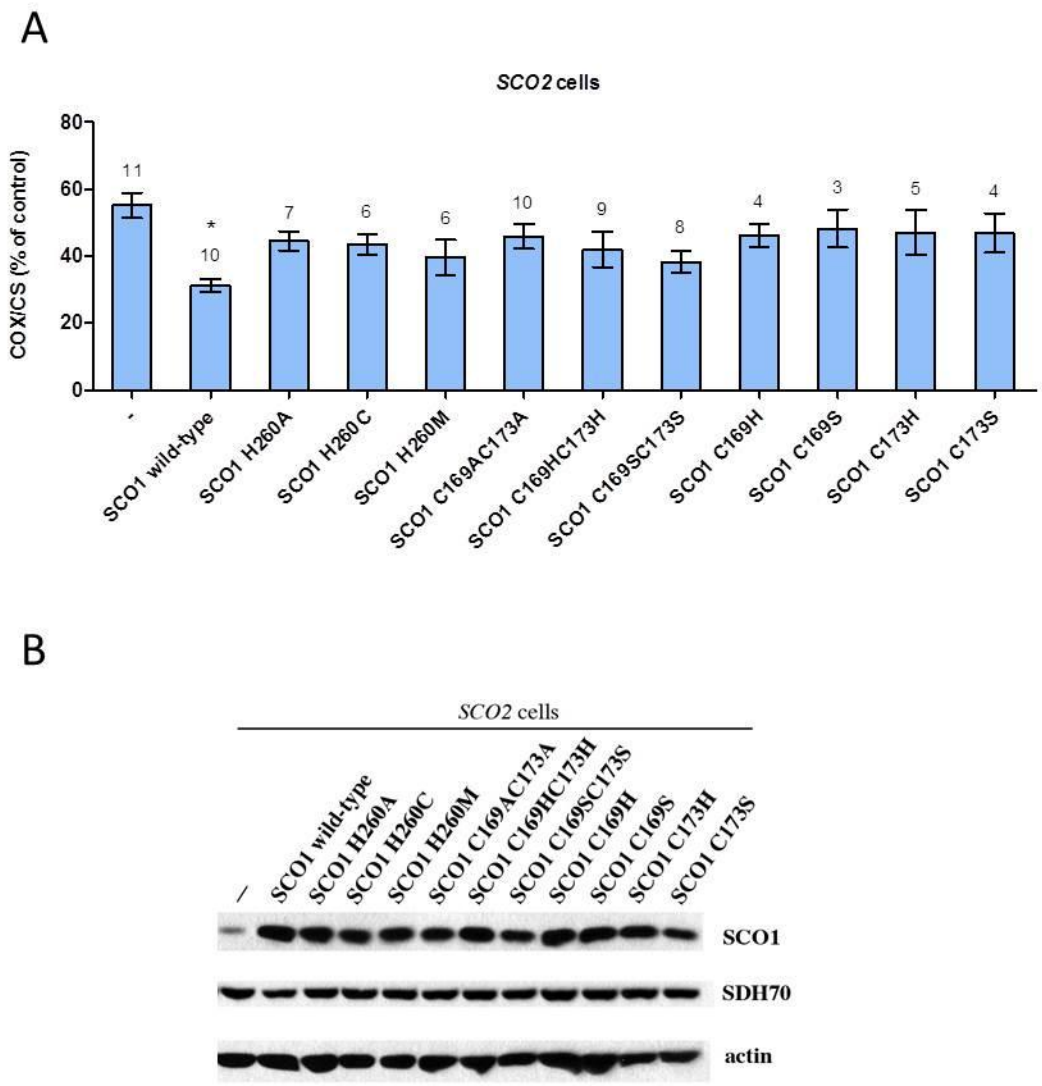

Figure 3.6 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO2 patient fibroblasts. **A.** *SCO2* patient fibroblasts stably expressing a given SCO1 variant were generated via their retroviral transduction and subsequent drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in matched control cells. The number of replicates for each experimental group is indicated above the mean +/- the standard error. Statistical analysis identified a significant difference in COX activity between untransduced patient fibroblasts and those overexpressing wild-type SCO1 (*, $p<0.0001$). **B.** Western blot analysis of the steady-state levels of SCO1 in *SCO2* patient fibroblasts alone (-) and in those overexpressing a given SCO1 variant. SDH70, a subunit of Complex II of the mitochondrial respiratory chain, and actin served as internal loading controls.

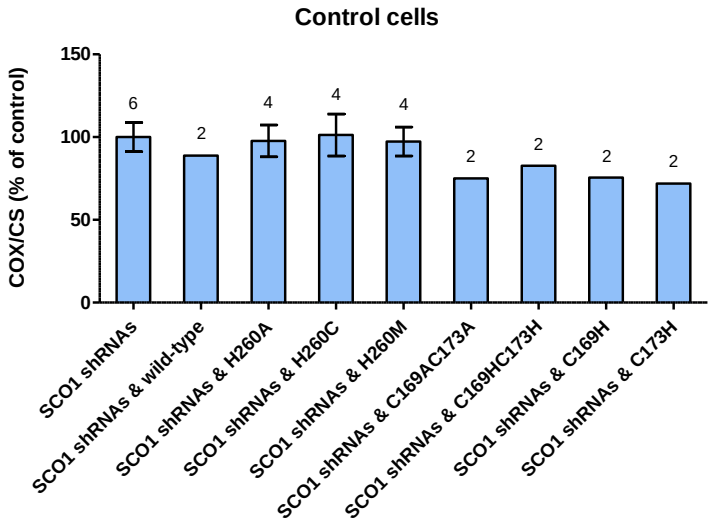

Figure 3.7 Phenotypic effect of overexpressing SCO1 variants on COX activity in control fibroblasts with SCO1 knockdown. Control fibroblasts stably expressing a *SCO1* shRNA were retroviral transduced with a given SCO1 variant followed by dual drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in control cells which had not been transduced with retrovirus. The number of replicates for each experimental group is indicated above the mean +/- the standard error.

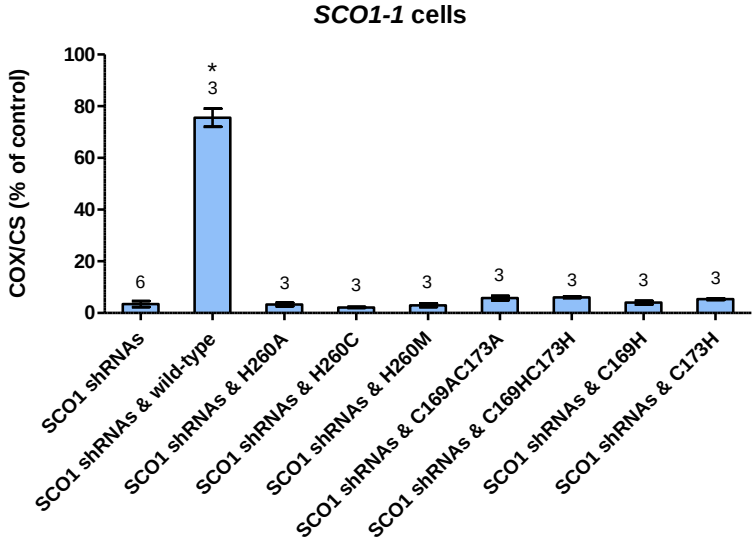

Figure 3.8 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO1-1 patient fibroblasts with SCO1 knockdown. *SCO1-1* patient fibroblasts which stably expressing a *SCO1* shRNA were retroviral transduced with a given SCO1 variant, followed by dual drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of the average value in matched control cells. The number of replicates for each experimental group is indicated above the mean +/- the standard error.

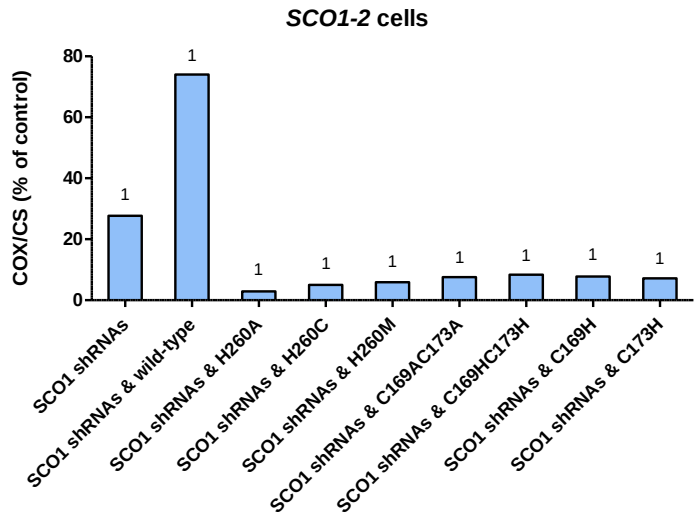

Figure 3.9 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO1-2 patient fibroblasts with SCO1 knockdown. *SCO1-2* patient fibroblasts stably expressing a *SCO1* shRNA were retroviral transduced with a given SCO1 variant, followed by dual drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS), and expressed as a percentage of average value in matched control cells. The number of replicates for each experimental group is indicated above the mean.

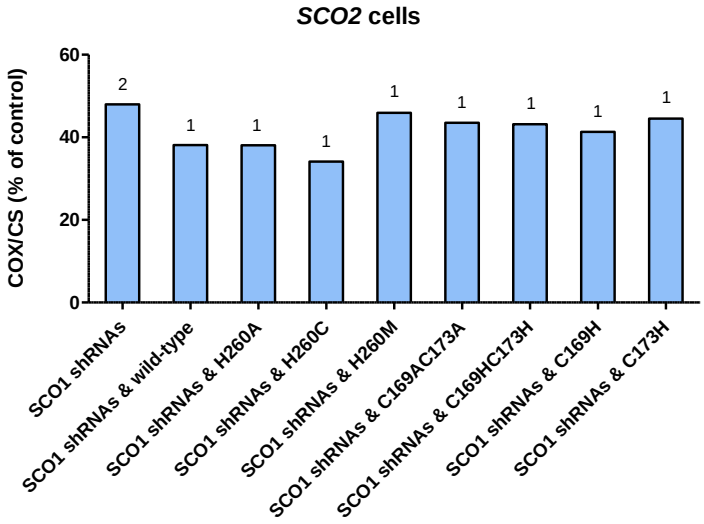

Figure 3.10 Phenotypic effect of overexpressing SCO1 variants on COX activity in SCO2 patient fibroblasts with SCO1 knockdown. *SCO2* patient fibroblasts stably expressing a *SCO1* shRNA were retroviral transduced with a given SCO1 variant, followed by dual drug selection. COX activity in these cell lines was measured spectrophotometrically, normalized to CS activity (i.e. COX/CS) and expressed as a percentage of average value in matched control cells. The number of replicates for each experimental group is indicated above the mean.

3.3 Characterization of the copper binding properties of soluble C-terminal SCO1 mutants

Because a SCO1 point mutant that cannot bind copper would be predicted to have the same effect on COX assembly as one that binds copper too tightly to transfer it to COX II, I decided to examine the copper-binding properties of these C-terminal SCO1 mutants *in vitro*. Soluble *SCO1* variants were generated that lacked the N-terminal mitochondrial targeting sequence and the single transmembrane helix (lacking the first 111 amino acids), to avoid the inherent difficulty associated with membrane protein purification. These soluble SCO1 variant proteins were fused with a 6x histidine-tag, purified on a Ni-NTA column and dialyzed in PBS in the presence or absence of EDTA using an established protocol (Horng *et al.*, 2005). Wild-type SCO1 and a H260A mutant served as a positive and negative control, respectively, for these analyses, because their copper content following dialysis in the absence or presence of EDTA, a Cu(II) chelator, has previously been reported (Horng *et al.,* 2005). Unlike the previous study (Horng *et al.,* 2005), the H260A mutant I purified and dialyzed in PBS alone bound much less copper (0.4 vs 0.7 mol. Eq.) (Figure 3.11). While I achieved a comparable result when the wild-type protein was dialyzed in PBS alone (0.8 vs 1 mol. Eq.), its copper content in two of the three purifications was significantly lower than the published value (0.8±0.6, n=3). The low total amount of bound copper may be explained by several factors. First, there may not have been enough copper ions present during SCO1 synthesis, particularly in the form of Cu(I). Second, other divalent metal ions like nickel may be occupying the copper-binding site. Third, the purified SCO1 protein preparation may be contaminated with other proteins. I therefore carried out several experiments to investigate whether any of these factors was adversely affecting my results.

3.3.1 The effect of copper supplementation on the copper-binding properties of SCO1

An *E. coli* system was used to produce a large quantity of each soluble SCO1 variant because it offers a method of inducing high levels of protein expression (Brondyk, 2009). There are two possible ways in which copper may have been limiting to SCO1 during its folding; there was not enough copper in the media at the time of induction, or copper uptake by *E. coli* from the media was impaired. Copper uptake is tightly regulated in *E. coli*, and its

cytoplasm contains nearly no free copper and lacks copper-binding proteins (Rensing and Grass, 2003). Although the addition of copper to a bacterial culture can be used to overcome copper limitation in *E. coli*, it also slows down and can even inhibit bacterial growth (Grey and Steck, 2001). To determine the optimal concentration of copper required in the growth media in relation to induction of SCO1 expression and its subsequent metallation, I varied the amount of IPTG and $CuSO_4$ in cultures expressing wild-type SCO1. The data showed that the total amount of copper bound to the wild-type protein was not improved by changing the concentration of either $CuSO_4$ or IPTG (data not shown), suggesting that the amount of copper in the bacterial culture was not limiting to the metallation of SCO1.

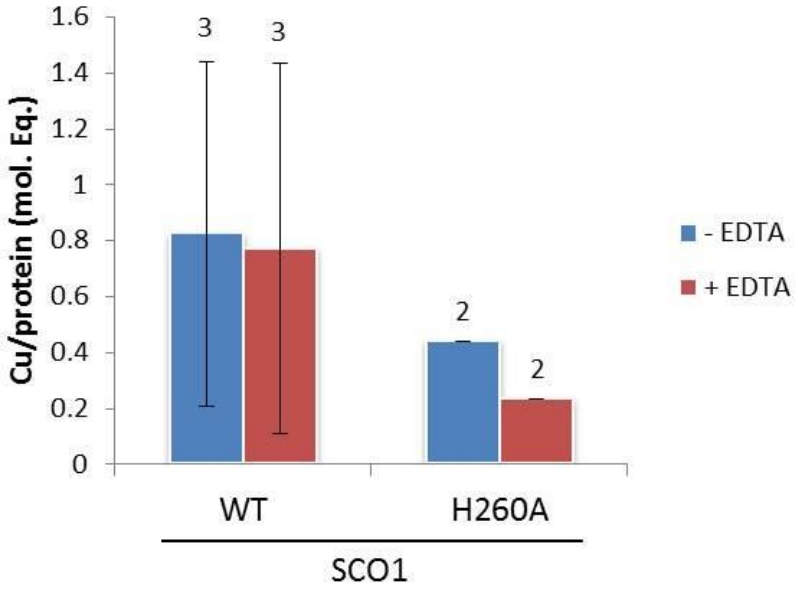

Figure 3.11 The total amount of copper bound by wild-type SCO1 and the H260A mutant purified from E. coli. Soluble SCO1 variants were overexpressed in *E. coli* and purified using a Ni-NTA column. Eluates were dialyzed overnight at 4°C in PBS with or without 1 mM EDTA. The copper content of wild-type SCO1 and the H260A mutant was measured by UV-vis spectroscopy. The number of replicates for each experimental group is indicated above the mean, and standard errors are presented for the wild-type isolates.

Next, to circumvent the possibility of restricted copper uptake by the *E. coli* culture, copper was added to the cell pellet at the point of lysis. However, I observed that even the addition of small micromolar concentrations of copper to the cell lysate resulted in non-specific copper-binding. This situation is illustrated by the SCO1 C169AC173A mutant, which has

previously been shown to be an apo-protein (Horng *et al.*, 2005), yet here clearly contains copper after its purification from a copper supplemented lysis step (Figure 3.12). One of the most likely causes of this non-specific copper-binding is the 6x histidine-tag fused at the N-terminus of these SCO1 variants.

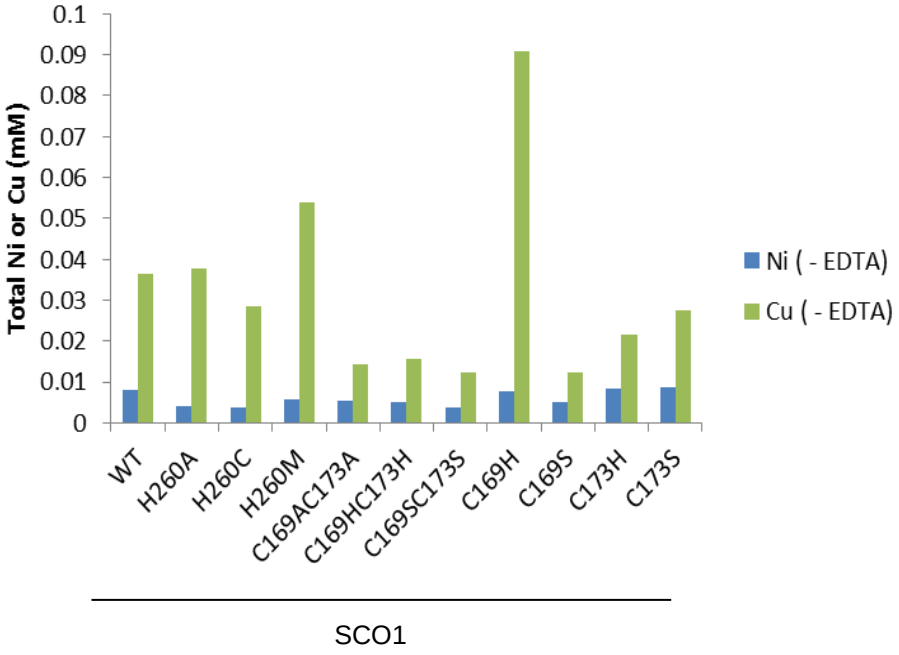

Figure 3.12 Total amounts of Ni and Cu bound by soluble SCO1 C-terminal variants dialyzed in PBS lacking EDTA. Soluble SCO1 variants were overexpressed in *E. coli* and purified using a Ni-NTA column. Eluates were dialyzed overnight in PBS alone at 4°C. Total amounts of bound Ni and Cu bound were measured post-dialysis by ICP-OES.

3.3.2 Determination of metal ion contamination in purified SCO1

SCO1 is able to bind both Cu(I) and Cu(II) (Horng *et al.*, 2005), indicating that SCO1 might have affinity for other metal ions with the same valencies. To avoid metal contamination, I therefore restricted the metal source at all levels of protein purification. As shown in Figure 3.12, SCO1 variants were still contaminated with low levels of Ni(II), which most likely came from the Ni-NTA column during protein purification. No other metal ions were detected in the purified protein samples.

3.3.3 Determination of the relative purity of isolated SCO1 variants

The purity of the recombinant SCO1 preparation might also be contributing to the low mol. Eq. of bound copper we calculated for the protein. To investigate this possibility, fractions were collected at each step of the expression and purification process (detail of purification process is included in section 2.6.3). The majority of non-specific proteins were in the flow through and little residual protein was detected at the wash step (Figure 3.13). However, elution fraction 1 was significantly contaminated compared to elution fraction 2. Since both elution fractions were pooled for subsequent copper measurements, these non-specific proteins contribute to the total protein concentration of a given sample and therefore to a lower mol. Eq. of copper bound to each SCO1 molecule.

3.3.4 Quantification of total copper bound to soluble SCO1 C-terminal variants

To minimize the effect of contaminating Ni, the amount of residual bound Ni was subtracted from the total amount of bound copper in the pooled eluate, and the corrected copper values for each soluble SCO1 variant were expressed as a percentage of that for the wild-type protein (Figure 3.14). After dialysis against EDTA, the SCO1 C169H mutant retained the most copper, and bound roughly 2 times more copper than the wild-type protein. In contrast, the other two SCO1 cysteine to histidine variants (C173H and C169HC173H) bound less than 20% of the copper detected in the wild-type protein, suggesting that copper coordination by Cys169 and Cys173 may be different. The three cysteine to serine mutants (C169SC173S, C169S and C173S) abolished copper-binding, and after dialysis each variant retained less than 30% of its original copper complement. None of the histidine point mutants increased the ability of SCO1 to bind copper when compared to the wild-type protein.

Consistent with previous data (Horng *et al.*, 2005), purified wild-type SCO1 exhibited a chromophore in the visible region of the absorption spectrum with maxima at 360 and 480 nm (data not shown). This absorption spectrum reflects the presence of bound Cu(II) ions in SCO1 (Basumallick *et al.*, 2005; Horng *et al.*, 2005; Lieberman *et al.*, 2001). However, there was no corresponding chromophore found in the SCO1 C169H mutant, strongly suggesting that this mutant protein preferentially binds Cu(I) ions.

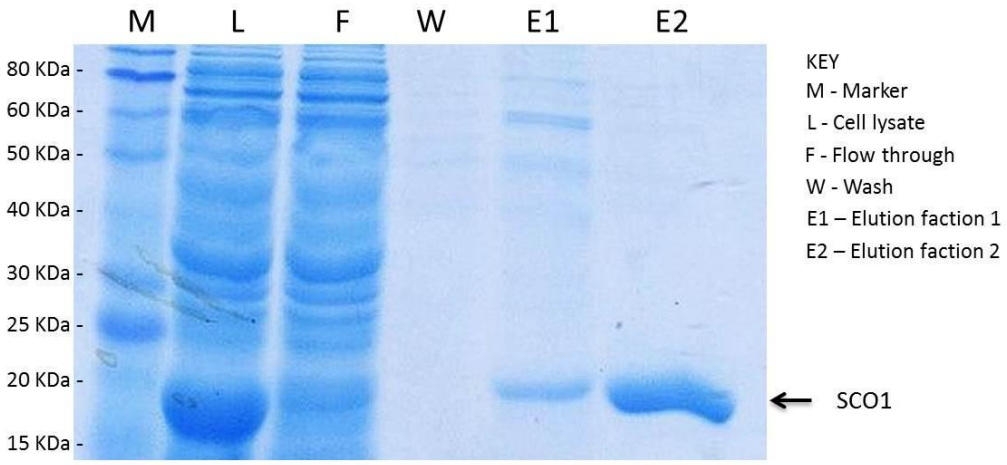

Figure 3.13 Expression and purification scheme for soluble SCO1 truncates. After IPTG induction, cells were harvested, lysed and loaded onto a Ni-NTA column. Fractions were collected at each step of the expression and purification process, denatured and separated on a 12% SDS-PAGE gel. A representative gel was stained with coomassie brilliant blue. The arrow denotes the soluble SCO1 truncate (details regarding the purification process are included in section 2.6.3).

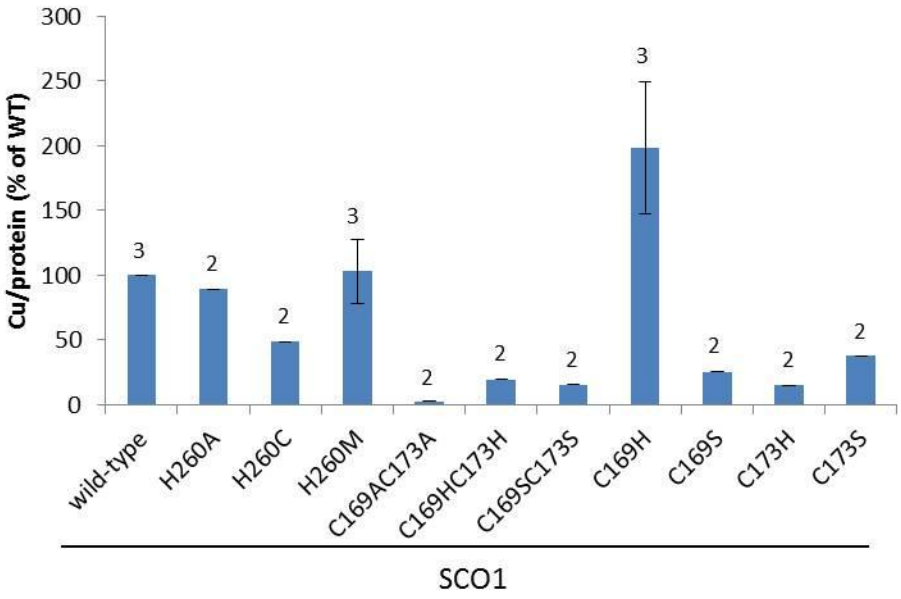

Figure 3.14 The total amount of copper bound by soluble SCO1 variants purified from E. coli. The copper content of soluble SCO1 variants was measured by UV-vis spectroscopy, corrected for residual bound Ni, and expressed as a percentage of wild-type SCO1. The number of replicates for each experimental group is indicated above the mean +/- the standard deviation.

4 DISCUSSION

4.1 Evaluating the significance of the N terminus to SCO1 function

In this section of my thesis studies, I investigated a possible role for the N-terminus of SCO1 in copper transport across the inner membrane for its eventual delivery to COX II. I found that the two cysteine residues (Cys45 and Cys61) within the N-terminus of SCO1 are dispensable, because single or double cysteine to alanine point mutants (SCO1 C45A, C61A and C45AC61A) behaved like the wild-type protein, rescuing the COX deficiency in *SCO1* patient fibroblasts and exacerbating the COX deficiency in *SCO2* patient fibroblasts. To further explore the importance of the N-terminus of SCO1 to protein function, I analyzed three truncation mutants of SCO1 in which the N-terminus was progressively shortened (Δ17, Δ37 and Δ57) following the predicted mitochondrial targeting sequence. Although SCO1Δ17 and SCO1Δ37 functionally complemented the COX deficiency in *SCO1* patient fibroblasts, again arguing that this region of the protein is dispensable for its function, Western blot analysis revealed multiple immunoreactive bands specific to SCO1. These bands could not be rationally explained based on the predicted cleavage site of the targeting sequence, and implied that our truncation mutants adversely affected protein import and processing. Consistent with this idea, another M.Sc. student in the lab (Aren Boulet) has since determined empirically by mass spectrometry that the targeting sequence is much larger than the one predicted *in silico*, and is found at amino acid position 69. Thus, my findings do not provide any support for the previous proposal that the matrix-localized, N-terminal tail of SCO1 has evolved to provide the protein with features unique from SCO2 that are critical to its function (Leary *et al.*, 2004).

4.2 Functional characterization of C-terminal SCO1 mutants

It is not experimentally trivial to establish that one or both SCO proteins physically transfers copper to COX II *in vivo*. Because SCO1 functions downstream of SCO2 during COX assembly (Leary *et al.*, 2007; Leary *et al.*, 2009), which suggests it is most likely to catalyze the metallation of COX II, my M.Sc. thesis studies focused on mutating its copper-binding residues (Cys169, Cys173, His260). My goal was to generate a protein variant that bound copper with greater affinity than the wild-type protein, thus preventing it from transferring the copper to COX II, an effect we predicted would impair the ability to assemble

COX. My functional characterization of all SCO1 mutants indicates that their ability to function as a COX assembly factor was severely impaired. In fact, SCO1 variants with mutations in any of these three amino acids, alone or in combination, failed to rescue the COX defect when overexpressed in *SCO1* fibroblasts, and even exacerbated the COX deficiency in cells from one of the two *SCO1* pedigrees.

The importance of the three Cu(I)-binding amino acids has been shown in several studies (Lode *et al.*, 2000; Nittis *et al.*, 2001). In yeast, alanine substitutions of any of these residues negated the ability of SCO1 to complement the growth of the *SCO1*Δ strain (Nittis *et al.*, 2001). In *SCO1-1* human fibroblasts, overexpressing either SCO1 H260A or C169AC173A mutant proteins also failed to rescue the COX deficiency, and the ability of the H260A variant to bind copper was severely perturbed (Horng *et al.*, 2005). Therefore, it is reasonable to conclude that alanine substitutions of these three residues abrogate the ability of SCO1 to bind copper, and negatively affect its function. Serine is not able to bind copper, although it is structurally similar to cysteine. As expected, the three SCO1 mutants with serine substitutions (C169S, C173S and C169SC173S) generated for our experiments were not able to complement the COX defect in *SCO1* patient fibroblasts and did not exacerbate the COX deficiency in *SCO2* patient fibroblasts. Other mutants (H260C, H260M, C169H, C173H and C169HC173H) that we rationalized might have increased binding affinities for copper also failed to rescue the COX deficiency in either *SCO1* patient background. The inability of these SCO1 variants to functionally complement the COX deficiency was not attributable to a destabilizing effect, as Western blot analysis demonstrated that all mutants were robustly expressed in control and patient cells. Because SCO1 functions as a homodimer *in vivo* (Leary *et al.*, 2004), it is possible that the effect of SCO1 variants on COX activity was being titrated by allelic complementarity upon their oligomerization with endogenous, wild-type or mutant SCO1 protein in control and *SCO* patient fibroblasts. To address this possibility, I stably knocked down endogenously expressed *SCO1* by targeting the 3' UTR of its mRNA and overexpressed a cDNA encoding each *SCO1* variant of interest. The fact that I obtained the same results argues that the ability of SCO1 point mutants with cysteine or histidine substitutions to affect COX assembly was not being counteracted by the presence of endogeous SCO1 protein. However, these experiments did not allow me to distinguish between a mutation that adversely

affects copper binding by altering the charge or the space of the binding pocket, from one that enhanced copper binding affinity and in turn prevented metal transfer to COX II.

The M294V mutation of SCO1 carried by the *SCO1-2* patient fibroblasts has a modest effect on the ability of SCO1 to function as a COX assembly factor, when compared to the P174L mutant expressed in *SCO1-1* patient cells (Leary *et al.*, 2013a). Fibroblasts from patient *SCO1-1* exhibit a reduction in fully assembled COX and an increased accumulation of the S2 assembly intermediate (Leary *et al.*, 2004). The P174L substitution is adjacent to the CxxxC motif of SCO1, and structural and biochemical studies argue that this mutation significantly impairs the ability of SCO1 to interact with COX17 (Banci *et al.*, 2007b; Cobine *et al.*, 2006b). Patient cells harbouring M294V substitution have higher residual levels of COX activity, and do not accumulate the S2 intermediate (Leary *et al.*, 2013a). Immunoblot analysis of *SCO1-1* and *SCO1-2* fibroblasts showed that the abundance of the M294V mutant was much lower than that of the P174L mutant (Leary *et al.*, 2007; Leary *et al.*, 2004), implying that the pathology of M294V mutation is most likely caused by protein destabilization. Consistent with the M294V mutation being a relatively milder substitution, its overexpression completely rescued the COX deficiency in both *SCO1* pedigrees (Leary *et al.*, 2013a), while P174L SCO1 partially restored COX activity in *SCO1-1* patient cells. Our analysis of copper-binding mutants further supports this idea; while residual COX activity was not altered by overexpressing any of the C-terminal SCO1 mutants in the *SCO1-1* patient background, it was further reduced in *SCO1-2* patient fibroblasts. Since SCO1 functions as a homodimer (Leary *et al.*, 2004), overexpressing a non-functional form of the protein would be predicted to have a greater effect in *SCO1-2* patient cells which express very little residual SCO1 M294V mutant, compared to *SCO1-1* patient cells which express reasonable amounts of largely non-functional SCO1 P174L.

Control fibroblasts contain wild-type levels of SCO1 and SCO2. Stable knockdown of SCO1 to 15-20% of its levels in parental cells does not affect COX content, indicating that only a small fraction of the total protein pool is required to promote COX assembly (Leary *et al.*, 2007). Consistent with this idea and previously published data (Leary *et al.*, 2004), overexpression of SCO1 variants in control cells failed to alter COX activity. Since these transduced control fibroblasts have a mixed population of endogenous, wild-type SCO1 and exogenous mutant protein, we envision that a sufficient number of homodimers of wild-type SCO1 remain in these cells to allow for normal rates of COX assembly.

COX activity is severely decreased in *SCO2* patient backgrounds upon the overexpression of wild-type SCO1 or SCO1 P174L (Cobine *et al.*, 2006b; Leary *et al.*, 2004). Although the underlying mechanism(s) remains unknown, there are several possibilities that may explain this observation. First, COX17 is required to load both SCO1 and SCO2 with copper (Banci *et al.*, 2008a; Banci *et al.*, 2007b; Horng *et al.*, 2004), and its ability to metallate the limiting amounts of mutant SCO2 may be compromised by overexpressing SCO1. Second, while endogenously expressed SCO1 and SCO2 are found as homodimers *in vivo* (Leary *et al.*, 2004), overexpression of SCO1 may result in the formation of unproductive SCO1/SCO2 heterodimers. Such an effect would be particularly deleterious to COX assembly in *SCO* patient backgrounds that express very low levels of mutant SCO protein. Unexpectedly, none of the SCO1 variants generated for my thesis work were capable of producing an equivalent phenotype. Thus as with control fibroblasts, it is likely that *SCO2* patient fibroblasts express enough wild-type SCO1 to buffer against the expression of non-functional SCO1 variants.

4.3 Determination of the copper-binding properties of C-terminal SCO1 mutants

Because our *in vivo* studies could not distinguish between a mutant SCO1 protein with impaired copper-binding properties from one that binds copper too tightly to transfer it to COX II, we next sought to directly investigate the copper-binding properties of C-terminal SCO1 variants *in vitro*. These analyses showed that the SCO1 C169H mutant binds significantly more Cu(I) than the wild-type protein. The fact that the other two cysteine to histidine mutants, SCO1 C173H and C169HC173H, had a compromised ability to coordinate copper suggests that these two conserved cysteines might have different roles in copper coordination. The Cys169 may serve as a switch to allow the protein to load or release copper. Therefore, it is conceivable that substituting Cys169 to histidine might allow for an enhanced ability to coordinate copper while impairing subsequent transfer of the metal ion. The ability of the C169H mutant to bind Cu(I) therefore warrants further investigation. Cysteines contain a thiol group (-SH), which has a high affinity for copper. This thiol group can form a Cu-S bond between cysteine and copper and allow for metal ion coordination. Previous studies of BsSco, the *Bacillus subtilis* homologue of human SCO1, have found there is a difference between the two Cu-S (cysteine) bonds from cysteine residues in the CxxxC motif (Andruzzi *et al.*, 2005).

It has been observed that large couplings assignable to the second cysteine residue of the copper binding site lead to a strong Cu-S bond, while the first cysteine has a relatively shorter and weaker Cu-S interaction (Andruzzi *et al.*, 2005). Additional evidence comes from a recent study showing that Cys169 is the capture ligand of Cu(II). Cu(II) ion is initially bound to Cys169, with subsequent reorganization resulting in Cys173 binding (Blundell *et al.*, 2013). Collectively, these observations suggest that Cys169 has a relatively more critical role than Cys173 during the initial steps of copper binding.

Several studies have shown that the copper content of wild-type SCO1 when purified as a soluble protein is about 1.0 Cu atom/monomer (Beers *et al.*, 2002; Horng *et al.*, 2005; Nittis *et al.*, 2001). A few experimental factors, such as the copper concentration in the culture media, the poly-histidine tag used for purification and the nickel derived from Ni-NTA may all have had a slight negative effect on the copper content of the SCO1 protein I purified in my thesis studies. Increasing the exogenous levels of copper in medium was able to improve the copper metallation of SCO1 protein by 0.3 molar equivalent, especially in the yeast expression system (Beers *et al.*, 2002), and the poly-histidine tag was shown to be responsible for 20% of the copper bound by SCO1 (Horng *et al.*, 2005; Nittis *et al.*, 2001). A small amount of Ni(II) ions but no other metal atom were detected in the SCO1 protein (Horng *et al.*, 2005). Although I used a previously published method (Horng *et al.*, 2005) to express and purify wild-type SCO1 and SCO1 variants of interest, the copper content of the wild-type protein reported herein is much lower than it is in the literature. In addition to minor Ni(II) contamination and some non-specific copper binding by the poly-histidine tag, I had difficulty obtaining adequate amounts of highly purified, metallated SCO1 protein. Although such issues have not been reported in other, related studies, I assume that the lower purity of my wild-type SCO1 isolates is a major contributing factor to the suboptimal total copper content of these samples.

Another possibility is to employ a yeast-based expression system, which has been used previously for human SCO1 protein expression, purification and subsequent characterization of its copper-binding properties (Horng *et al.*, 2005). The cytoplasm of yeast contains Cup1 and Crs5 (copper resistant suppressor), both of which are abundant metallothioneins that bind Cu(I) (Rae *et al.*, 1999). As a consequence, it is a highly competitive, copper limiting environment. When SCO1 protein is purified from the yeast cytoplasm, the presence of bound copper in the protein can therefore be used to indicate its affinity for copper. SCO1 has been shown to be an

apo-protein in yeast cytoplasm unless it is co-expressed with COX17, which results in its full metallation (Horng *et al.*, 2005). However, one limitation of this experimental approach, and of the prokaryotic expression system (irrespective of the technical difficulties I encountered), is that the copper-binding properties of soluble SCO1 variants may not reflect those of the full-length proteins, especially when expressed in their native milieu.

4.4 Future directions

Several observations have shown that SCO1 and SCO2 interact with each other to promote COX assembly and regulate the rate of copper efflux from the cell (Leary *et al.*, 2004; Leary *et al.*, 2009). While it is known that copper-binding is essential to each of these roles, the nature of the relationship between copper-binding and protein function remains unclear. One purpose of my M.Sc. thesis was to investigate the significance of the N-terminus of SCO1 to copper mobilization from the matrix to the IMS for its eventual transfer to COX II. Based on my data, there is little evidence to suggest that the N-terminus imparts functional attributes that make SCO1 unique from SCO2; however, it is clear that the originally predicted cleavage site was wrong, and further investigation of deletion mutants lacking the ~20-30 amino acids that follow the cleavage site we have recently mapped but that precede the transmembrane domain will be required prior to making any definitive conclusions.

While our focus on copper-binding residues could be rationalized at the time, it ignored the millions of years of evolution that have resulted in the copper-binding properties of the SCO1 protein. It is clear from my thesis work that there is limited value in continued targeting of the cysteine of the CxxxC motif or the conserved histidine. Therefore, future work on this front may consider the value of introducing point mutations in other highly conserved amino acids of SCO1 that are close to the CxxxC motif and His260, including Phe166, Tyr163, Val172, Leu177, Phe200, Ile257 and Ile262 (Williams *et al.*, 2005). While laborious, if a mutant could be identified that locked SCO1 in a copper-loaded state, it could be exploited to understand how copper binding affects SCO1 function both in the context of COX II metallation and the regulation of cellular copper homeostasis.

Future work also must improve the purification of soluble SCO1 truncates. The main problem in my experiment was contamination with other untagged proteins. While a poly-

histidine tag was used to facilitate the purification of SCO1, cellular proteins that contain two or more adjacent histidine residues can also bind to the Ni-NTA (Schmitt *et al.*, 1993) and therefore introduce significant contamination into the protein isolate. It has been reported that several methods can reduce non-specific binding of untagged proteins and improve the level of purity (Bornhorst and Falke, 2000). First, increasing the concentration of imidazole in the wash buffer will effectively remove non-specifically bound proteins from Ni-NTA column (Bornhorst and Falke, 2000), because imidazole is a functional group of histidine and has a higher affinity for nickel relatively to histidine. Second, a wash buffer with a pH lower than that of the binding buffer may also elute untagged proteins (Bornhorst and Falke, 2000). Addition of other agents in the wash buffer may also help to reduce non-specific protein binding without substantially affecting the binding of the tagged protein to the column. These agents include low levels (up to 1%) of the non-ionic detergents Triton X-100 or Tween 20 in the protein buffers, or salt such as NaCl (up to 500 mM), or low concentration of ethanol (up to 20%) (Bornhorst and Falke, 2000). The effects of manipulating these buffer components should be evaluated experimentally to achieve optimum purification of SCO1.

The Cu(I) binding properties of SCO1 mutant proteins, especially C169H, also need to be addressed in the future. This is worthwhile for a SCO1 mutant protein that preferentially binds Cu(I) over Cu(II), or vice versa, would be a powerful tool to explore the mechanisms that govern the biogenesis of the binuclear Cu_A site and the mitochondrial regulation of copper efflux from the cell.

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