

The Human Cytochrome *c* Oxidase Assembly Factors SCO1 and SCO2 Have Regulatory Roles in the Maintenance of Cellular Copper Homeostasis

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DOI 10.1016/j.cmet.2006.12.001

SUMMARY

Human SCO1 and SCO2 are metallochaperones that are essential for the assembly of the catalytic core of cytochrome *c* oxidase (COX). Here we show that they have additional, unexpected roles in cellular copper homeostasis. Mutations in either SCO result in a cellular copper deficiency that is both tissue and allele specific. This phenotype can be dissociated from the defects in COX assembly and is suppressed by overexpression of SCO2, but not SCO1. Overexpression of a SCO1 mutant in control cells in which wild-type SCO1 levels were reduced by shRNA recapitulates the copper-deficiency phenotype in SCO1 patient cells. The copper-deficiency phenotype reflects not a change in high-affinity copper uptake but rather a proportional increase in copper efflux. These results suggest a mitochondrial pathway for the regulation of cellular copper content that involves signaling through SCO1 and SCO2, perhaps by their thiol redox or metal-binding state.

INTRODUCTION

Copper is an essential micronutrient required by proteins that function in a wide range of metabolic pathways, including mitochondrial respiration, free radical scavenging, and neurotransmitter biosynthesis (Hamza and Gitlin, 2002). Its properties as a transition metal, however, allow copper to generate potentially cytotoxic free radicals when free within the cell. Cellular mechanisms have therefore evolved to ensure that copper is safely delivered to discrete subcellular locations by specific chaperones for

either storage or incorporation into target molecules (Rees and Thiele, 2004; Tao and Gitlin, 2003). Accordingly, there is essentially no free copper in the cytoplasm under normal physiological conditions (Rae et al., 1999).

Two cuproproteins, Cu/Zn superoxide dismutase (SOD1) and cytochrome *c* oxidase (COX), are metallated in the mitochondrial intermembrane space; however, the cellular chaperones that deliver copper to mitochondria have yet to be identified. In higher eukaryotes, COX is composed of 13 structural subunits that are encoded in both the nuclear and mitochondrial genomes. Highly conserved domains within two of the mitochondrially encoded subunits (I and II) of COX contain copper centers (Cu_A and Cu_B) that are essential for enzyme catalysis. Metallation of these sites occurs during assembly of the holoenzyme complex and is dependent, at least in part, on a bioactive pool of copper within the mitochondrial matrix (Cobine et al., 2004, 2006a).

Several accessory factors that are essential for copper delivery to COX have been identified (reviewed in Cobine et al., 2006c), and pathogenic mutations in two of these, SCO1 and SCO2, have thus far been described (Jaksch et al., 2000; Papadopoulou et al., 1999; Valnot et al., 2000). Patients present with early-onset, tissue-specific clinical phenotypes with fatal outcomes as a result of a severe, isolated COX deficiency; however, SCO2 mutations are associated primarily with neonatal encephalomyopathy, while mutations in SCO1 cause a neonatal hepatopathy and ketoacidotic coma. The distinct clinical phenotypes are not a result of tissue-specific expression of the two genes, as SCO1 and SCO2 are ubiquitously expressed and exhibit a similar expression pattern in different human tissues (Papadopoulou et al., 1999). All reported SCO2 patients carry at least one E140K missense allele. Patients homozygous for the E140K mutation have a delayed onset of the disease pathology and a more prolonged course of disease as compared

to compound heterozygotes (Jaksch et al., 2001a). *SCO1* mutations have only been reported in a single pedigree (Valnot et al., 2000) in which affected individuals were compound heterozygotes, with a nonsense mutation on one allele and a P174L missense mutation on the second allele.

Molecular genetic and biochemical analyses of *SCO1* and *SCO2* patient cell lines have demonstrated that human SCOs have essential, nonoverlapping functions in the biogenesis of the Cu_A site that depend on their ability to bind both Cu(I) and Cu(II) (Hornig et al., 2005; Leary et al., 2004). Their precise molecular function, however, remains unknown and underscores one of the most puzzling questions in the field—namely, how do mutations in ubiquitously expressed housekeeping genes give rise to tissue-specific clinical phenotypes? Here we demonstrate that mutations in *SCO1* and *SCO2* produce significant reductions in cellular copper content that are both tissue and allele specific, suggesting a mitochondrial pathway for the regulation of cellular copper homeostasis.

RESULTS

Tissue-Specific Differences in COX Deficiency in *SCO1* and *SCO2* Patients

To investigate the molecular genetic basis of the tissue-specific COX deficiencies in *SCO* patients, we first assessed COX assembly in mitochondria isolated from the liver, heart, and skeletal muscle of *SCO1* and *SCO2* patients and age-matched controls by blue native PAGE (BN-PAGE) analysis. In *SCO2* patients, COX was almost undetectable in heart and skeletal muscle; however, the amount of fully assembled holoenzyme in liver was comparable to that in controls (Figure 1A), consistent with a recent report (Stiburek et al., 2005). In contrast, both liver and skeletal muscle from the *SCO1* patient exhibited a severe deficiency in COX assembly.

Immunoblot analysis of the steady-state levels of the mutant SCO proteins showed that levels of *SCO1* P174L were only slightly reduced in skeletal muscle and were in the control range in liver (Figure 1B), consistent with the expression of a single, stable missense allele. In contrast, *SCO2* patient tissues exhibited a drastic reduction in the amount of mutant *SCO2* in skeletal muscle, liver (Figure 1B), and heart (data not shown). The relatively normal assembly of COX in the liver of *SCO2* patients is not therefore simply a function of higher residual levels of mutant *SCO2* protein, which appears unstable in all tissues. Furthermore, it could not be attributed to a compensatory upregulation of other factors (*SCO1*, *COX11*, and *COX17*) involved in mitochondrial copper delivery to COX (Figures 1B and 1C; Leary et al., 2004).

Striking differences in *SCO2* content were, however, observed among control tissues (Figure 1B). By analyzing incremental amounts of mitochondrial protein from heart, skeletal muscle, and liver of a representative control, we estimate that the *SCO2* content of control liver is 3- to 5-fold higher than in heart or skeletal muscle (Figure 1D).

This compares with a mitochondrial COX content of 300 U/mg in liver versus 1350 U/mg in heart, suggesting that *SCO2* may have other functions in the liver in addition to COX assembly. The above data show that the severity of the COX assembly defect in *SCO* patient tissues is consistent with the observed clinical phenotypes, but they do not explain why COX assembly is severely affected in the heart and skeletal muscle of *SCO2* patients but not in the liver.

Mutations in *SCO1* and *SCO2* Produce a Severe Copper Deficiency in Affected Tissues

Several studies have shown rescue of the COX deficiency in *SCO2* fibroblasts and myoblasts and its partial rescue in *SCO1* fibroblasts following supplementation of culture media with copper salts (Jaksch et al., 2001b; Leary et al., 2004; Salviati et al., 2002). Reversal of the hypertrophic cardiomyopathy in a *SCO2* patient has also been reported following administration of copper histidine (Cu-His) (Freisinger et al., 2004). These observations raise the possibility that alterations in tissue copper content could be responsible for tissue-specific differences in the severity of COX deficiency in *SCO* patients. To test this hypothesis, we measured the total cellular copper content in skeletal muscle, heart, and liver from *SCO* patients and controls (Figure 2 and data not shown). In the normal liver, copper content declines rapidly during early development from several hundred $\mu\text{g/g}$ dry weight in neonates to a mean of $18.6 \pm 3.9 \mu\text{g/g}$ dry weight beyond 6 months of age (Figure 2A, $n = 38$), whereas copper levels do not change significantly with age in either skeletal muscle ($7.6 \pm 1.0 \mu\text{g/g}$, $n = 23$) or heart ($8.7 \pm 0.5 \mu\text{g/g}$, $n = 9$) (Figures 2B and 2C). The *SCO1* patient exhibited a severe copper deficiency in both liver and skeletal muscle (33 and $1.7 \mu\text{g/g}$ dry weight, respectively), with copper content being well below the lowest level measured in age-matched controls (Figures 2A and 2C). Mutations in *SCO2* also resulted in a severe reduction in the copper content of patient heart to about 25% of the mean control value (Figure 2B). Changes in skeletal muscle copper content in *SCO2* patients were more variable: 2 of 4 patients were on the low end of the normal range, one was severely deficient, and one was in the normal range (Figure 2C). The copper content in *SCO2* patient liver was on the low end of the normal range (Figure 2A); however, it was about 16-fold higher than the mean value observed in patient skeletal muscle (92.3 versus $5.6 \mu\text{g/g}$ dry weight) and 40-fold higher than the value observed in patient heart. This copper level is also about 2-fold greater than that found in *SCO2* fibroblasts treated chronically with $300 \mu\text{M}$ Cu-His (data not shown), which results in a full rescue of the COX deficiency (Jaksch et al., 2001b). We conclude that mutations in *SCO1* or *SCO2* can produce severe, tissue-specific reductions in total cellular copper levels that would serve to exacerbate the severity of the COX deficiency. Of the tissues we were able to analyze, only the liver of *SCO2* patients appears to contain sufficient copper to promote *SCO2*-independent COX assembly in neonatal life.

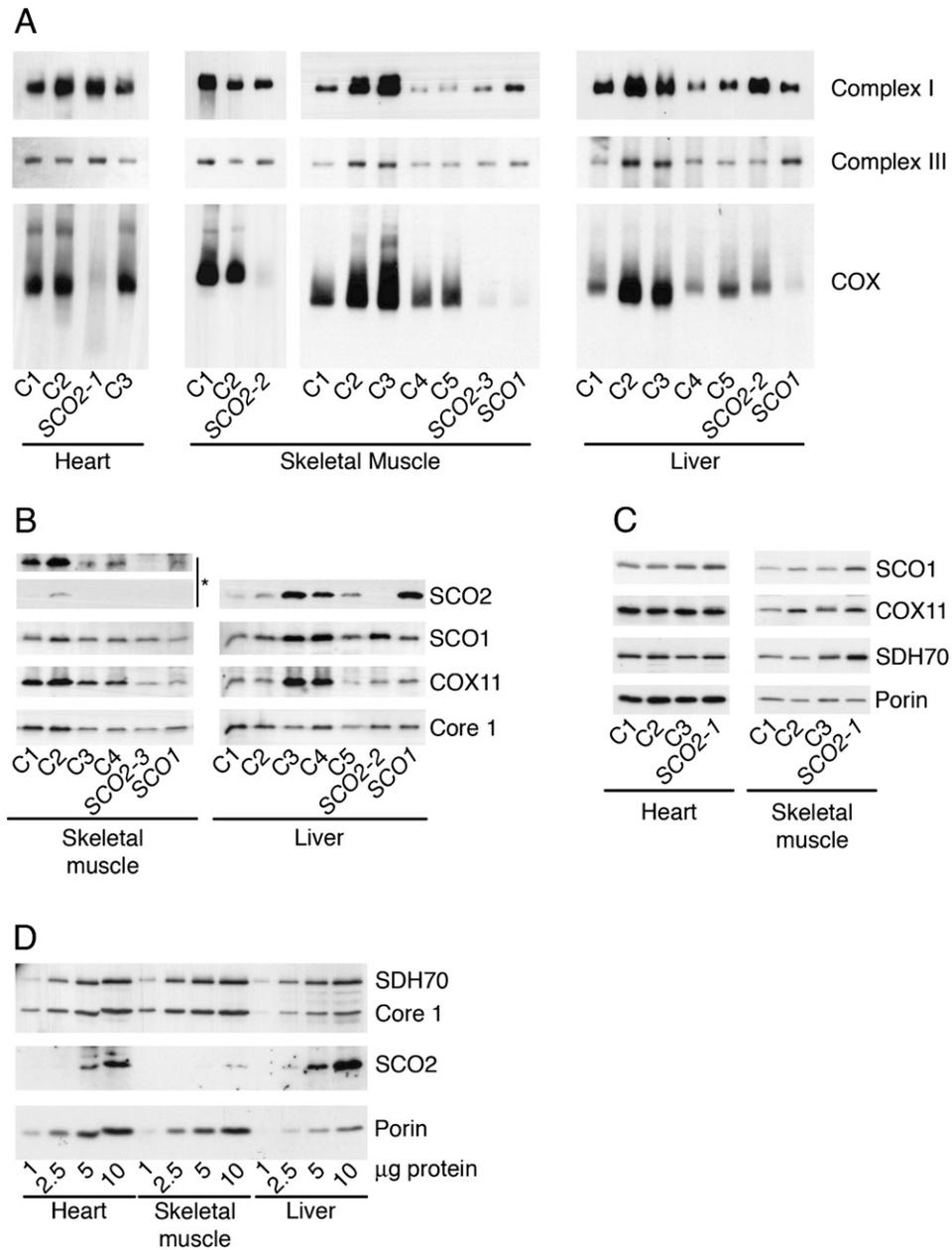


Figure 1. Tissue-Specific COX Assembly Defects in *SCO1* and *SCO2* Patients

(A) Mitochondrial extracts (5 μ g) from control, *SCO1*, and *SCO2* patient heart, skeletal muscle, and liver were fractionated on BN-PAGE gels, and membranes were blotted with antibodies specific to individual structural subunits of complex I (anti-39 kDa), complex III (anti-core 1), and COX (anti-COX IV). *SCO2-1* and *SCO2-2* are affected siblings (E140K/L151P) and are unrelated to *SCO2-3* (E140K/R171W).

(B) Mitochondrial extracts (15 μ g) from control, *SCO1*, and *SCO2* patient skeletal muscle and liver were fractionated on 15% acrylamide gels under denaturing conditions, and membranes were blotted with polyclonal antisera to detect SCO1, SCO2, and COX11. Core 1, SDH70, and porin served as internal loading controls. Exposure times for the visualization of all proteins were identical in both liver and skeletal muscle, allowing for direct comparison of differences in their abundance between the two tissues. The asterisk beside the upper panel for skeletal muscle highlights the longer exposure time that is necessary to visualize SCO2 in all of the controls.

(C) Protein extracts were prepared and analyzed exactly as described in (B), except that, due to limiting amounts of *SCO2* patient heart sample, a total of only 3.5 μ g protein was loaded per lane.

(D) Incremental amounts of mitochondrial protein (1–10 μ g) from heart, skeletal muscle, and liver from a representative control were analyzed as described in (B).

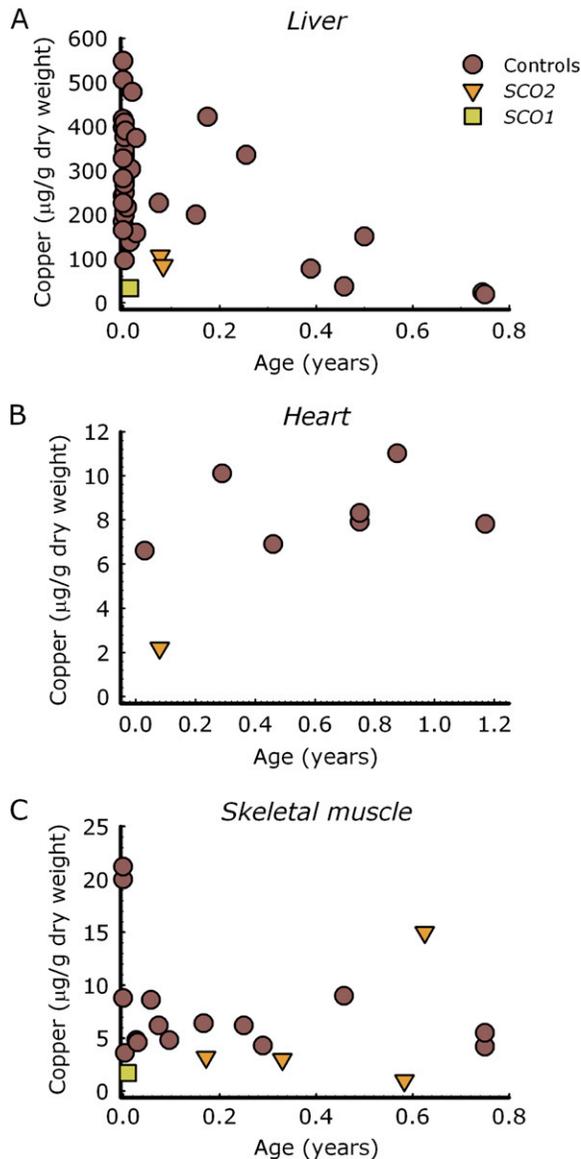


Figure 2. Mutations in *SCO1* and *SCO2* Produce Cellular Copper-Deficiency Phenotypes in Affected Tissues

Copper levels were measured in control, *SCO1*, and *SCO2* patient tissues by ICP-MS or ICP-OES and are expressed as a function of tissue dry weight. In both patient backgrounds, the levels of zinc and iron were within the control range (data not shown). Mutations in the *SCO2* patients included E140K/R171W (skeletal muscle), E140K/L151P (liver), E140K/C133S (liver and heart), E140K/R90X (skeletal muscle), and E140K/E140K (skeletal muscle) [n = 2]).

Cellular Copper Deficiency in Fibroblasts from Patients with Other COX Assembly Defects

To validate and extend the results obtained from tissue copper measurements, we measured total cellular copper content in fibroblast lines derived from all of our patients with mutations in COX assembly factors. Consistent with the tissue analyses, *SCO1* patient fibroblasts exhibited the most severe copper-deficiency phenotype of all patient

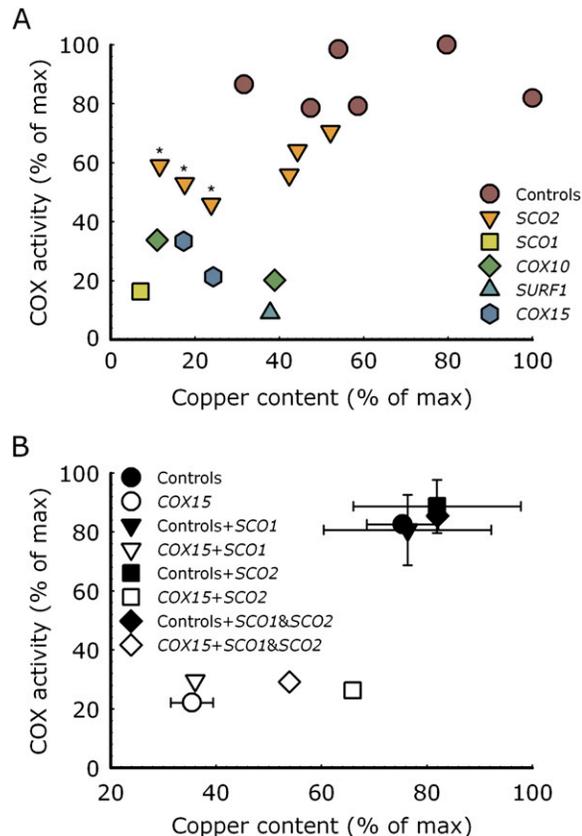


Figure 3. Mutations in a Number of COX Assembly Factors Produce a Cellular Copper-Deficiency Phenotype in Patient Fibroblasts that Is Suppressed by Overexpression of *SCO2*

(A) Copper content was quantified in cell pellets from control and patient (*SCO1*, *SCO2*, *COX10*, *COX15*, and *SURF1*) fibroblasts by ICP-OES. A fraction of the total cellular material was also used to measure COX and citrate synthase (CS) enzyme activities. Both COX activity and cellular copper content were expressed as a percentage of the maximum control value after normalization to CS activity or total cellular zinc levels. Expressing cellular copper levels as a function of either dry weight or total cellular sulfur content did not influence the results. *SCO2* patients carrying a single missense allele are denoted by an asterisk.

(B) Control and *COX15* patient fibroblasts were transduced with cDNAs expressing *SCO1*, *SCO2*, or *SCO1* and *SCO2*. Following positive drug selection, copper levels in these cells were compared with those in untransduced cells. The percentage of maximum control values for both COX activity and cellular copper content were calculated for baseline cells and for cells in which each construct or group of constructs was overexpressed (mean ± SEM, n = 2–4 for total number of controls per group; n = 5 for *COX15* baseline cells).

backgrounds (Figure 3A). Analysis of several *SCO2* patient cell lines also revealed a clear genotype-phenotype correlation with respect to cellular copper deficiency: patients carrying two missense alleles were on the low end of the normal range, while those carrying a single missense allele were severely copper deficient (% of maximum control: 46.2 ± 3.7 versus 17.7 ± 4.3 respectively, n = 3 for each group). Reduced cellular copper levels in both *SCO1* and *SCO2* patient backgrounds were further supported

Table 1. Cellular Copper Levels and COX Activity in Control, SCO1, and SCO2 Patient Fibroblasts

Cell Line	Baseline		SCO1 ^a		SCO2 ^b		SCO1/SCO2 ^a		SCO1 P174L ^a		SCO2 E140K ^c	
	Copper	COX	Copper	COX	Copper	COX	Copper	COX	Copper	COX	Copper	COX
Controls	67.1 ± 3.9	88.6 ± 2.1	79.4 ± 13.5	90.0 ± 7.3	78.5 ± 5.8	95.2 ± 3.3	75.2 ± 9.8	96.2 ± 3.1	64.6 ± 11.0	85.8 ± 4.4	86.1 ± 8.5	91.7 ± 4.8
SCO2	40.6 ± 6.9	51.7 ± 1.6	40.0	21.3	65.3 ± 10.3	89.0 ± 5.1	32.0	17.7	46.9	33.9 ± 2.9	44.0 ± 9.6	86.6 ± 8.2
SCO1	11.3 ± 3.0	16.9 ± 6.4	14.0	100.0	34.1 ± 5.8	4.9 ± 1.9	36.0	9.5	14.6	36.6 ± 4.8	24.5	1.8

COX activity was normalized to citrate synthase activity, and cellular copper content was expressed as a function of total cellular zinc levels (mean ± SEM, wherever applicable). Expressing cellular copper levels as a function of either dry weight or total cellular sulfur content did not influence the results. All values are expressed as a percentage of the maximum control value within a given group. Baseline measurements of COX activity and copper content were made in both primary and immortalized fibroblasts. Although copper levels were higher in immortalized cells from both control and patient fibroblasts, the ratio of copper to zinc and the relationship between copper levels in controls and patients remained constant. Cellular copper content was therefore expressed as a function of zinc to allow for inclusion of all the data. Multiple measurements (n = 7, minimum) were made in baseline cells (6 controls, 4 SCO2 patients [two missense alleles, n = 3; one missense allele, n = 1], and 1 SCO1 patient). In all overexpression experiments, single measurements of COX activity and copper levels were made in 4 controls.

^a 2 SCO2 patients (n = 1 for one and two missense alleles), SCO1 patient (n = 1).

^b 6 SCO2 patients (n = 3 for each group), SCO1 patient (n = 3).

^c 6 SCO2 patients (n = 3 for each group), SCO1 patient (n = 2).

by the diminished activity of SOD1, another cellular protein that requires copper for catalytic activity (SCO1 patient, 51.3 ± 6.4 [n = 7]; SCO2 patients [two missense alleles], 78.2 ± 3.3 [n = 2, 4 replicates each]; SCO2 patients [one missense allele], 55.1 ± 11.6 [n = 1, 4 replicates] % of total cellular SOD).

Unexpectedly, fibroblast lines from COX10 and COX15 patients, whose COX deficiency results from a defect in heme A biosynthesis, also showed reduced cellular copper levels, with one of the two COX10 fibroblast lines and both of the COX15 fibroblast lines being severely copper deficient (Figure 3A). To test whether the copper deficiency in COX15 cells could be rescued by altering SCO levels, we overexpressed SCO1 or SCO2 alone or in combination (Figure 3B). Cellular copper levels were unaffected by overexpressing SCO1; however, the cellular copper deficiency was rescued by overexpressing SCO2, an effect that was attenuated when SCO1 and SCO2 were overexpressed together. Overexpression of the SCOs had no effect on COX activity. In contrast, overexpression of COX15, while rescuing the COX defect, did not suppress the copper deficiency (data not shown). These data suggest that the function of a number of COX assembly factors ties into a common signaling pathway involved in the regulation of cellular copper homeostasis that is distinct from the COX assembly pathway. The ability of SCO2 overexpression to suppress the cellular copper-deficiency phenotype further suggests that it has a prominent role in this signaling pathway.

Dissociation of the COX Assembly Defect from the Copper Deficiency in SCO Fibroblasts

To further define the molecular genetic basis of mitochondrial regulation of cellular copper homeostasis, we focused our attention on SCO patient cell lines. We first tested whether overexpression of cDNAs that either restore COX activity in patient cells or act as dominant

negatives (Leary et al., 2004) could suppress the cellular copper-deficiency phenotype (Table 1). Overexpression of a wild-type SCO2 cDNA restored COX activity and rescued the cellular copper deficiency in SCO2 patient fibroblasts, while cellular copper levels were unchanged by overexpression of either full-length SCO1 or a SCO1/SCO2 chimera, both of which exert a strong dominant-negative phenotype at the level of COX activity. In contrast, the severe copper deficiency in SCO1 patient fibroblasts was not suppressed by SCO1 overexpression, despite the complete rescue of COX activity. Cellular copper levels were partially restored, however, in SCO1 patient fibroblasts overexpressing either SCO2 or the SCO1/SCO2 chimera, both of which exacerbate the COX deficiency in these cells (Table 1). These data provide further evidence that the copper deficiency in both SCO backgrounds is attributable not to changes in COX content per se but rather to some additional aspect of SCO protein function that is compromised in these patients.

The Copper-Deficiency Phenotype in SCO1 Patient Fibroblasts Can Be Recapitulated in a Control Cell Line

To test whether SCO1 P174L exerts its effects on cellular copper homeostasis by acting as a dominant negative, we overexpressed SCO1 P174L in control, SCO1, and SCO2 patient fibroblasts and measured cellular copper content and COX activity (Table 1). While overexpression of SCO1 P174L altered COX activity in both SCO patient backgrounds, no significant changes were observed in cellular copper levels. Pairwise comparisons in controls also revealed that cellular copper content was unaffected by overexpressing SCO1 P174L (data not shown). These data suggested that the manifestation of the cellular copper-deficiency phenotype might depend on the relative levels of wild-type to mutant SCO1. To investigate this possibility, we used an RNAi approach in a control fibroblast line in

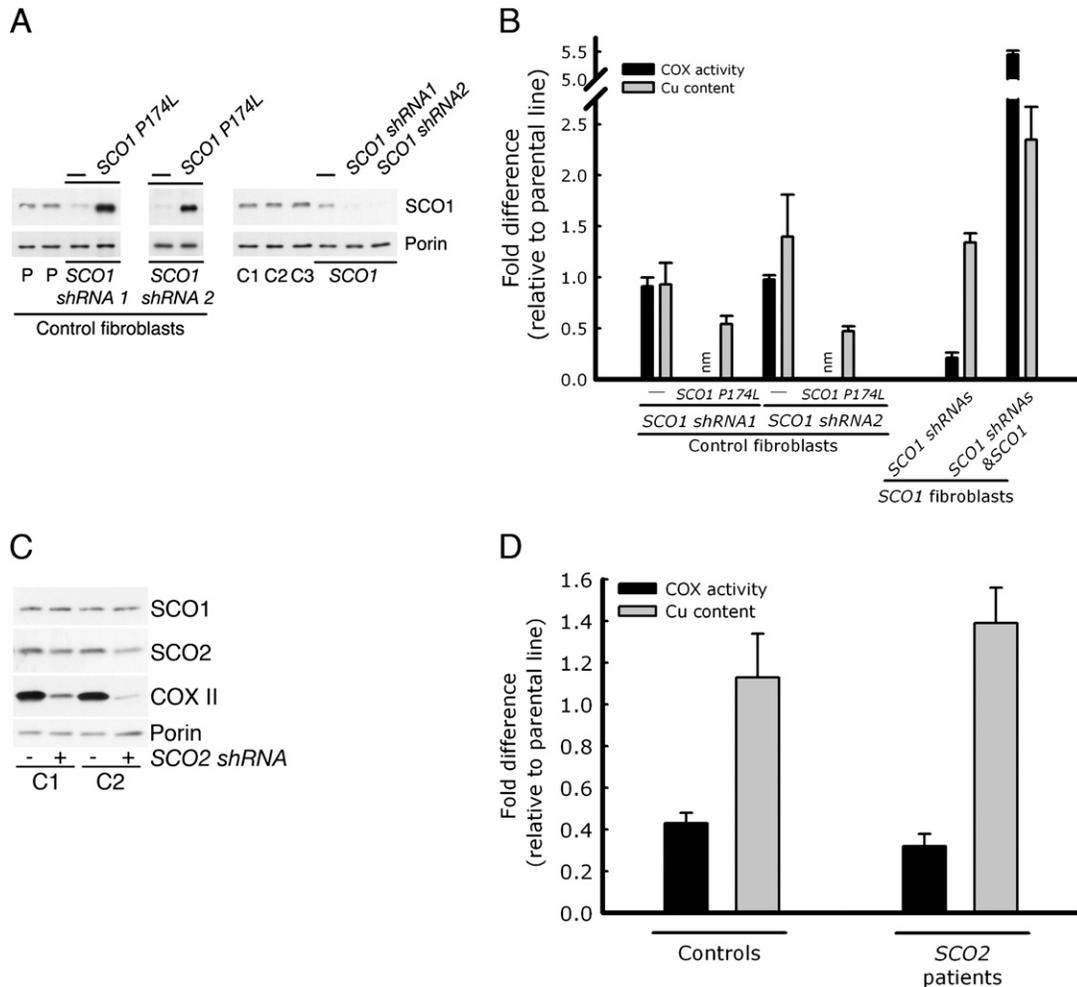


Figure 4. SCO1 P174L Modulates Cellular Copper Levels

(A) Immunoblot analysis of mitoplasts (20 μ g) from individual clones from a control fibroblast line (P) overexpressing one of two different SCO1 shRNA constructs (1 and 2) alone (–) or in combination with SCO1 P174L. Levels of SCO1 in controls (C1–C3), SCO1 patient fibroblasts, and bulk cultures of SCO1 patient fibroblasts overexpressing one of two different SCO1 shRNA constructs are also shown. Porin served as an internal loading control.

(B) Cellular copper levels and COX activity were measured in five control clones that showed the greatest knockdown of SCO1 protein levels with each SCO1 shRNA construct and in bulk cultures of SCO1 patient fibroblasts overexpressing the SCO1 shRNA constructs alone (n = 6) or in combination with a wild-type SCO1 cDNA (n = 3). Values for both parameters are expressed relative to the untransduced parental lines (mean \pm SEM). Although COX activity was not measured (nm) in control SCO1 shRNA clones overexpressing SCO1 P174L, COX II protein levels were identical to those found in the parental line (data not shown).

(C) Immunoblots of mitoplasts (20 μ g) showing representative knockdown of SCO2 protein levels in bulk cultures of two control lines overexpressing a SCO2 shRNA construct. Blots were also probed with COX II and SCO1 antisera. Porin served as an internal loading control.

(D) COX activity and cellular copper levels were measured in control (n = 4) and SCO2 patient fibroblasts (two missense alleles, n = 3; one missense allele, n = 2) overexpressing the SCO2 shRNA construct and are expressed relative to values in untransduced parental lines (mean \pm SEM).

an attempt to recapitulate the copper-deficiency phenotype. A number of clones from two shRNA constructs that were targeted to the 3' UTR of the SCO1 mRNA exhibited a knockdown of SCO1 protein levels to approximately 15%–20% of that in the parental line (Figure 4A). Residual SCO1 protein content in these clones was sufficient to maintain both total COX content and cellular copper levels; however, overexpression of the SCO1 P174L variant reduced cellular copper content by about 50% (Figure 4B). The reciprocal experiment, in which the levels of SCO1 P174L

were knocked down in the SCO1 patient background, exacerbated the COX deficiency but resulted in a 40% increase in total cellular copper content (Figures 4A and 4B). Overexpression of wild-type SCO1 in this genetic context further increased cellular copper levels to 245% of parental levels and completely rescued the COX deficiency (Figure 4B). We conclude that the P174L missense mutation abrogates an aspect of SCO1 function that is necessary for the normal regulation of cellular copper homeostasis.

Rescue of COX Activity but Not Cellular Copper Levels by Overexpression of the E140K Allele in SCO2 Fibroblasts

The cellular copper deficiency in SCO2 patients could be the result of the marked reduction in the level of functional SCO2 protein or the presence of low residual levels of mutant SCO2 protein. To distinguish between these two possibilities, we overexpressed the common E140K variant in control, SCO1, and SCO2 patient fibroblasts and measured COX activity and cellular copper levels (Table 1). While SCO2 E140K overexpression rescued the COX deficiency in SCO2 patient fibroblasts, cellular copper levels were unchanged. Overexpression of SCO2 E140K in SCO1 patient fibroblasts reduced COX activity to near zero but was less effective in raising cellular copper content than wild-type SCO2 (Table 1). Taken together, these data provide genetic evidence that, when expressed in appreciable amounts, the common E140K SCO2 variant can function as a COX assembly factor; however, its role in the mitochondrial regulation of cellular copper homeostasis is compromised.

To further confirm that SCO2 variants do not adversely affect cellular copper homeostasis per se, we used RNAi to knock down the residual levels of mutant protein in SCO2 patient fibroblasts. Given the difficulty associated with detecting mutant SCO2 by immunoblot analysis, we also knocked down SCO2 in control fibroblasts to test the efficiency of the knockdown. Two representative control lines expressing the most effective SCO2 shRNA construct showed a decrease in SCO2 abundance of up to 50% without any changes in the levels of SCO1 (Figure 4C). Although this led to a marked reduction in COX II protein content and a 60% decrease in COX activity, cellular copper levels were relatively unchanged (Figure 4D). The reduction in COX activity in SCO2 patient fibroblasts overexpressing the SCO2 shRNA construct was comparable to that observed in the controls, suggesting a similar knockdown of mutant SCO2 protein levels; however, this was associated with a small increase in cellular copper content. These data further suggest that the cellular copper deficiency in SCO2 patient fibroblasts is mainly due to a lack of wild-type SCO2 protein as opposed to aberrant signaling through mutant SCO2 variants.

The Copper Deficiency in SCO2 Fibroblasts Can Be Partially Rescued by Overexpression of CTR1

To test whether the mutations in SCO1 or SCO2 were exerting their effects on cellular copper content by altering the abundance of other proteins known to be important to copper trafficking and metabolism within the cell, we measured the steady-state levels of CTR1, Atox1, Murr1 (COMMD), Wnd (ATP7B), and Mnk (ATP7A). No consistent differences were observed in the levels of these factors (Figures 5A and 5B). CTR1 maturation was, however, altered in SCO1 patient liver, with the molecular weight of the immunoreactive band being consistent with the presence of the unglycosylated precursor protein. Immunoblot analysis also indicated that there was some variability in

the levels of the fully glycosylated, mature form of CTR1 in SCO1 patient fibroblasts (arrow, Figures 5B and 5D), an observation that was further confirmed by its resistance to cleavage with endoglycosylase H (data not shown; Klomp et al., 2003). To evaluate whether SCO mutations affected high-affinity copper uptake, we overexpressed CTR1 in control, SCO1, and SCO2 patient fibroblasts (Figure 5C). Overexpression of CTR1 resulted in a partial rescue of the copper-deficiency phenotype in SCO2 patient fibroblasts. Those cell lines carrying two missense alleles were particularly responsive to the manipulation of CTR1 copy number, with cellular copper levels increasing by 79% relative to untransduced parental lines. In contrast, cellular copper levels were unchanged in SCO1 patient fibroblasts, despite the fact that CTR1 was clearly overexpressed (Figure 5D).

To test whether CTR1 trafficking was compromised as a result of the SCO1 mutation, a fully functional, myc-tagged human CTR1 (Lee et al., 2002; Petris et al., 2003) was overexpressed in control and SCO1 patient fibroblasts to visualize its cellular localization in response to manipulation of the copper content of the culture medium. Rapid internalization of CTR1-myc was observed in both cell lines in response to a 15 min exposure to 100 μ M Cu-His (data not shown), suggesting that high-affinity copper uptake by endocytosis of the transporter is not affected in the SCO1 patient cells. This is further supported by comparable sensitivity of control, SCO1, and SCO2 patient fibroblasts to cisplatin (data not shown), a drug whose cytotoxicity is a function of CTR1 abundance and its localization to the plasma membrane (Guo et al., 2004; Ishida et al., 2002).

The Copper Deficiency in SCO1 and SCO2 Fibroblasts Results from Increased Copper Efflux

To directly investigate altered copper handling in SCO patients, we conducted uptake and retention experiments in fibroblasts grown in serum-free medium in the presence of trace amounts of radioactive copper (^{64}Cu). Initial rates (0–10 min) of ^{64}Cu uptake in SCO1 and SCO2 patient fibroblasts were not significantly different when compared to the mean value for control fibroblasts (Figure 6A). To evaluate the effects of SCO mutations on copper efflux, control, SCO1, and SCO2 patient fibroblasts were incubated in serum-free medium containing ^{64}Cu for 2 hr ($t = 0$), at which point cells were washed in phosphate-buffered saline (PBS) and then chased in regular medium for up to 12 hr. Relative to controls, both SCO1 and SCO2 patient fibroblasts exhibited an enhanced rate of copper efflux (Figure 6B). This phenotype was most pronounced in SCO1 patient fibroblasts, where ^{64}Cu content was below background levels at the end of the chase. In SCO2 patient fibroblasts, there was a clear genotype-phenotype correlation in the rate of ^{64}Cu efflux; the patient cell line carrying a single missense allele (SCO2-5) was more severely affected than the patient cell line carrying two missense alleles (SCO2-6). Collectively, these results strongly suggest that SCO mutations cause cellular copper deficiency through aberrant signaling that ultimately

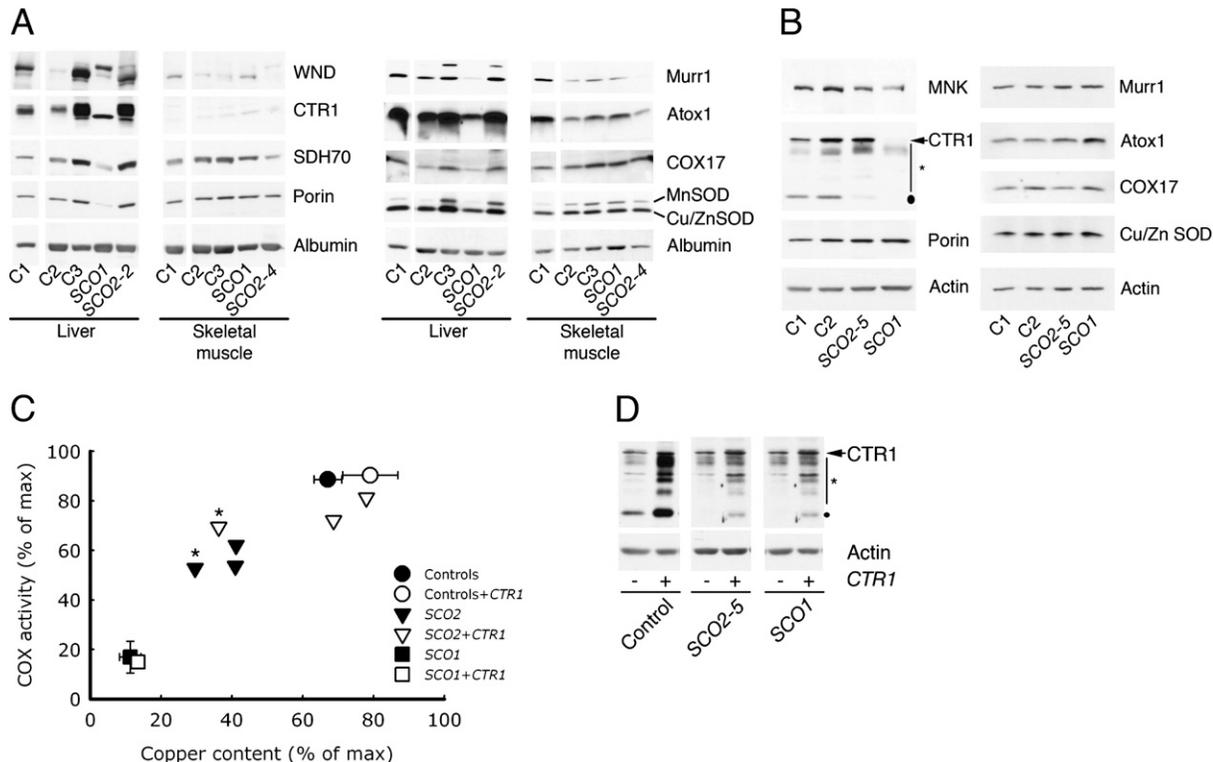


Figure 5. The Copper Deficiency in *SCO1* Patient Fibroblasts Is Not Explained by a Defect in High-Affinity Uptake

(A and B) Whole-cell extracts (10 μ g) from control (C1–C3) and *SCO* patient tissues (A) and fibroblasts (B) were fractionated on 5%–20% gradient gels under denaturing conditions and blotted with polyclonal antisera raised against several proteins important in cellular copper trafficking (CTR1, COX17, Atox1, Murr1, CTR1, Mnk, and Wnd). For CTR1, the fully glycosylated, mature form of the transporter is highlighted by the arrow and the precursor protein by the circle. Glycosylated intermediates are indicated by an asterisk. Membranes were also blotted with antibodies specific for MnSOD, Cu/ZnSOD, porin, and SDH70. Albumin served as the loading control for samples that were boiled prior to electrophoresis, while actin was used to normalize the loading of gels in which samples were denatured at 37°C.

(C) Control, *SCO1*, and *SCO2* patient fibroblasts were transduced with a *CTR1* cDNA, and, following positive drug selection, cellular copper levels and COX activity were measured and expressed as a percentage of maximum control values. Single measurements were made in all cell lines (controls, $n = 4$; *SCO2* patients, $n = 3$ [two missense alleles, $n = 2$; one missense allele, $n = 1$]; mean \pm SEM). The *SCO2* patient carrying a single missense allele is denoted by an asterisk.

(D) Whole-cell protein extracts (20 μ g) were denatured and fractionated, and membranes were blotted to detect CTR1 abundance. Representative immunoblots are shown for control, *SCO1*, and *SCO2* patient fibroblasts alone (–) and overexpressing CTR1 (+). Actin served as an internal loading control.

results in the misregulation of factors important to the retention of copper within the cell.

DISCUSSION

This study demonstrates that the human COX assembly factors *SCO1* and *SCO2* have additional and unexpected regulatory roles in the maintenance of cellular copper homeostasis. Several lines of evidence support this conclusion. First, affected tissues and fibroblast cell lines from *SCO1* and *SCO2* patients exhibit significant reductions in total cellular copper content, and there is a clear genotype-phenotype relationship in *SCO2* fibroblast lines between the number of missense alleles present and cellular copper levels. Second, the copper-deficiency phenotype can clearly be dissociated from the COX assembly defect in both *SCO1* and *SCO2* fibroblast lines.

Third, the reduction in cellular copper levels in fibroblasts from a patient with mutations in *COX15*, another COX assembly factor, can be rescued by overexpressing *SCO2*. Finally, pulse-chase experiments with ^{64}Cu show that the cellular copper deficiency in *SCO1* and *SCO2* patient cell lines results from an inability to retain ^{64}Cu rather than a defect in its uptake by high-affinity transporters.

We have previously shown that human *SCO1* and *SCO2* are copper-binding proteins (Hornig et al., 2005) whose cooperative interaction is necessary for the formation of the Cu_A site on COX II, an essential step in the assembly of the catalytic core of the holoenzyme (Leary et al., 2004). The present study shows that their additional role in the regulation of cellular copper homeostasis also likely involves an interaction between the two molecules, which does not completely overlap with their function as COX assembly factors.

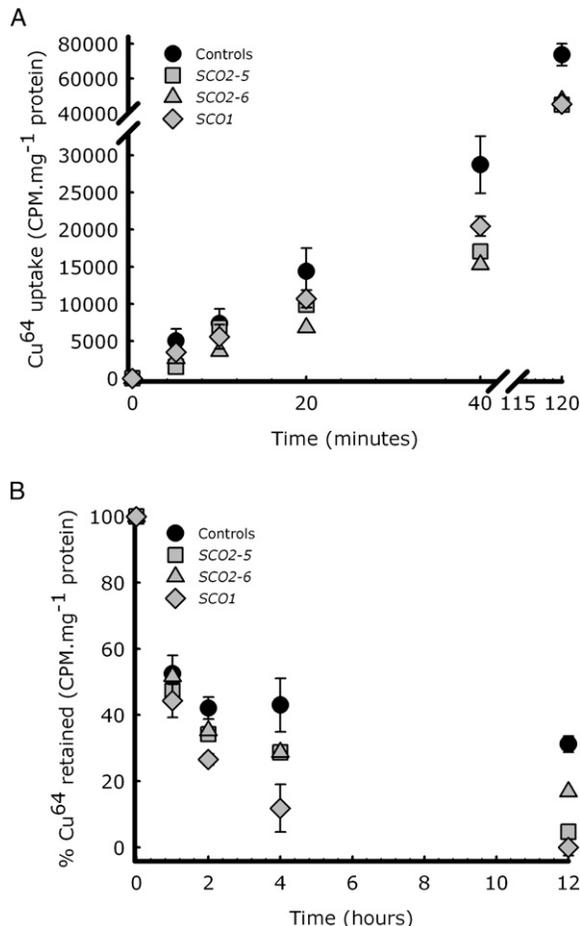


Figure 6. The Copper Deficiency in *SCO1* and *SCO2* Patient Fibroblasts Results from an Enhanced Rate of Copper Efflux

The kinetics of ^{64}Cu uptake (A) and efflux (B) were measured in control (mean \pm SEM, $n = 4$), *SCO2* ($n = 2$, from patients expressing either one or two missense alleles), and *SCO1* (mean \pm SEM, $n = 3$) patient fibroblasts as described in [Experimental Procedures](#). In all cases, ^{64}Cu levels were normalized for protein. In the retention experiments, the amount of ^{64}Cu per mg protein is expressed as a percentage of the appropriate value at $t = 0$.

The observation that overexpression of *SCO2*, but not *SCO1*, can suppress the cellular copper deficiency in fibroblasts from *SCO2*, *SCO1*, and *COX15* patients suggests that the *SCO*-dependent regulation of cellular copper levels depends, at least in part, on the activity of wild-type *SCO2*. Cellular copper content is positively correlated with steady-state *SCO2* protein levels in neonatal control liver, skeletal muscle, and heart, lending support to the idea of a causal relationship. Indeed, the comparatively high levels of *SCO2* in neonatal liver may be necessary to maintain high copper levels at this stage of development. The demonstration that overexpression of the *SCO2* E140K mutant rescues COX assembly, but not the copper deficiency, in *SCO2* patient fibroblasts clearly shows that the two functions of *SCO2* can be dissociated and that the instability of the mutant protein is the primary

cause of the assembly defect. The cellular copper deficiency then presumably results from some aspect of *SCO2* function that is missing in the E140K mutant but that is relatively unimportant in its cooperative interaction with *SCO1* in copper delivery to the Cu_A site.

How might the signal from *SCO2* be transduced? *SCO2* is present at wild-type levels in *SCO1* patient tissues and cells, yet they are severely copper deficient, suggesting that an abnormal signal is generated from the *SCO1* P174L mutant that cannot be modulated by normal levels of *SCO2*. Consistent with this interpretation, overexpression of *SCO2* only partially restores cellular copper content in *SCO1* patient fibroblasts, but it completely rescues the copper-deficiency phenotype in *COX15* and *SCO2* patient fibroblasts, both of which contain normal amounts of wild-type *SCO1*. Furthermore, the rescue of cellular copper levels in *COX15* patient fibroblasts overexpressing *SCO2* is attenuated if *SCO1* is simultaneously overexpressed. Together, these results argue that *SCO1* signaling is modified by, and acts downstream of, *SCO2*. Both *SCO*s have a conserved CXXXC copper-binding site, and they share an overall structural similarity to thio-redoxin reductases ([Chinenov, 2000](#)). A recent solution and crystal structure of the soluble domain of the copper conformer of human *SCO1* showed that the conserved cysteines on human *SCO1* can undergo thiol redox chemistry and suggested that oxidized *SCO1* may be an intermediate in the transfer of copper to the Cu_A site ([Banci et al., 2006](#)). One possibility, therefore, is that the function of *SCO2* is to catalyze the oxidation of *SCO1*, thereby facilitating copper transfer to its substrate. Mutant *SCO2* cannot fulfill this function efficiently, and, as a consequence, the proportion of reduced, copper-bound *SCO1* increases, signaling a state of cellular copper overload. The P174L mutation severely compromises the COX17-dependent metallation of *SCO1* ([Cobine et al., 2006b](#)), and we favor the idea that the mutation stabilizes a conformation mimicking the copper-bound form of the wild-type protein, the end result being the transduction of the same copper overload signal.

Although the exact nature of signaling mechanisms that are disturbed by the mutant *SCO* proteins remains to be determined, kinetic studies of ^{64}Cu uptake and efflux clearly show that reduced cellular copper levels are caused by an inability to retain copper rather than a defect in its high-affinity uptake and that the severity of this defect is determined by the specific mutations in a given patient background. A previous report suggested that copper uptake was increased in primary fibroblasts from *SCO2* patients without a change in retention time and that copper content was increased in primary *SCO2* myoblasts ([Jaksch et al., 2001b](#)). In that study, the evaluation of copper uptake and retention was based entirely on endpoint measurements, and uptake was inferred from a very long (24 hr) pulse. In contrast, our measurements were kinetic, and we were careful to measure initial rates to specifically evaluate high-affinity copper uptake.

The altered kinetics of copper efflux provide a mechanistic explanation for the observed correlation between

cellular copper levels and the number of missense alleles in different SCO2 patient fibroblast lines. It also explains why overexpression of the high-affinity copper uptake transporter CTR1 was only effective in rescuing the copper deficiency in cells with the slowest rate of copper efflux: SCO2 cells carrying two missense alleles. A defect in copper retention most likely reflects altered trafficking of a copper handling protein in response to aberrant signaling from mutant SCOs. Mnk is the only protein in fibroblasts that is known to lower abnormally high copper levels, by cycling between the *trans*-Golgi network and the plasma membrane (Hung et al., 1997; Petris et al., 1996). We have not been able to detect gross differences in Mnk localization by indirect immunofluorescence in control and SCO fibroblasts cultured under basal conditions or in the presence of either Cu-His or BCS (T.Y. Tao, J.D. Gitlin, S.C.L., and E.A.S., unpublished data). Only a small percentage of the total cellular Mnk pool, however, is required to effect rapid changes in cellular copper status (Pase et al., 2004), and this may escape detection using this method.

The observation that cellular copper homeostasis is disturbed in patients with COX assembly defects provides some insight into the tissue-specific etiology of disease progression. As the activity of COX is itself reduced in copper-deficiency states (Lee et al., 2001; Nose et al., 2006; Prohaska, 1983), lower cellular copper levels would serve to further exacerbate the COX deficiency in patients with existing COX assembly defects. Of the tissues that we and others (Jaksch et al., 2001b; Papadopoulou et al., 1999; Stiburek et al., 2005; Vesela et al., 2004) have been able to study in SCO patients, the liver and kidney in SCO2 patients are the only ones that maintain near normal levels of COX assembly and activity. Although pathological examination of the liver in SCO2 patients has revealed some abnormalities (enlargement and congestion; Papadopoulou et al., 1999), there is no indication of liver failure in SCO2 patients, all of whom die from cardiac insufficiency. Our results suggest that hepatic copper content in SCO2 neonates is sufficiently high to drive COX assembly.

The copper deficiency in patients with mutations in the SCOs or other COX assembly factors would be predicted to produce some of the features of nutritional copper deficiency, the extent of which would of course depend on the degree to which different tissues are copper deficient. Nutritional copper deficiency is associated with iron-deficiency anemia and with iron overload in the gut, liver, and spleen (Fox, 2003). Interestingly, severe, transfusion-dependent anemia has been reported in two patients with COX10 mutations (Antonicka et al., 2003), the etiology of which has remained obscure. It will therefore be important to create animal models to fully investigate the physiological relevance of SCO-dependent regulation of cellular copper homeostasis on iron metabolism.

In conclusion, this study has uncovered roles in cellular copper homeostasis for two metallochaperones, SCO1 and SCO2, that were previously known only to function in the assembly of the catalytic core of COX. We suggest

a model whereby SCO2 activity modulates either the thiol redox or metallation state of SCO1, which then acts as a molecular signal to regulate cellular copper efflux. These findings add two members to a growing list of bifunctional proteins that participate in cellular copper metabolism, including Mnk, Wnd, and XIAP (Hung et al., 1997; Mufti et al., 2006; Petris et al., 1996). Future studies will clarify the molecular mechanisms involved in SCO-dependent regulation of cellular copper homeostasis.

EXPERIMENTAL PROCEDURES

Tissue Culture

Primary fibroblasts from control, SCO1 (Valnot et al., 2000), and SCO2 (Jaksch et al., 2000, 2001a; Leary et al., 2006; Sacconi et al., 2003) patients were immortalized as previously described (Leary et al., 2004). All cells were grown in high-glucose DMEM supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO₂ and were tested to ensure that they were mycoplasma free (MycAlert, Cambrex) prior to harvesting.

Electrophoresis and Immunoblotting

Protocols for native and denaturing gel electrophoresis have been described elsewhere (Leary et al., 2004). Blots were decorated with monoclonal antibodies raised against porin (Calbiochem), 39 kDa, SDH70, COX IV, and core 1 (Molecular Probes) or polyclonal antisera raised against MnSOD, Cu/ZnSOD (Stressgen), *c-myc* (Santa Cruz), albumin (ICN), COX11, SCO1 (Leary et al., 2004), SCO2 (Jaksch et al., 2001b), CTR1 (Nose et al., 2006), Mnk, Atx1, Murr1, and Wnd (kind gift of J.D. Gitlin, Washington University School of Medicine). Polyclonal antiserum was raised against His-tagged, full-length recombinant COX17 in rabbits and then affinity purified (Pierce). Following incubation with the relevant secondary antibody, immunoreactive proteins were detected by luminol-enhanced chemiluminescence (Pierce).

Elemental Analyses

Tissue samples and cell pellets were digested in 40% nitric acid by boiling for 1 hr in capped, acid-washed tubes; diluted in ultra-pure, metal-free water; and analyzed by either ICP-OES (PerkinElmer, Optima 3100XL) or ICP-MS versus acid-washed blanks. Concentrations were determined from a standard curve constructed with serial dilutions of two commercially available mixed metal standards (Optima). Blanks of nitric acid with and without "metal spikes" were analyzed to ensure reproducibility.

⁶⁴Cu Experiments

Cells were plated in regular medium at 50%–75% confluency in 60 mm dishes 24 hr prior to experimentation. The following day, cells were washed twice in room-temperature PBS and incubated in Opti-MEM supplemented with 2 μM CuCl₂ for 30 min prior to the addition of 10 μCi/ml ⁶⁴Cu (Université de Sherbrooke). For uptake experiments, cells were harvested at the indicated time points by washing three times with ice-cold PBS and lysing in PBS supplemented with 1% Triton X-100, 0.1% SDS, and 1 mM EDTA. Cell lysates were counted using a γ counter (Canberra), corrected for ⁶⁴Cu decay, and normalized for protein content. Incubation of a representative plate from each cell line in ice-cold Opti-MEM for 15 min on ice was used as a background correction for nonspecific adherence of ⁶⁴Cu to either the plate or the cells. For retention experiments, cells were treated as described above and incubated in ⁶⁴Cu-containing Opti-MEM for 2 hr (t = 0), at which point plates were rinsed twice with room-temperature PBS and replenished with DMEM supplemented with 10% FBS and a 1% penicillin/streptomycin mixture (GIBCO) for up to 12 hr. Cells were harvested and counted at various time points as described above.

Miscellaneous Procedures

RNAi constructs were designed using a web-based algorithm (<http://www.genscript.com/rnai.html#design>). Two pairs of shRNA oligonucleotides targeting different regions of the 3'UTR of the SCO1 mRNA were ultimately selected: 5'-GATCTGTTATTAGCCACCAAGAAGCTTTGATATCCGAGTCTTGGTGGCTAATAATTTTTTC-3' and 5'-TCGAGAAAAAATTATTAGCCACCAAGAAGCTCGGATATCAAAGTTCTTGGTGGCTAATAACA-3', and 5'-GATCTGTATATAGGCTCCTATGCGATTGATATCCGTCGCATAGGAGCCTATATATTTTTTC-3' and 5'-TCGAGAAAAAATATATAGGCTCCTATGCGACGGATATCAATCGCATAGGAGCCTATATACA-3'. For shRNA knockdown of SCO2 mRNA, a pair of oligonucleotides, 5'-GATCTGTGTCAGTGACCAAGCAGGATGATATCCGCTCGTCTTGGTCCACTGATTTTTTC-3' and 5'-TCGAGAAAAATCAGTGGACCAAGCAGGATCAACTCGTCTTGGTCCACTGACA-3', which target a sequence contained within the first exon, were selected. Primers were allowed to self-anneal, digested with BglII and XhoI, and ligated into a commercially available retroviral expression vector (pSUPER, Invitrogen). SCO1 P174L and SCO2 E140K point mutants were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (Hornig et al., 2005), while wild-type cDNAs were amplified by RT-PCR. All constructs were cloned into retroviral expression vectors. Phoenix amphotropic cells (G. Nolan, Stanford University) were used to transiently produce and package all individual human cDNA constructs of interest; subsequent infection and selection of cell lines were performed as previously described (Leary et al., 2004). Isolated mitochondria were prepared and protein concentration, COX, and citrate synthase activities were measured as described previously (Leary et al., 2004).

ACKNOWLEDGMENTS

The authors would like to acknowledge all of the patients and their families, without whom this study would not have been possible. We thank S. Labbé for coordinating synthesis and delivery of ⁶⁴Cu from the core facility at the Université de Sherbrooke. Polyclonal CTR1 antiserum was a kind gift of D.J. Thiele (Duke University), while Mnk, Wnd, Atox1, and Murr1 polyclonal antibodies were kindly provided by J.D. Gitlin (Washington University). M. Tarnopolsky (McMaster University) provided tissues from a SCO2 patient. This research was supported by grants from the CIHR (E.A.S.), MDA (E.A.S. and S.C.L.), and NIH (#CA61286, D.R.W.). S.C.L. was supported by a postdoctoral fellowship from the CIHR. E.A.S. is an International Scholar of the Howard Hughes Medical Institute and a Senior Investigator of the CIHR.

Received: September 25, 2006

Revised: November 23, 2006

Accepted: December 7, 2006

Published: January 2, 2007

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