Human Molecular Genetics, 2009, Vol. 18, No. 12 **2230–2240**
doi:10.1093/hmg/ddp158
Advance Access published on March 31, 2009

Human SCO2 is required for the synthesis of CO II and as a thiol-disulphide oxidoreductase for SCO1

Scot C. Leary[1,†], Florin Sasarman[1,†], Tamiko Nishimura[1] and Eric A. Shoubridge[1,2,*]

[1]Montreal Neurological Institute, McGill University, 3801 University Street, Montreal, Quebec, Canada H3A 2B4 and [2]Department of Human Genetics, McGill University, Montreal, Canada H3A 2B4

Received February 6, 2009; Revised and Accepted March 25, 2009

Human *SCO1* and *SCO2* code for essential metallochaperones with ill-defined functions in the biogenesis of the Cu_A site of cytochrome *c* oxidase subunit II (CO II). Here, we have used patient cell lines to investigate the specific roles of each SCO protein in this pathway. By pulse-labeling mitochondrial translation products, we demonstrate that the synthesis of CO II is reduced in *SCO2*, but not in *SCO1*, cells. Despite this biosynthetic defect, newly synthesized CO II is more stable in *SCO2* cells than in control cells. RNA_i-mediated knockdown of mutant SCO2 abolishes CO II labeling in the translation assay, whereas knockdown of mutant SCO1 does not affect CO II synthesis. These results indicate that SCO2 acts upstream of SCO1, and that it is indispensable for CO II synthesis. The subsequent maturation of CO II is contingent upon the formation of a complex that includes both SCO proteins, each with a functional CxxxC copper-coordinating motif. In control cells, the cysteines in this motif in SCO1 exist as a mixed population comprised of oxidized disulphides and reduced thiols; however, the relative ratio of oxidized to reduced cysteines in SCO1 is perturbed in cells from both *SCO* backgrounds. Overexpression of wild-type SCO2, or knockdown of mutant SCO2, in *SCO2* cells alters the ratio of oxidized to reduced cysteines in SCO1, suggesting that SCO2 acts as a thiol-disulphide oxidoreductase to oxidize the copper-coordinating cysteines in SCO1 during CO II maturation. Based on these data we present a model in which each SCO protein fulfills distinct, stage-specific functions during CO II synthesis and Cu_A site maturation.

INTRODUCTION

Cytochrome *c* oxidase (COX) catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen, a biochemical reaction that contributes to the proton gradient essential for aerobic ATP production. In humans, COX is a multimeric protein complex comprised of 13 structural subunits that is embedded in the inner mitochondrial membrane. Its core is composed of the three mitochondrially encoded subunits (CO I–III). Highly conserved domains in subunits I and II contain the two heme (a, a_3) and two copper (Cu_A, Cu_B) moieties that are critical for catalytic activity (1,2). The nuclear-encoded subunits surround the catalytic core, conferring structural stability, and providing sites for allosteric modulation of enzyme activity (3). Assembly of individual structural subunits into a functional holoenzyme is a complex process, with the biogenesis of three discrete assembly intermediates, termed S1–S3, preceding the formation of the fully mature oxidase (4). At least 20 of the 30 genetic complementation groups associated with isolated COX deficiency in yeast encode factors dedicated to various aspects of holoenzyme assembly (5,6). Roughly half of these genes have human homologues.

To date, pathogenic mutations in six genes encoding COX assembly factors have been identified, all of which are associated with early onset, autosomal recessive disorders with fatal clinical outcomes: *LRPPRC* (7), *COX10* (8), *COX15* (9), *SURF1* (10,11), *SCO1* (12) and *SCO2* (13,14). Mutations in *FASTKD2* (15) and *ETHE1* (16,17) also produce severe, tissue-specific COX deficiencies, although neither gene product is thought to directly participate in holoenzyme assembly. *SCO1* and *SCO2* are paralogous genes that code for integral membrane metallochaperones with poorly understood roles in the biogenesis of the Cu_A site of CO II. They share a high degree of sequence similarity, particularly in the C-terminal region, which protrudes into the mitochondrial

*To whom correspondence should be addressed. Tel: +1 5143988523; Fax: +1 5143981509; Email: eric@ericpc.mni.mcgill.ca
†The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

intermembrane space (IMS), and contains a conserved CxxxC copper-binding motif (14,18–20). Both genes were first identified in yeast as high copy suppressors of a *COX17Δ* strain; however, only the deletion of *SCO1* results in a respiratory phenotype (21), and a function for yeast SCO2 is yet to be described. In contrast, both *SCO1* and *SCO2* are essential in humans, with mutations in either gene causing a severe COX deficiency. *SCO2* mutations are associated primarily with neonatal encephalocardiomyopathy, while *SCO1* mutations cause neonatal hepatic failure and ketoacidotic coma. These distinct clinical phenotypes are not the 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 (14).

All reported *SCO2* patients carry an *E140K* missense mutation. In general, patients are either homozygous for this mutation or are compound heterozygotes, although hemizygosity at the *SCO2* genomic locus has been reported (22). Patients homozygous for the *E140K* mutation have a delayed onset of the disease pathology and a more prolonged course of disease (23). *SCO1* mutations have only been reported in a single pedigree. Patients were compound heterozygotes with a nonsense mutation on one allele and a *P174L* missense mutation on the second allele (12).

Although functional studies (24,25) and phylogenetic analyses (26,27) support the notion that SCO proteins fulfill multiple functions, it remains unclear why some eukaryotes only need a single SCO protein for COX assembly while others require two SCO proteins. Molecular, genetic and biochemical analyses of *SCO1* and *SCO2* patient cell lines have demonstrated that SCO proteins have independent but cooperative functions in the maturation of the binuclear, mixed valence Cu_A site in CO II at the S2 stage of holoenzyme assembly (19). The ability of each SCO protein to bind both Cu(I) and Cu(II) is essential for its function (28–30). Cu(I) binding requires the cysteine thiols in the CxxxC motif as well as a conserved axial histidyl ligand (30–32). Both the *P174L* mutation in SCO1 and the *E140K* mutation in SCO2 are adjacent to their respective CxxxC motifs, and are thought to compromise either copper binding or transfer by altering the redox state of the cysteine thiols. Both SCO proteins receive copper from COX17 (28,33,34). SCO1 in turn has been proposed to transfer copper to newly synthesized CO II in a reaction that depends on the ability of the cysteine thiols in the CxxxC motif to undergo redox chemistry (28). It is not clear, however, what role SCO2 plays in this process. We therefore investigated the relative roles of both SCO proteins in the biogenesis of the Cu_A site of CO II and show that while only SCO2 is required for CO II synthesis, both SCO proteins are required for copper-loading during its subsequent maturation, with SCO2 acting as a thiol-disulphide oxidoreductase to oxidize the copper-binding cysteines of SCO1.

RESULTS

SCO2 mutations impair CO II synthesis and delay its turnover

To investigate the importance of SCO2 function in CO II synthesis and maturation, we pulse-labeled, then chased, the mitochondrial translation products in control and *SCO2* myoblasts (Fig. 1A and B). The rate of CO II synthesis was reduced in *SCO2* cells [64.0 $\pm$ 5.3% of control ($n = 7$)], but the stability of the newly synthesized polypeptide increased 2-fold [202.0 $\pm$ 11.6% of control ($n = 7$)]. In contrast, we observed a severe reduction in the stability of newly synthesized CO II, without a significant change in its rate of synthesis, in fibroblasts derived from a *SCO1* patient (Fig. 1C and D) (34).

To exclude the possibility that these specific phenotypes could be explained by the difference in the severity of the COX deficiency in the patient cell lines (24% of control in *SCO1*, 51% in *SCO2*), we performed similar experiments in fibroblasts from *SURF1*, *COX10* and *COX15* patients, in which residual COX activity varied from 9% to 52% of control. Mutations in all three genes reduced the stability of nascent CO I and CO III, while those in *COX10* and *COX15* also decreased the rate of CO II and CO III synthesis (Supplementary Material, Fig. S1). We conclude that the magnitude of the COX deficiency *per se* does not determine the nature of the defect in the expression of the mitochondrially encoded COX subunits, and that of the assembly factors we analyzed, only mutations in *SCO1* and *SCO2* alter both the rate of CO II synthesis and turnover.

SCO2 is a molecular chaperone for CO II

The distinct effects of mutations in *SCO1* or *SCO2* on the synthesis and turnover of CO II suggest that SCO2 acts upstream of SCO1 on the newly synthesized polypeptide. To test whether SCO2 is essential for CO II synthesis, we transiently knocked it down in control and *SCO2* myoblasts (Fig. 2). SCO2 protein was reduced to 5–10% of parental levels in control cells (Fig. 2A and C), and it was undetectable in *SCO2* cells (Fig. 2C). Knockdown of SCO2 in control cells reduced the rate of CO II synthesis by approximately 50%, and resulted in a relative stabilization of newly synthesized CO II (171% of parental; Fig. 2B). CO II synthesis was undetectable in the patient cells, and this was associated with a severe reduction in COX content and a marked accumulation of the S2 assembly intermediate (Fig. 2B and D). These data argue that the reduced rates of CO II synthesis and turnover in *SCO2* patient cells result from low levels of SCO2 protein, and are not attributable to the mutant protein *per se*. We conclude that SCO2 plays an essential role in the synthesis of CO II.

The low residual levels of mutant SCO2 in the patient background (19,35,36) argue that the E140K mutation perturbs the structure of the protein, which in turn promotes its turnover. *In vitro*, E140K SCO2 binds roughly half the molar equivalents of Cu(I) when compared with wild-type SCO2 (37), suggesting that copper-binding is critical to both protein stability and function. To further address the importance of copper binding to SCO2 function during CO II synthesis *in vivo*, we performed mitochondrial translation experiments in control and *SCO2* myoblasts overexpressing wild-type or mutant SCO2 proteins (Supplementary Material, Fig. S2). In *SCO2* cells, the defect in CO II synthesis was functionally complemented by overexpressing either wild-type or E140K SCO2 (% of parental; 172 and 140, respectively), while it was exacerbated by overexpressing a SCO2 variant incapable of

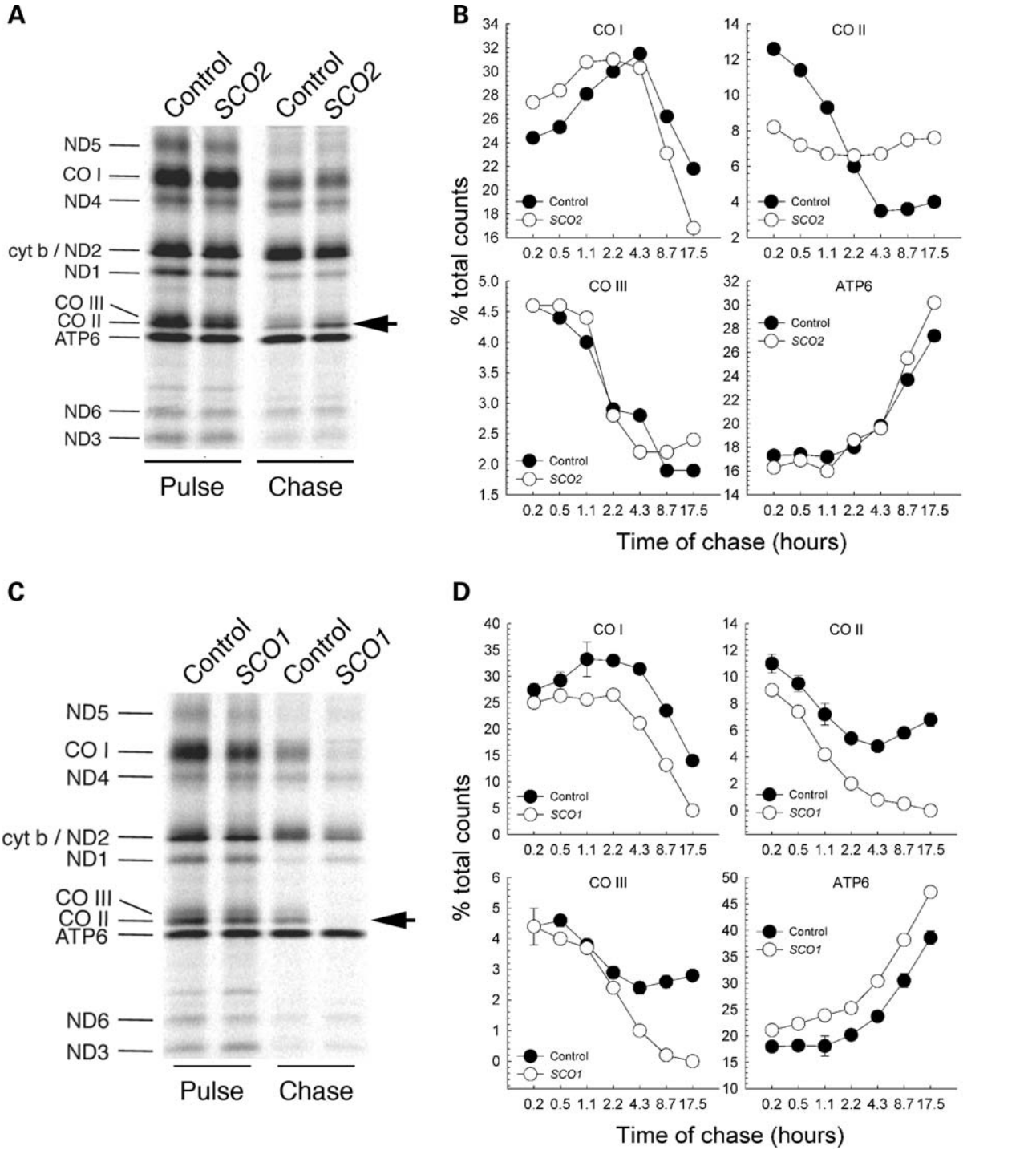

Figure 1. *SCO2* mutations result in a specific defect in CO II expression, impairing its rate of productive synthesis and delaying its subsequent turnover. (**A, C**) Mitochondrial translation products were pulse-labeled for 1 h and then chased for 17.5 h in control and *SCO2* myoblasts (A), and in control and *SCO1* fibroblasts (C), as described in the Materials and Methods section. Equal amounts of protein (50 μg) were then denatured in sample loading buffer by sonication, and fractionated by SDS–PAGE on a 12–20% gradient gel, which was then dried for autoradiography. The mitochondrial translation products comprising six Complex I subunits (ND1–ND6), one Complex III subunit (cyt b), three Complex IV subunits (CO I–III) and one Complex V subunit (ATP6) are identified on the left. The arrow on the right denotes radiolabeled CO II. (**B, D**) Mitochondrial translation products were pulse-labeled for 1 h and then serially sampled over a 17.5-h chase to assess the relative stability of CO I, CO II and CO III. For all samples, the abundance of each mitochondrially encoded COX structural subunit was quantified using ImageQuant software (Molecular Dynamics/GE Healthcare), and then divided by the total counts within the lane to normalize for differences in loading. ATP6 levels, which are selectively stabilized by the pre-treatment with chloramphenicol, and therefore appear to increase during the chase period, are plotted for comparative purposes. All data points for control and *SCO2* myoblasts, and for *SCO1* fibroblasts, were collected from a single experiment, while those for the control fibroblast line represent the mean ± SEM of three independent experiments. The raw data for *SCO1* fibroblasts and for one of the replicates of the control fibroblast line presented in panel (D) have been published elsewhere (34), but have been re-analyzed as described above and are presented here to allow for a direct comparison with the phenotypic effects of *SCO2* mutations on CO II synthesis and its subsequent turnover.

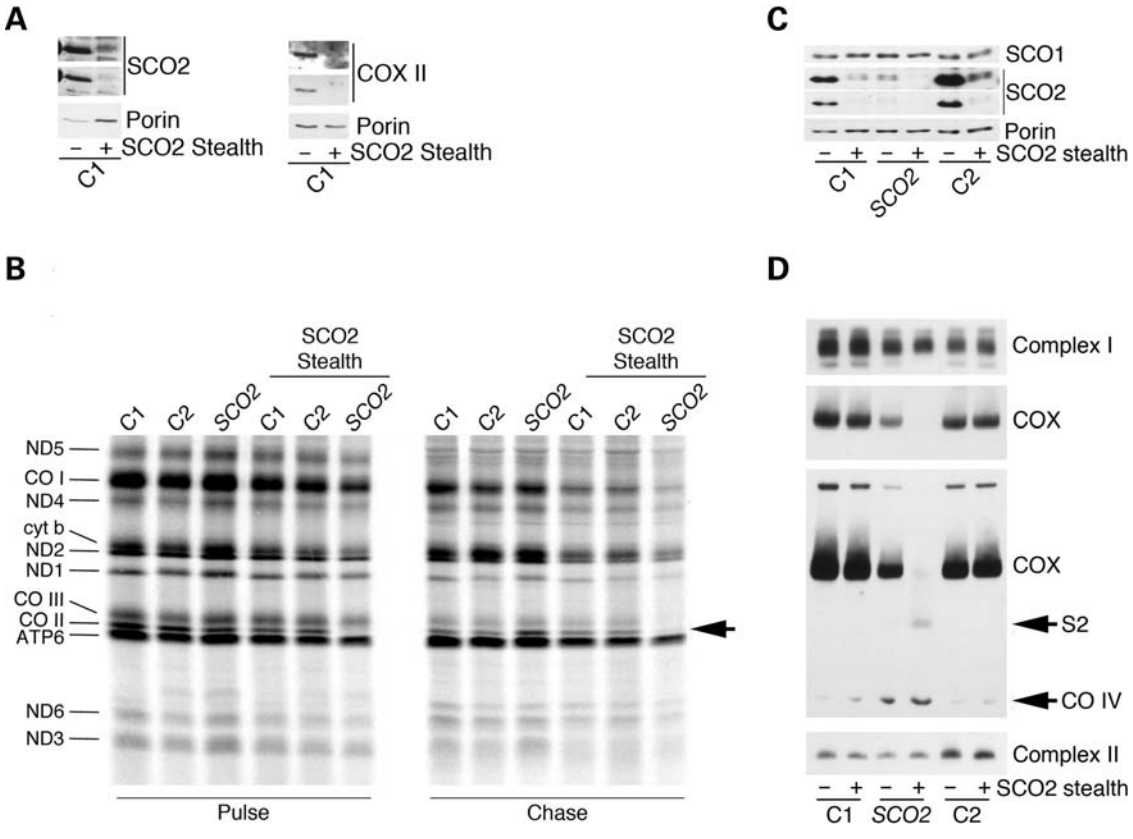

Figure 2. SCO2 is required as a chaperone for newly synthesized CO II. Control (C1, C2) and *SCO2* myoblasts alone, and those in which the steady-state levels of SCO2 were transiently knocked down using Stealth siRNA (Invitrogen), were used for western blotting (**A, C**), pulse-chase labeling of mitochondrial translation products (**B**) and native PAGE analyses (**D**). The results of two independent experiments are shown (A, B versus C, D). Comparable knockdown of SCO2 was achieved in both experiments [compare C1 in A (left hand side) versus in C], and the residual COX activity in *SCO2* myoblasts was <5% of that of the parental line (data not shown); however, a more severe reduction in COX content was observed in controls in the first experiment [compare CO II steady-state levels (A, right hand side) versus holoenzyme content (D) in C1]. The arrow on the right in (B) denotes radiolabeled CO II. In (D) unassembled CO IV and the S2 assembly intermediate are indicated.

binding copper, the result of Cys to Ala substitutions in the CxxxC motif (61% of parental) (30). These data suggest that productive CO II synthesis depends not only on SCO2 abundance but also on the ability of the cysteines in the CxxxC motif to either bind copper or undergo redox chemistry.

SCO1 function is not necessary for CO II synthesis

Although the mitochondrial translation experiments in patient cell lines do not point to a role for SCO1 in the synthesis of CO II, it is possible that SCO1 facilitates this process in a manner that is not perturbed by the *P174L* missense mutation. To firmly rule out a role for SCO1 in CO II synthesis, we pulse-labeled mitochondrial translation products in *SCO1* fibroblasts in which the mutant protein had been stably knocked down below immunologically detectable levels (Fig. 3A and B) (25). The rate of CO II synthesis was not different than in the parental cells (98% of parental); however, the stability of the newly synthesized polypeptide was further reduced (60% of parental; Fig. 3A). This resulted in reduced levels of fully assembled COX and a relative enrichment in the abundance of the S2 assembly intermediate (Fig. 3C). These data confirm that CO II synthesis does not require SCO1 function, and provide additional evidence that SCO1 plays a crucial role in stabilizing CO II during its subsequent maturation.

To further dissect the relative roles of SCO1 and SCO2 in CO II synthesis and turnover, we tested the effect of overexpressing wild-type or P174L SCO1 in control and *SCO2* myoblasts (Fig. 4). Overexpression of either SCO1 construct in the *SCO2* patient background exacerbated the defect in CO II synthesis (% of parental; 57 and 58, respectively), leading to a decrease in the amount of fully assembled COX, and a relative increase in the abundance of the S2 assembly intermediate (Fig. 4B); however, only overexpression of wild-type SCO1 reduced the accumulation of newly synthesized CO II in the chase (% of parental; 36 versus 77). These data suggest that the relative ratios of the SCO proteins are crucial for the correct processing and maturation of CO II, and further support a model in which SCO1 functions downstream of SCO2.

Overexpression of SCO2 or a SCO1 copper-binding mutant reduces the initial turnover rate of newly synthesized CO II in the *SCO1* background

To investigate the basis for enhanced turnover of the newly synthesized CO II polypeptide in *SCO1* cells, we manipulated the relative ratio of the two SCO proteins by overexpressing

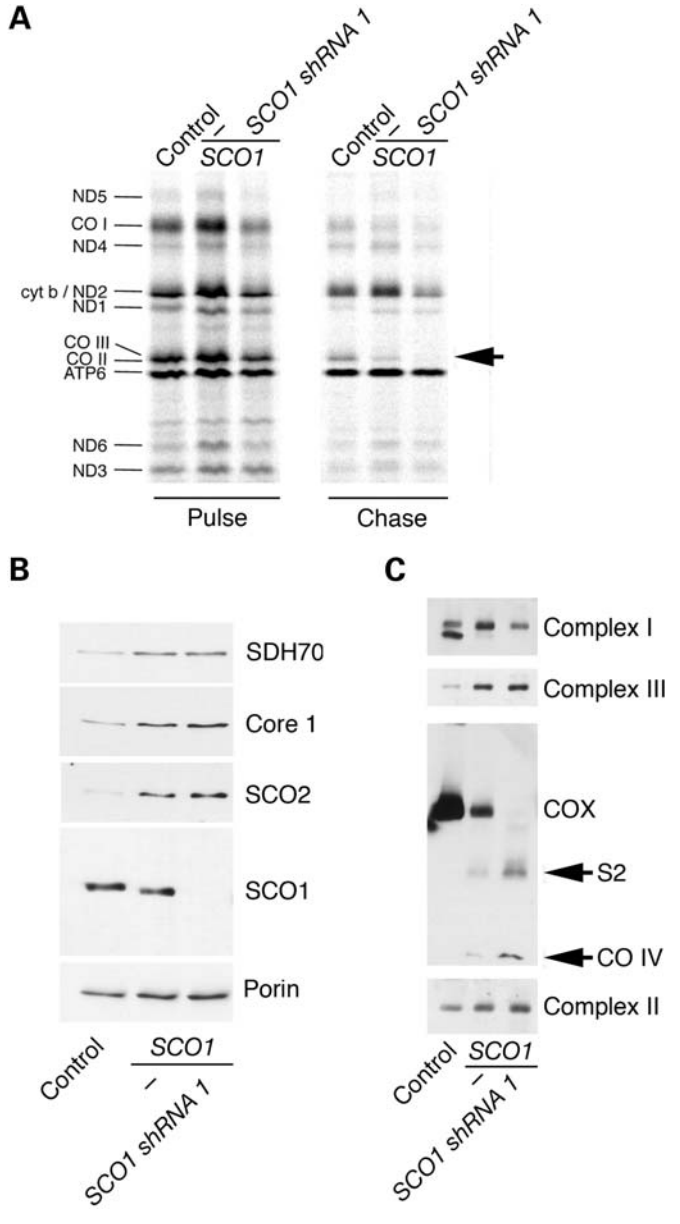

Figure 3. SCO1 is required for CO II maturation but not for polypeptide synthesis. SCO1 steady-state levels were stably knocked down by shRNA in *SCO1* fibroblasts (25), which effectively generated a *SCO1* null background. Control and *SCO1* fibroblasts alone (−), along with the *SCO1* null background, were then used for pulse-chase labeling (**A**), western blotting (**B**) and native PAGE (**C**) analyses. The arrow on the right in (A) denotes radiolabeled CO II. In (C) unassembled CO IV and the S2 assembly intermediate are indicated.

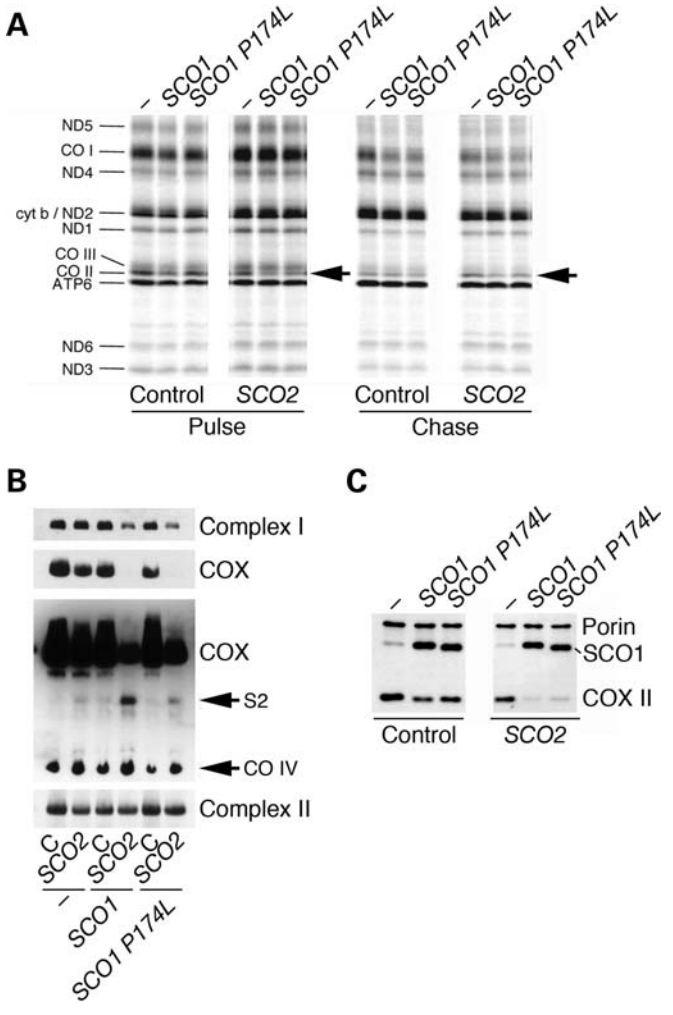

Figure 4. The downstream function of SCO1 during CO II maturation is tightly coupled to that of SCO2. Control and *SCO2* myoblasts alone (−), and those overexpressing either wild-type or P174L SCO1, were used for pulse-chase labeling (**A**), native PAGE analyses (**B**) and western blotting (**C**). The arrows on the right in (A) denote radiolabeled CO II. In (B) unassembled CO IV and the S2 assembly intermediates are indicated.

either wild-type SCO2 or a SCO1 variant incapable of binding copper (30) (Fig. 5). Control and *SCO1* fibroblasts overexpressing wild-type and P174L SCO1 were included in these analyses as positive controls (34). Mitochondrial translation products were pulsed, and the label was chased for 1, 2 and 17.5 h. No changes in the rate of CO II synthesis were observed as a result of overexpressing any of the SCO variants. Overexpression of wild-type SCO2 or the SCO1 copper-binding mutant also failed to increase the overall stability of CO II at the endpoint of the chase (17.5 h); however, both of these SCO variants delayed the initial rate of CO II turnover, the kinetics of which were comparable to those in *SCO1* fibroblasts overexpressing wild-type SCO1 over the first 2 h of the chase (Fig. 5A). Overexpression of each variant also led to a relative enrichment in the abundance of the S2 assembly intermediate (Fig. 5B). These data suggest that the stabilization of newly synthesized CO II is contingent upon the formation of a complex that includes SCO1 and SCO2. The delay in the initial turnover rate of CO II in *SCO1* fibroblasts overexpressing a SCO1 copper-binding mutant further suggests that the redox-active cysteines of the CxxxC motif of SCO1 are essential for the maturation of CO II from this complex.

The redox state of the copper-coordinating cysteines in SCO1 is perturbed in both *SCO* patient backgrounds

The data presented thus far suggest that progression through the S2 stage of holoenzyme assembly is stalled in *SCO*

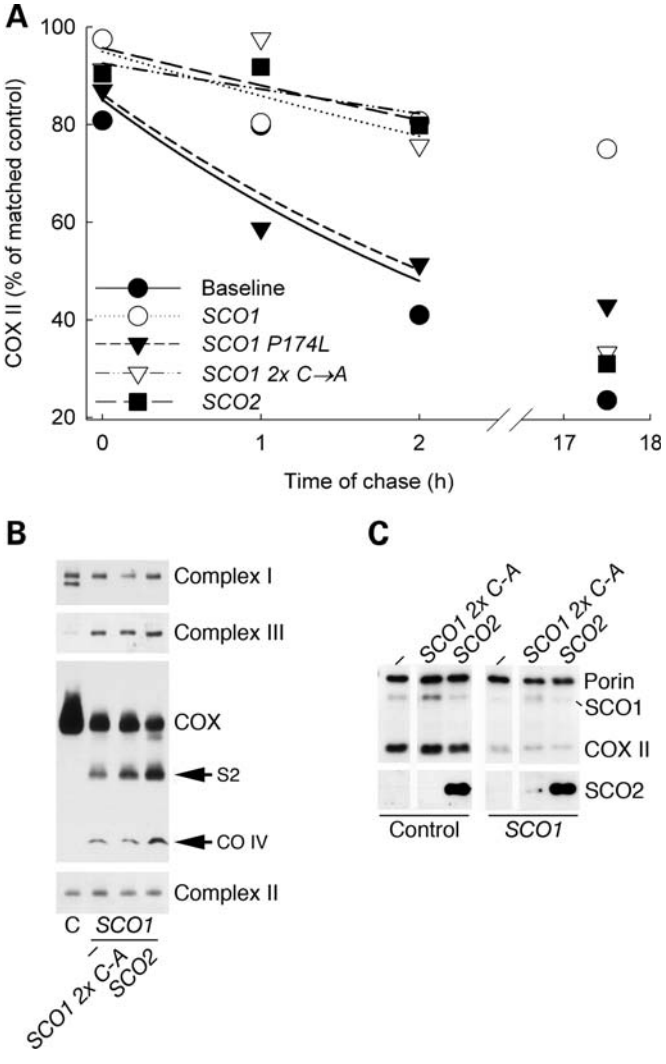

Figure 5. Overexpression of wild-type SCO2 or a SCO1 copper-binding mutant delays the initial kinetics of CO II turnover in the *SCO1* background, and leads to the accumulation of the S2 assembly intermediate. (**A**) Mitochondrial translation products were pulse-labeled and chased for 1, 2 and 17.5 h in control and *SCO1* fibroblasts alone (baseline), and in control and *SCO1* fibroblasts overexpressing SCO2, wild-type SCO1, P174L SCO1 or SCO1 2× C→A, a SCO1 variant incapable of binding copper (30). (**B**) Native PAGE analysis was conducted using control and *SCO1* fibroblasts alone (−), and *SCO1* fibroblasts overexpressing SCO2 or SCO1 2× C→A. Unassembled CO IV and the S2 assembly intermediates are indicated. Holoenzyme assembly was unaffected in controls overexpressing either of these constructs (data not shown). (**C**) Western blot analysis demonstrating stable overexpression of either SCO2 or SCO1 2× C→A in control and *SCO1* fibroblasts.

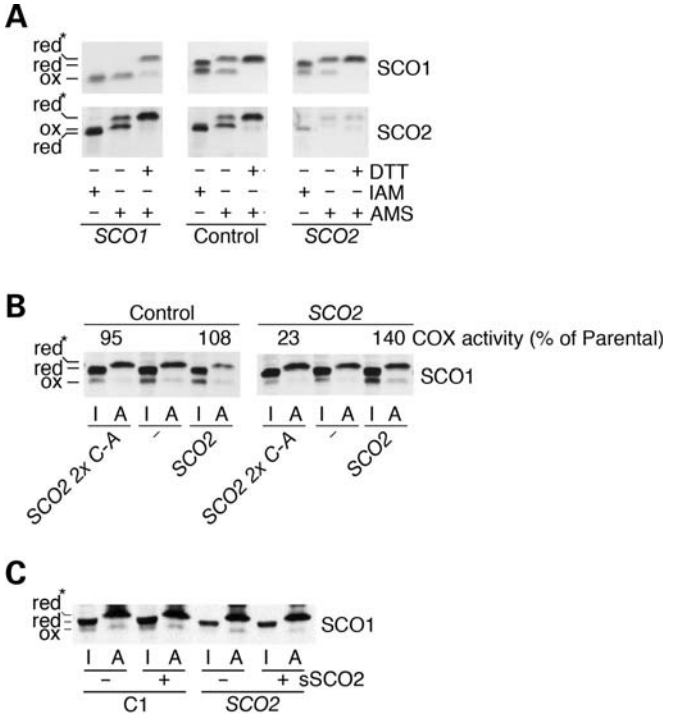

Figure 6. SCO2 acts as a thiol-disulphide oxidoreductase to oxidize the cysteines of SCO1 during the maturation of CO II. (**A**) Isolated mitochondria (2 mg/ml) from control, *SCO1* and *SCO2* fibroblasts were first incubated in the presence or absence of the reductant dithiothreitol (DTT), followed by a second incubation with one of the two alkylating agents to irreversibly modify those cysteines containing reduced thiols; iodoacetamide (IAM) or 4-acetoamido-4'-maleimidylstilbene-2,2'-disulphonic acid (AMS; see the Materials and Methods section). Species of SCO1 and SCO2 containing oxidized (ox) cysteines were then resolved from those with reduced (red) cysteines by non-reducing SDS–PAGE, and detected by immunoblotting with the appropriate polyclonal antisera. Red* refers to reduced thiols that were modified in the presence of AMS. (**B**) Whole cell extracts (2 mg/ml) from control and *SCO2* myoblasts alone (−), and those overexpressing SCO2 or SCO2 2× C→A, a SCO2 variant incapable of binding copper (30) were treated with either IAM (I) or AMS (A) and analyzed as described in (A). (**C**) Whole cell extracts (2 mg/ml) from control and *SCO2* myoblasts alone (−), and those treated with siRNA (Invitrogen) to transiently knockdown SCO2 abundance (see Fig. 2C lanes 5 versus 6 for control myoblasts, and 3 versus 4 for *SCO2* myoblasts) were treated with either IAM (I) or AMS (A) and analyzed as outlined in (A).

patient cells because of the impaired ability of the cysteines in the CxxxC motif of SCO1 to undergo changes in redox chemistry. To test whether the redox state of the cysteines of SCO1 is significantly perturbed in *SCO* patient backgrounds, we isolated mitochondria from control, *SCO1* and *SCO2* fibroblasts and incubated them in buffer alone or buffer containing the reductant dithiothreitol (DTT). We then treated them with iodoacetamide (IAM), an agent that irreversibly alkylates cysteine thiols, or 4-acetoamido-4'-maleimidylstilbene-2,2'-disulphonic acid (AMS), an alkylating agent that adds a 0.5 kDa moiety per free thiol group, and fractionated the samples under non-reducing conditions to resolve the oxidized and reduced species (Fig. 6A). P174L SCO1 is present essentially as a single population containing oxidized disulphides in *SCO1* fibroblasts (reduced:oxidized cysteines; 5.4% of control). In contrast, SCO1 exists as a mixed population consisting of species with either reduced thiols or oxidized disulphides in both control and *SCO2* fibroblasts; however, the proportion of the total pool of SCO1 that contains reduced thiols is enriched in the *SCO2* patient background (reduced:oxidized cysteines; 193.0% of control).

The over-representation of SCO1 species that contain reduced thiols in the *SCO2* patient background suggests that SCO2 acts as a thiol-disulphide oxidoreductase to regulate the redox state of the cysteines in SCO1. To test this hypothesis, we treated control and *SCO2* fibroblasts (data not shown) and myoblasts (Fig. 6B) overexpressing wild-type SCO2 or

a SCO2 variant incapable of binding copper (30) with either IAM or AMS and fractionated them under non-reducing conditions. Only the overexpression of wild-type SCO2 in the patient background enriched for the presence of SCO1 species containing oxidized disulphides [reduced:oxidized cysteines (% of parental): SCO2 2× C→A, 96.8; SCO2, 37.6]. Consistent with a role for SCO2 in oxidizing the cysteines in the CxxxC motif of SCO1, knockdown of mutant SCO2 in the patient background (Fig. 2D) further enriched for the presence of reduced thiols in the total SCO1 pool [Fig. 6C; reduced:oxidized cysteines (% of control): *SCO2*, 208.3; *SCO2* + sSCO2, 339.0]. These data argue that SCO2 is a bona fide thiol-disulphide oxidoreductase, and that its ability to oxidize the cysteine thiols of SCO1, which is critical to CO II maturation (Fig. 3), depends on its CxxxC copper-binding motif.

DISCUSSION

This study demonstrates that human SCO1 and SCO2 have tightly coupled, but distinct, stage-specific functions in the maturation of the Cu_A site in CO II. First, distinct defects in both CO II synthesis and turnover are observed in cells derived from *SCO1* and *SCO2* patients, but not in those from patients with other isolated COX deficiencies. Second, RNA_i knockdown experiments and overexpression studies show that SCO2, but not SCO1, is required for productive CO II synthesis. Third, while both SCO proteins participate in the maturation of CO II, the biogenesis of the Cu_A site depends on the ability of SCO2 to oxidize the copper-binding cysteines in SCO1. Finally, genetic manipulations that exacerbate defects in CO II expression in either patient background lead to the increased abundance of the S2 assembly intermediate.

Knockdown of SCO2 in control cells recapitulated the biosynthetic defect in CO II observed in *SCO2* cells, while the lack of immunologically detectable mutant protein in *SCO2* cells treated with RNA_i effectively eliminated productive CO II synthesis. We propose that the association of CO II with SCO2 is required immediately following its synthesis, perhaps for the recruitment of SCO1, otherwise the newly synthesized protein is rapidly turned over. This may involve physical interactions with COX18 or COX20, additional COX assembly factors with roles in inserting CO II into the membrane or chaperoning it during its subsequent maturation (38). Alternatively, the steady-state levels of SCO2 may directly affect the synthesis of CO II mRNA. Although this mechanism seems less likely given that the CxxxC motif of SCO2 is localized to the IMS and translation occurs in the mitochondrial matrix, we cannot exclude the possibility that a decrease in SCO2 abundance downregulates the expression of a specific CO II translational activator.

The *E140K* mutation, which is common to all reported *SCO2* patients, lies adjacent to the conserved CxxxC copper-binding motif (13,14,36). Although recombinant E140K SCO2 binds copper relatively weakly *in vitro* (37), overexpression of the mutant protein in *SCO2* cells functionally complements the COX deficiency (25), a result that we show here is directly attributable to its ability to rescue the defect in CO II synthesis. In contrast, overexpression of a SCO2 variant incapable of binding copper (30) exacerbates the CO II biosynthetic defect in *SCO2* cells. These results suggest that normal rates of CO II synthesis depend on the redox-active cysteines in the CxxxC motif of SCO2, likely for association with, and direct transfer of copper to, newly synthesized CO II. They are also consistent with earlier proposals that the mutant protein retains appreciable function, and that the predominant effect of the missense mutation is to destabilize the protein (22,25,36). It is clear, however, from the RNA_i knockdown experiments (Fig. 2C and D, lanes 2 versus 3) that E140K SCO2 is not functionally equivalent to the wild-type protein, since roughly equimolar amounts of SCO2 protein in the control knockdown cells are able to sustain greater levels of holoenzyme activity and content when compared with the patient background alone.

In addition to its role in CO II synthesis, the rapid and complete stabilization of nascent CO II in *SCO2* cells (Fig. 1B) indicates that SCO2 function is also required during CO II maturation. The ability to recapitulate this relative stabilization in two control myoblast lines in which the levels of SCO2 were severely reduced by RNA_i further argues that this stage of CO II maturation is directly affected by SCO2 abundance. However, its CxxxC motif is also clearly relevant as newly synthesized CO II fails to accumulate in *SCO2* myoblasts overexpressing a SCO2 variant incapable of binding copper (30). While the wild-type function of SCO2 is contingent upon its ability to bind both Cu(I) and Cu(II) (30), its metallation state within the IMS remains unknown. Its copper-binding cysteines, like those of E140K SCO2, are reduced (Fig. 6A) and therefore primed for ligand-exchange reactions with their copper donor COX17 (33). Why then are there such low residual levels of mutant protein, given the relatively mild effects of the *E140K* mutation on SCO2 function? One possible explanation is that its stability is dependent on being copper-loaded and, analogous to the *P174L* mutation in SCO1, the E140K substitution compromises the ability of SCO2 to interact with COX17.

What specific function does SCO2 fulfill in the early stages of CO II maturation? In addition to their CxxxC motifs, SCO proteins contain a highly conserved thioredoxin fold (39), which was recently shown to confer thiol-disulphide oxidoreductase activity in PrrC, the SCO orthologue in *Rhodobacter* (24) and in SCO1 in *Thermus thermophilus* (40). Although purified, soluble truncates of human SCO1 and SCO2 do not exhibit such an activity *in vitro* (36,41), our results demonstrate that the cysteines in SCO1 are disproportionately reduced in *SCO2* cells. Consistent with a role for SCO2 as a thiol-disulphide oxidoreductase that regulates the redox state of the cysteines in SCO1, knockdown of mutant SCO2 in the patient background further enriches for the presence of reduced thiols in SCO1. Overexpression of wild-type SCO2, but not a SCO2 copper-binding mutant, in *SCO2* fibroblasts and myoblasts increases the relative proportion of disulphides in SCO1, arguing that the CxxxC motif in SCO2 is essential to catalyze the oxidation of the cysteine thiols in the CxxxC motif of SCO1 as CO II is matured. In *SCO2* cells, the cysteines of the mutant protein are disproportionately reduced relative to those of SCO2 in control cells, and limit its ability to maintain the appropriate stoichiometry of

oxidized to reduced cysteines in SCO1. The lack of comparable effects in control cells in these experiments suggests, however, that only a fraction of the total SCO2 within the inner mitochondrial membrane is required to fulfill this aspect of its function. While SCO1 is clearly not acting in a reciprocal manner on the copper-binding cysteines of SCO2, our data do not exclude the possibility that it also possesses a thiol-disulphide oxidoreductase activity that is important in regulating the redox state of the cysteines of other proteins within the IMS (41).

The results presented in this study allow us to propose the following model for the relative roles of human SCO1 and SCO2 in regulating the synthesis, maturation and insertion of CO II into the nascent holoenzyme complex (Fig. 7). First, SCO2 interacts with newly synthesized CO II, either as it is inserted into the inner membrane or immediately thereafter in a manner that depends on its ability to bind copper. Second, the physical interaction between SCO2 and CO II triggers the recruitment of SCO1 to the SCO2–CO II complex, and metallation of SCO1 by COX17. Third, copper is delivered to CO II sequentially by each SCO protein to form the Cu_A site, an event that results in the dissociation of the ternary complex and incorporation of the mature polypeptide into the nascent holoenzyme complex. Sequential delivery of copper during Cu_A site biogenesis was recently described for a bacterial CO II *in vitro* (40), and although we cannot rule out the possibility that two copper atoms are simultaneously donated to human CO II *in vivo* to form the Cu_A site, we favor a scenario in which SCO2 donates its copper initially, with its cysteine thiols being oxidized in the process. After transfer of copper from SCO1 to CO II, SCO2 re-oxidizes the cysteines in SCO1. Alternatively, SCO2 may induce disulphide bond formation in copper-loaded SCO1, thereby facilitating copper transfer from SCO1 to CO II. Either mechanism, however, would effectively prime the cysteines of both proteins for their subsequent metallation by COX17 and another round of copper delivery to CO II.

The proposed metallation steps involving COX17 have been substantiated by recent *in vitro* studies from Bertini and colleagues (28,33). In fact, the reduced population of cysteines in SCO2 that we observe in control and *SCO* patient backgrounds meets the demonstrated requirement for reduced thiols in order for SCO2 to participate in ligand exchange reactions with COX17. While SCO1 can be metallated by COX17 *in vitro* irrespective of the redox state of its cysteines (33), the disproportionate reduction of the cysteines in SCO1 in *SCO2* cells, and the associated defects at the level of CO II expression, suggest that COX17 transfers Cu(I) to the oxidized cysteines of SCO1 *in vivo*. The fact that the cysteines in P174L SCO1 are less amenable to chemical reduction by DTT (Fig. 6A, data not shown) further argues that the accessibility of the CxxxC motif is sterically hindered by the missense mutation, an observation that may explain why ligand-exchange reactions between mutant SCO1 and COX17 are severely compromised (34,42).

Although Rodel and colleagues have shown that yeast SCO1 and SCO2 interact with CO II when overexpressed (43,44), thus far we have failed to demonstrate physical interactions between any of these three proteins in humans.

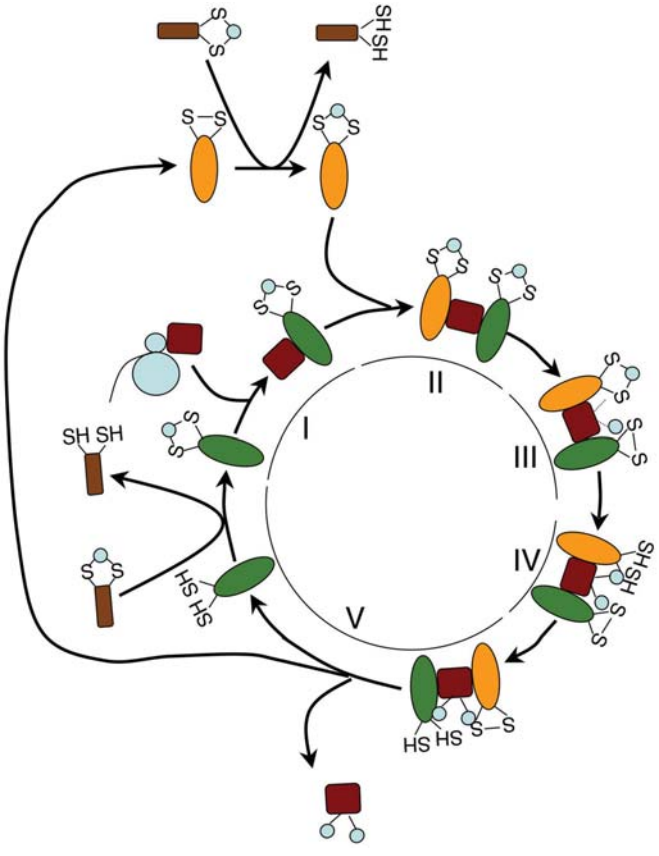

Figure 7. SCO-dependent regulation of CO II synthesis and maturation. Proposed model of the individual steps that require SCO protein function (I–V), from synthesis of CO II to its insertion into the assembling holoenzyme (CO II, maroon squares; SCO1, orange ovals; SCO2, green ovals; COX17, brown rectangles). Normal rates of productive CO II synthesis require the presence of sufficient, copper-loaded SCO2 (I). Association of SCO2 with CO II triggers the COX17-dependent metallation of SCO1, and its recruitment to form a SCO1–SCO2–CO II ternary complex (II). Copper is then transferred sequentially to CO II by each SCO protein (III and IV), with SCO2 being the initial Cu(I) donor. SCO2 subsequently acts as a thiol-disulphide oxidoreductase to oxidize the cysteines of SCO1, a reaction that ultimately primes the cysteines of both proteins for a subsequent round of CO II synthesis and maturation (V). Full maturation of the Cu_A site and re-priming of the redox state of the cysteines of each SCO protein both serve to trigger the dissociation of CO II from the ternary complex, and its insertion into the nascent holoenzyme.

However, the dominant-negative effects on CO II expression and holoenzyme assembly we observe upon overexpression of several SCO variants in each patient background provide strong genetic evidence for interactions between SCO1, SCO2 and CO II. The increased accumulation of the S2 assembly intermediate in these genetic contexts further suggests that maturation of the Cu_A site and insertion of CO II into the nascent holoenzyme complex occur concurrently. What could account for the apparent discord between the two *SCO* patient backgrounds with respect to the amount of newly synthesized CO II that accumulates in the chase relative to the abundance of the S2 assembly intermediate detected by native PAGE? In *SCO2* cells, the disproportionate accumulation of newly synthesized CO II in the chase relative to the S2 assembly intermediate suggests that CO II is initially stabilized by another upstream factor (e.g. COX20), and that

its interaction with SCO2 is rate-limited by its reduced abundance as well as being slowed down kinetically by the *E140K* mutation; however, once mutant SCO2 associates with CO II, formation of the ternary complex, metallation of CO II and subsequent insertion of the protein into the assembling holoenzyme occurs very rapidly. In contrast, the ternary complex is formed in the *SCO1* patient background, but the *P174L* mutation severely compromises the metallation of mutant SCO1 by COX17 (34,42), which in turn stalls holoenzyme assembly and leads to the accumulation of the S2 assembly intermediate owing to an inability to fully mature the Cu_A site in CO II. Although this model suggests that either apo- or partially metallated CO II is recognized by the mitochondrial quality control system and rapidly degraded, the molecular signal that triggers the turnover of the nascent polypeptide remains unknown.

The number of *SCO* genes contained in prokaryotic and eukaryotic genomes varies widely, and some organisms lacking an aa_3-type oxidase with a Cu_A site nonetheless express one or more SCO proteins (26,27). Although phylogenetic analyses and functional studies support the idea that SCO proteins fulfill multiple functions, it is not clear why certain eukaryotes only need a single SCO protein for COX assembly while others require two SCO proteins. One possibility is that organisms expressing a single SCO protein have another enzyme within the IMS that acts as either the thiol-disulphide oxidoreductase or the copper donor during Cu_A site formation (40). Alternatively, organisms may encode a single SCO protein that fulfills both of these functions. Over evolutionary time, duplication of such an 'ancestral' *SCO* gene in certain organisms may reflect the selective advantage conferred by parsing out its various functions to multiple gene products. Irrespective of the selective pressure(s) that drove the independent duplication of the *SCO* gene in multiple lineages, the present study clarifies the relative roles of human SCO1 and SCO2 in CO II synthesis and maturation. The dynamic, multifaceted role for human SCO2 may be facilitated by the greater conformational changes observed in the quaternary structure of its apo- and copper-loaded conformers relative to those of SCO1 (29).

MATERIALS AND METHODS

Tissue culture and cell lines

Primary control, *SCO1* (12) and *SCO2* [E140K/R90X (13)], patient cells were immortalized and cultured as previously described (19). For stable overexpression and knockdown experiments, the production of virus, infection and selection of cells was done as described elsewhere (25). For transient knockdown experiments, three double-stranded RNA_i duplexes against human SCO2 mRNA were designed using Block-iT RNA_i Express (https://rnaidesigner.invitrogen.com/rnaiexpress), and synthesized and modified using Stealth RNA_i technology. The sequences of the sense strands were: *SCO2-A*, 5′-UCCAGGACUUCCACCCAAGACUGUU-3′; *SCO2-B*, 5′-GGACUACAUCGUGGACCACUCCAUU-3′; and *SCO2-C*, 5′-CUGACGGCCUCUUCACGGAUUACUA-3′. The antisense strands were the reverse complement of the aforementioned sense strands.

Stealth RNA_i duplexes were transiently transfected into immortalized myoblasts at 50% confluency using Lipofectamine RNA_iMAX (Invitrogen), according to the manufacturer's specifications. The efficacy of the three Stealth RNA_i duplexes was evaluated by western blot analysis, and maximal knockdown was achieved by transfection of cells with 13.5 nM of *SCO2-B* on Days 1, 3 and 6 for their eventual harvest on Day 10.

Native and denaturing polyacrylamide gel electrophoresis

Native and denatured extracts were prepared from isolated mitochondria or digitonized mitoplasts, and fractionated by blue-native and reducing SDS–PAGE, respectively, as previously described (19). For non-reducing SDS–PAGE experiments, isolated mitochondria (2 mg/ml) were resuspended in a standard import buffer (45) supplemented with 0.1 mg/ml bovine serum albumin (BSA), 0.5 mM PMSF (Sigma) and 1× protease inhibitor cocktail (Roche) in the presence or absence of 50 mM DTT (EM Biosciences) and incubated for 20 min at room temperature. Mitochondria were then pelleted by centrifugation at 8000*g* for 5 min at 4°C, and lysed in a buffer [50 mM Tris (pH 7.5), 1% SDS, 1 mM ethylene diaminetetraacetic acid, 0.1 mg/ml BSA, 0.5 mM PMSF and 1× protease inhibitor cocktail] containing either 50 mM IAM (Sigma) or 25 mM 4-acetoamido-4'-maleimidylstilbene-2,2'-disulphonic acid (AMS; Invitrogen). Following a 1 h incubation at room temperature, an equal volume of 2× sample loading buffer (Biorad) minus reductant was added, samples were boiled for 5 min and loaded onto 15% Tris–HCl Criterion gels (Biorad) that had been pre-run at 120 V for 30 min. Manipulation of whole cells was identical to that described above for mitochondria, with the exception that the centrifugation step was at 14 000*g* for 2 min at 4°C. Both native and denaturing gels were transferred to nitrocellulose membranes under semi-dry conditions, membranes were decorated with primary and secondary antibodies, and immunoreactive bands were detected by enhanced chemiluminescence as previously described (25).

In vitro labeling of mitochondrial translation products

Cells were labeled in 6 cm dishes for 60 min at 37°C and 5% CO_2, with 200 μCi/ml of a [^{35}S]-methionine and cysteine mixture obtained from either GE Healthcare (PRO-MIX) or Perkin-Elmer (Easy Tag^{TM} EXPRESS), in methionine- and cysteine-free DMEM (Gibco) containing 10% dialyzed fetal bovine serum (Gibco) and 100 μg/ml of anisomycin (Sigma), a reversible inhibitor of cytoplasmic translation. Prior to labeling, cells were incubated for 23 h in 40 μg/ml chloramphenicol (Sigma) to inhibit mitochondrial translation and allow for the accumulation of nuclear-encoded respiratory chain subunits, which effectively promotes the stabilization of the newly synthesized, labeled mtDNA-encoded subunits. Total cellular protein (50 μg) was resuspended in loading buffer [93 mM Tris–HCl (pH 6.7), 7.5% glycerol, 1% SDS, 0.25 mg/ml bromophenol blue and 3% β-mercaptoethanol], sonicated for 5 s, loaded and run on either 12–20% or 15–20% gradient gels. Gels were subsequently dried under vacuum at 60°C and the [^{35}S]-labeled mitochondrial

translation products were detected through direct autoradiography. For all samples, the abundance of each mitochondrial translation product was quantified using ImageQuant software (Molecular Dynamics/GE Healthcare), and then divided by the total counts within the lane to normalize for differences in loading.

Miscellaneous

All cell lines were tested to ensure that they were mycoplasma-negative prior to experimental manipulation (MycoAlert, Cambrex). Protein concentration, COX and citrate synthase activities were quantified as previously described (25). For non-reducing SDS–PAGE analyses, the abundance of SCO1 species containing oxidized disulphides and reduced thiols was quantified densitometrically for multiple exposures within the linear range of the film using ImageQuant software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

We acknowledge helpful discussions with Drs A. Vila (University of Rosario, Argentina), J. Herrmann (Technical University of Kaiserslautern, Germany), P. Cobine (Auburn University, USA) and D. Winge (University of Utah, USA).

Conflict of Interest statement. None declared.

FUNDING

This study was supported by grants from the Canadian Institutes of Health Research (E.A.S.) and Muscular Dystrophy Association (E.A.S. and S.C.L.). E.A.S. is an International Scholar of the Howard Hughes Medical Institute.

REFERENCES

1. Capaldi, R.A. (1990) Structure and assembly of cytochrome *c* oxidase. *Arch. Biochem. Biophys.*, **280**, 252–262.
2. Yoshikawa, S., Shinzawa-Itoh, K. and Tsukihara, T. (1998) Crystal structure of bovine heart cytochrome *c* oxidase at 2.8 A resolution. *J. Bioenerg. Biomembr.*, **30**, 7–14.
3. Kadenbach, B., Huttemann, M., Arnold, S., Lee, I. and Bender, E. (2000) Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome *c* oxidase. *Free Radic. Biol. Med.*, **29**, 211–221.
4. Nijtmans, L.G., Taanman, J.W., Muijsers, A.O., Speijer, D. and Van den Bogert, C. (1998) Assembly of cytochrome-c oxidase in cultured human cells. *Eur. J. Biochem.*, **254**, 389–394.
5. Barrientos, A., Barros, M.H., Valnot, I., Rotig, A., Rustin, P. and Tzagoloff, A. (2002) Cytochrome oxidase in health and disease. *Gene*, **286**, 53–63.
6. Grivell, L.A., Artal-Sanz, M., Hakkaart, G., de Jong, L., Nijtmans, L.G., van Oosterum, K., Siep, M. and van der Spek, H. (1999) Mitochondrial assembly in yeast. *FEBS Lett.*, **452**, 57–60.
7. Mootha, V.K., Lepage, P., Miller, K., Bunkenborg, J., Reich, M., Hjerrild, M., Delmonte, T., Villeneuve, A., Sladek, R., Xu, F. *et al.* (2003) Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc. Natl Acad. Sci. USA*, **100**, 605–610.
8. Valnot, I., von Kleist-Retzow, J.C., Barrientos, A., Gorbatyuk, M., Taanman, J.W., Mehaye, B., Rustin, P., Tzagoloff, A., Munnich, A. and Rotig, A. (2000) A mutation in the human heme A:farnesyltransferase gene (COX10) causes cytochrome *c* oxidase deficiency. *Hum. Mol. Genet.*, **9**, 1245–1249.
9. Antonicka, H., Mattman, A., Carlson, C.G., Glerum, D.M., Hoffbuhr, K.C., Leary, S.C., Kennaway, N.G. and Shoubridge, E.A. (2003) Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy. *Am. J. Hum. Genet.*, **72**, 101–114.
10. Zhu, Z., Yao, J., Johns, T., Fu, K., De Bie, I., Macmillan, C., Cuthbert, A.P., Newbold, R.F., Wang, J., Chevrette, M. *et al.* (1998) SURF1, encoding a factor involved in the biogenesis of cytochrome *c* oxidase, is mutated in Leigh syndrome. *Nat. Genet.*, **20**, 337–343.
11. Tiranti, V., Hoertnagel, K., Carrozzo, R., Galimberti, C., Munaro, M., Granatiero, M., Zelante, L., Gasparini, P., Marzella, R., Rocchi, M. *et al.* (1998) Mutations of SURF-1 in Leigh disease associated with cytochrome *c* oxidase deficiency. *Am. J. Hum. Genet.*, **63**, 1609–1621.
12. Valnot, I., Osmond, S., Gigarel, N., Mehaye, B., Amiel, J., Cormier-Daire, V., Munnich, A., Bonnefont, J.P., Rustin, P. and Rotig, A. (2000) Mutations of the *SCO1* gene in mitochondrial cytochrome *c* oxidase deficiency with neonatal-onset hepatic failure and encephalopathy. *Am. J. Hum. Genet.*, **67**, 1104–1109.
13. Jaksch, M., Ogilvie, I., Yao, J., Kortenhaus, G., Bresser, H.G., Gerbitz, K.D. and Shoubridge, E.A. (2000) Mutations in SCO2 are associated with a distinct form of hypertrophic cardiomyopathy and cytochrome *c* oxidase deficiency. *Hum. Mol. Genet.*, **9**, 795–801.
14. Papadopoulou, L.C., Sue, C.M., Davidson, M.M., Tanji, K., Nishino, I., Sadlock, J.E., Krishna, S., Walker, W., Selby, J., Glerum, D.M. *et al.* (1999) Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. *Nat. Genet.*, **23**, 333–337.
15. Ghezzi, D., Saada, A., D'Adamo, P., Fernandez-Vizarra, E., Gasparini, P., Tiranti, V., Elpeleg, O. and Zeviani, M. (2008) FASTKD2 nonsense mutation in an infantile mitochondrial encephalomyopathy associated with cytochrome *c* oxidase deficiency. *Am. J. Hum. Genet.*, **83**, 415–423.
16. Tiranti, V., Viscomi, C., Hildebrandt, T., Di Meo, I., Mineri, R., Tiveron, C., Levitt, M.D., Prelle, A., Fagiolari, G., Rimoldi, M. *et al.* (2009) Loss of ETHE1, a mitochondrial dioxygenase, causes fatal sulfide toxicity in ethylmalonic encephalopathy. *Nat. Med.*, **15**, 200–205.
17. Tiranti, V., D'Adamo, P., Briem, E., Ferrari, G., Mineri, R., Lamantea, E., Mandel, H., Balestri, P., Garcia-Silva, M.T., Vollmer, B. *et al.* (2004) Ethylmalonic encephalopathy is caused by mutations in *ETHE1*, a gene encoding a mitochondrial matrix protein. *Am. J. Hum. Genet.*, **74**, 239–252.
18. Buchwald, P., Krummeck, G. and Rodel, G. (1991) Immunological identification of yeast SCO1 protein as a component of the inner mitochondrial membrane. *Mol. Gen. Genet.*, **229**, 413–420.
19. Leary, S.C., Kaufman, B.A., Pellecchia, G., Guercin, G.H., Mattman, A., Jaksch, M. and Shoubridge, E.A. (2004) Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. *Hum. Mol. Genet.*, **13**, 1839–1848.
20. Nittis, T., George, G.N. and Winge, D.R. (2001) Yeast Sco1, a protein essential for cytochrome c oxidase function is a Cu(I)-binding protein. *J. Biol. Chem.*, **276**, 42520–42526.
21. Glerum, D.M., Shtanko, A. and Tzagoloff, A. (1996) SCO1 and SCO2 act as high copy suppressors of a mitochondrial copper recruitment defect in *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **271**, 20531–20535.
22. Leary, S.C., Mattman, A., Wai, T., Koehn, D.C., Clarke, L.A., Chan, S., Lomax, B., Eydoux, P., Vallance, H.D. and Shoubridge, E.A. (2006) A hemizygous SCO2 mutation in an early onset rapidly progressive, fatal cardiomyopathy. *Mol. Genet. Metab.*, **89**, 129–133.
23. Jaksch, M., Horvath, R., Horn, N., Auer, D.P., Macmillan, C., Peters, J., Gerbitz, K.D., Kraegeloh-Mann, I., Muntau, A., Karcagi, V. *et al.* (2001) Homozygosity (E140K) in SCO2 causes delayed infantile onset of cardiomyopathy and neuropathy. *Neurology*, **57**, 1440–1446.
24. Badrick, A.C., Hamilton, A.J., Bernhardt, P.V., Jones, C.E., Kappler, U., Jennings, M.P. and McEwan, A.G. (2007) PrrC, a Sco homologue from *Rhodobacter sphaeroides*, possesses thiol-disulfide oxidoreductase activity. *FEBS Lett.*, **581**, 4663–4667.
25. Leary, S.C., Cobine, P.A., Kaufman, B.A., Guercin, G.H., Mattman, A., Palaty, J., Lockitch, G., Winge, D.R., Rustin, P., Horvath, R. *et al.* (2007) The human cytochrome *c* oxidase assembly factors SCO1 and SCO2 have

regulatory roles in the maintenance of cellular copper homeostasis. *Cell Metab.*, **5**, 9–20.
26. Arnesano, F., Banci, L., Bertini, I. and Martinelli, M. (2005) Ortholog search of proteins involved in copper delivery to cytochrome *c* oxidase and functional analysis of paralogs and gene neighbors by genomic context. *J. Proteome Res.*, **4**, 63–70.
27. Banci, L., Bertini, I., Cavallaro, G. and Rosato, A. (2007) The functions of Sco proteins from genome-based analysis. *J. Proteome Res.*, **6**, 1568–1579.
28. Banci, L., Bertini, I., Calderone, V., Ciofi-Baffoni, S., Mangani, S., Martinelli, M., Palumaa, P. and Wang, S. (2006) A hint for the function of human Sco1 from different structures. *Proc Natl Acad. Sci. USA*, **103**, 8595–8600.
29. Banci, L., Bertini, I., Ciofi-Baffoni, S., Gerothanassis, I.P., Leontari, I., Martinelli, M. and Wang, S. (2007) A structural characterization of human SCO2. *Structure*, **15**, 1132–1140.
30. Horng, Y.C., Leary, S.C., Cobine, P.A., Young, F.B., George, G.N., Shoubridge, E.A. and Winge, D.R. (2005) Human Sco1 and Sco2 function as copper-binding proteins. *J. Biol. Chem.*, **280**, 34113–34122.
31. Andruzzi, L., Nakano, M., Nilges, M.J. and Blackburn, N.J. (2005) Spectroscopic studies of metal binding and metal selectivity in Bacillus subtilis BSco, a homologue of the yeast mitochondrial protein Sco1p. *J. Am. Chem. Soc.*, **127**, 16548–16558.
32. Imriskova-Sosova, I., Andrews, D., Yam, K., Davidson, D., Yachnin, B. and Hill, B.C. (2005) Characterization of the redox and metal binding activity of BsSco, a protein implicated in the assembly of cytochrome *c* oxidase. *Biochemistry*, **44**, 16949–16956.
33. Banci, L., Bertini, I., Ciofi-Baffoni, S., Hadjiloi, T., Martinelli, M. and Palumaa, P. (2008) Mitochondrial copper(I) transfer from Cox17 to Sco1 is coupled to electron transfer. *Proc. Natl Acad. Sci. USA*, **105**, 6803–6808.
34. Cobine, P.A., Pierrel, F., Leary, S.C., Sasarman, F., Horng, Y.C., Shoubridge, E.A. and Winge, D.R. (2006) The P174L mutation in human Sco1 severely compromises Cox17-dependent metallation but does not impair copper binding. *J. Biol. Chem.*, **281**, 12270–12276.
35. Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J. and Zeman, J. (2005) Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. *Biochem. J.*, **392**, 625–632.
36. Jaksch, M., Paret, C., Stucka, R., Horn, N., Muller-Hocker, J., Horvath, R., Trepesch, N., Stecker, G., Freisinger, P., Thirion, C. *et al.* (2001) Cytochrome c oxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts. *Hum. Mol. Genet.*, **10**, 3025–3035.
37. Foltopoulou, P.F., Zachariadis, G.A., Politou, A.S., Tsiftsoglou, A.S. and Papadopoulou, L.C. (2004) Human recombinant mutated forms of the mitochondrial COX assembly Sco2 protein differ from wild-type in physical state and copper binding capacity. *Mol. Genet. Metab.*, **81**, 225–236.
38. Herrmann, J.M. and Funes, S. (2005) Biogenesis of cytochrome oxidase-sophisticated assembly lines in the mitochondrial inner membrane. *Gene*, **354**, 43–52.
39. Chinenov, Y.V. (2000) Cytochrome *c* oxidase assembly factors with a thioredoxin fold are conserved among prokaryotes and eukaryotes. *J. Mol. Med.*, **78**, 239–242.
40. Abriata, L.A., Banci, L., Bertini, I., Ciofi-Baffoni, S., Gkazonis, P., Spyroulias, G.A., Vila, A.J. and Wang, S. (2008) Mechanism of Cu(A) assembly. *Nat. Chem. Biol.*, **4**, 599–601.
41. Williams, J.C., Sue, C., Banting, G.S., Yang, H., Glerum, D.M., Hendrickson, W.A. and Schon, E.A. (2005) Crystal structure of human SCO1: implications for redox signaling by a mitochondrial cytochrome *c* oxidase 'assembly' protein. *J. Biol. Chem.*, **280**, 15202–15211.
42. Banci, L., Bertini, I., Ciofi-Baffoni, S., Leontari, I., Martinelli, M., Palumaa, P., Sillard, R. and Wang, S. (2007) Human Sco1 functional studies and pathological implications of the P174L mutant. *Proc. Natl Acad. Sci. USA*, **104**, 15–20.
43. Lode, A., Kuschel, M., Paret, C. and Rodel, G. (2000) Mitochondrial copper metabolism in yeast: interaction between Sco1p and Cox2p. *FEBS Lett.*, **485**, 19–24.
44. Lode, A., Paret, C. and Rodel, G. (2002) Molecular characterization of *Saccharomyces cerevisiae* Sco2p reveals a high degree of redundancy with Sco1p. *Yeast*, **19**, 909–922.
45. Steenaart, N.A. and Shore, G.C. (1997) Mitochondrial cytochrome *c* oxidase subunit IV is phosphorylated by an endogenous kinase. *FEBS Lett.*, **415**, 294–298.