The mitochondrial metallochaperone SCO1 maintains CTR1 at the plasma membrane to preserve copper homeostasis in the murine heart

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Abstract

SCO1 is a ubiquitously expressed, mitochondrial protein with essential roles in cytochrome c oxidase (COX) assembly and the regulation of copper homeostasis. SCO1 patients present with severe forms of early onset disease, and ultimately succumb from liver, heart or brain failure. However, the inherent susceptibility of these tissues to SCO1 mutations and the clinical heterogeneity observed across SCO1 pedigrees remain poorly understood phenomena. To further address this issue, we generated Sco1hrt/hrt and Sco1stm/stm mice in which Sco1 was specifically deleted in heart and striated muscle, respectively. Lethality was observed in both models due to a combined COX and copper deficiency that resulted in a dilated cardiomyopathy. Left ventricular dilation and loss of heart function was preceded by a temporal decrease in COX activity and copper levels in the longer-lived Sco1stm/stm mice. Interestingly, the reduction in copper content of Sco1stm/stm cardiomyocytes was due to the mislocalisation of CTR1, the high affinity transporter that imports copper into the cell. CTR1 was similarly mis-localized to the cytosol in the heart of knockin mice carrying a homozygous G115S substitution in Sco1, which in humans causes a hypertrophic cardiomyopathy. Our current findings in the heart are in marked contrast to our prior observations in the liver, where Sco1 deletion results in a near complete absence of CTR1 protein. These data collectively argue that mutations perturbing SCO1 function have tissue-specific consequences for the machinery that ultimately governs copper homeostasis, and further establish the importance of aberrant mitochondrial signaling to the etiology of copper handling disorders.

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Introduction

Copper is an essential micronutrient that can rapidly cycle between two redox states, Cu⁺ and Cu²⁺, thereby facilitating electron transfer. This property has been co-opted by a number of proteins which use copper as a cofactor to catalyze biochemical reactions critical to respiration, free radical detoxification, iron homeostasis, and neurotransmitter and collagen biosynthesis (1,2). Copper is also extremely toxic in excess because it can destabilize iron-sulphur clusters (3,4) and potentiate the generation of highly damaging free radicals (5,6). Conserved mechanisms therefore evolved to mitigate its toxic potential, and act in concert to regulate total and bioavailable copper stores such that the cell contains negligible amounts of the free metal ion (7). Genetic lesions or environmental insults that impair these copper handling mechanisms perturb copper homeostasis, and underlie a number of severe forms of human disease (8).

In mammals, copper enters the cell through the essential protein CTR1, the only known high-affinity copper importer (9,10). CTR1 functions as a homotrimer to facilitate copper translocation across the plasma membrane (11). Copper is then transferred to evolutionarily conserved, soluble chaperones that deliver it to various subcellular compartments (12–16). CTR1 abundance and localisation are tightly controlled to regulate the rate of copper import and, by extension, cellular copper homeostasis. Under conditions of copper excess, CTR1 can be internalized from the plasma membrane into endosomes (17), a mechanism that is thought to protect the cell from copper toxicity (18). Endosomally-localized CTR1 can then be targeted for degradation by the proteasome, or recycled back to the plasma membrane (19). However, the universality of this regulatory mechanism has been debated, given that CTR1 localisation is not always altered in response to increased copper concentrations (20). The P-type ATPases ATP7A and ATP7B normally reside in the trans-Golgi network where they promote the metallation of secreted cuproproteins (21–23). However, both proteins also undergo regulated trafficking to the cell periphery where they facilitate copper export (24,25). Pathogenic mutations in ATP7A and ATP7B severely perturb protein function and cause Menkes and Wilson disease, respectively (26,27).

There is a growing appreciation for the influence of mitochondria on how the cell senses its copper status and prioritizes its copper stores to maintain homeostasis (28). Mitochondria contain two enzymes that depend on copper for their activity; cytochrome c oxidase (COX) and a small, but physiologically significant fraction of copper-zinc superoxide dismutase (SOD1). COX contains two copper prosthetic groups (CuA, CuB) that are matured within the intermembrane space of the mitochondrion in biochemical reactions that require a surprisingly large number of accessory proteins termed COX assembly factors. Pathogenic mutations in genes encoding three of these factors, COA6, SCO1 and SCO2, impair copper delivery to the CuA site of COX and result in a severe, isolated COX deficiency that underlies a number of early onset forms of fatal human disease (29–31). SCO1 and SCO2 are paralogs with complementary but non-overlapping functions in this pathway that rely on the ability of each protein to bind copper (32,33). SCO1 gene mutations also impair the function of a redox-dependent, mitochondrial signaling pathway that is integral to the regulation of cellular copper homeostasis, and affected tissues in SCO1 and SCO2 patients are both COX and copper deficient (34–36).

Three pedigrees with pathogenic mutations in SCO1 have been described to date, each with a unique clinical phenotype and course of disease that selectively affects liver (30; P174L/R149X), heart (36; G132S/G132S) and/or brain (37; M294V/Y93X) function. How different mutations in a ubiquitously expressed gene produce such striking clinical heterogeneity remains an enduring mystery. Affected SCO1 patient tissues are severely copper deficient, and the copper deficiency in SCO1 and SCO2 patient fibroblasts is driven in large part by a disproportionate rate of copper efflux from the cell (34). In contrast, loss of SCO1 function in the liver of a mouse model of the human disease results in a severe copper deficiency that is caused by a significant reduction in CTR1 abundance, and therefore copper uptake (38). These observations suggest that differences may exist across cell types in how SCO1-dependent mitochondrial signaling is integrated into the regulatory framework that controls copper homeostasis. To determine if this aspect of SCO1 function does in fact differ across tissues, we investigated its importance to copper homeostasis in the developing and adult heart using two relevant mouse models. We found that deletion of Sco1 in both a heart and a striated muscle-specific knockout mouse was lethal, and that Sco1 null hearts were both COX and copper deficient. However, unlike our previous findings in the Sco1 null liver (38), the cardiac copper deficiency was caused by CTR1 mislocalisation rather than a reduction in its abundance. Disproportionate internalization of CTR1 in the absence of any changes in its steady-state levels was also observed in a Sco1<sup>G132S/G132S</sup> knockin mouse, an animal model of the human disease that results in an early onset, fatal cardiomyopathy in one of the three SCO1 pedigrees (36). These data collectively support the concept that copper homeostasis is regulated by redox-dependent signaling through SCO1 in a tissue-specific manner.

Results

Generation of a heart-specific Sco1 knockout mouse

The SCO1 patient homozygous for the G132S mutation exhibited defects in cardiac physiology and presented with a lethal, neonatal cardiomyopathy (36). To better understand the mechanisms underlying the tissue-specific pathology and progression of this disease, we generated a transgenic mouse model in which Sco1 was specifically deleted in the heart. Briefly, mice containing a pair ofloxP sites flanking the second exon of the Sco1 gene (Sco1<sup>loxfl/fl</sup>) (38) were crossed to mice in which Cre recombinase expression is driven by the α-myosin heavy chain promoter (Mhc-Cre<sup>Cre<sup>lox</sup>/</sup>Cre<sup>lox</sup>., Jackson #018972). Cre positive, F1 progeny (i.e. Mhc-Cre<sup>Cre<sup>lox</sup>/</sup>Cre<sup>lox</sup>/+, Sco1<sup>loxfl/fl</sup>) were then backcrossed to Sco1<sup>loxfl/fl</sup> animals to generate heart-specific Sco1 knockout mice (Mhc-Cre<sup>Cre<sup>lox</sup>/</sup>Cre<sup>lox</sup>/+, Sco1<sup>loxfl/fl</sup>), hereafter referred to as Sco1<sup>hbr/hbr</sup> mice. Sco1<sup>hbr/hbr</sup> mice were viable and all alleles were inherited at the expected Mendelian frequencies (data not shown). Although the birth weight of Sco1<sup>hbr/hbr</sup> mice was comparable to that of wild-type littermates (Supplementary Material, Fig. S1A), none survived beyond two days of age (Fig. 1A). PCR amplification of the Sco1 locus in a number of somatic tissues confirmed that Cre-mediated excision events were restricted to the myocardium (Supplementary Material, Fig. S1B), and Western blot analysis demonstrated that the hearts of Sco1<sup>hbr/hbr</sup> mice had very low levels of SCO1 protein as early as embryonic day 15 (E15) (Fig. 1B).

Sco1<sup>hbr/hbr</sup> mice have a dilated cardiomyopathy owing to a severe, isolated COX deficiency

To further investigate the underlying basis of lethality in these animals, we first examined the Sco1<sup>hbr/hbr</sup> heart histologically. Hematoxylin and eosin staining revealed an enlarged left
ventricle, indicative of a dilated cardiomyopathy (Fig. 1C). Negative Masson’s trichrome staining (Fig. 1D) suggests that the ventricular dilation occurred in the absence of significant fibrosis. Sco1<sup>hrt/hrt</sup> hearts also exhibited a severe COX deficiency when compared with wild-type hearts (Fig. 1E, Supplementary Material, Fig. S1C). The COX deficiency was not accompanied by a change in the activity of citrate synthase (Fig. 1E) or the abundance of other OXPHOS complexes (Fig. 1F, Supplementary Material, Fig. S1C), suggesting that mitochondrial content remained unchanged in hearts that lack SCO1 expression.

A combined COX and copper deficiency is observed in affected SCO1 patient tissues, and is a hallmark of the associated disease and its progression (36,38). Accordingly, the Sco1<sup>hrt/hrt</sup> heart had a slight, but statistically significant reduction in total copper content when compared with the wild-type heart (Fig. 1G), as well as a decrease in the total activity of superoxide

**Figure 1.** Sco1<sup>hrt/hrt</sup> mice present with a dilated cardiomyopathy owing to a severe, isolated COX deficiency. (A) Kaplan-Meier survival curve of wild-type (black) and Sco1<sup>hrt/hrt</sup> (grey) mice. (Controls, n = 40; Sco1<sup>hrt/hrt</sup>, n = 15). (B) Western blot analysis of SCO1 and COX IV abundance in wild-type and Sco1<sup>hrt/hrt</sup> hearts at E15, E17 and P1. GAPDH was used as a loading control. (C) P1 wild-type (left) and Sco1<sup>hrt/hrt</sup> (right) hearts were stained with hematoxylin and eosin. LV denotes left ventricle, scale bar represents 500 μm. (D) 20X magnification of P1 wild-type (left) and Sco1<sup>hrt/hrt</sup> (right) hearts stained with hematoxylin and eosin. Scale bar represents 50 μm. (E) COX (P = 0.04), total SOD (P = 0.002) and CS activities in P1 Sco1<sup>hrt/hrt</sup> hearts expressed as a percentage of wild-type littermates (COX and CS, n = 3 per genotype; total SOD, n = 6 per genotype). (F) BN-PAGE analysis of the abundance of oxidative phosphorylation complexes in a P1 wild-type and Sco1<sup>hrt/hrt</sup> heart. (G) Total copper levels in the heart (P = 0.03, n = 5 per genotype) and liver (P = 0.01, n = 3 per genotype) of P1 Sco1<sup>hrt/hrt</sup> mice expressed as a percentage of wild-type littermates. (H, I) Western blot analysis of ATP7A, CTR1 and CCS abundance in hearts (H) and livers (I) of P1 wild-type and Sco1<sup>hrt/hrt</sup> mice. Hearts from Ctr1<sup>flox/flox</sup> and Ctr1<sup>hrt/hrt</sup> mice (53) were used as a reference control to identify the glycosylated (arrow) and truncated (asterisk) forms of CTR1. Mouse embryonic fibroblasts treated with scrambled (Ccswt) or Ccs siRNA (CcssiRNA) were also included as controls to identify the authentic species of CCS protein, which is denoted by the unfilled arrow. GAPDH was used as a loading control.
dismutase (SOD) (Fig. 1D) that was directly attributable to a reduction in the activity of the copper-dependent SOD1 form of the enzyme (Supplementary Material, Fig. S1D). Immunoblot analysis demonstrated that the mild copper deficiency in Sco1hr/hrhearts was not caused by changes in the total abundance of the copper import protein CTR1 or the copper export protein ATP7A (Fig. 1H). While total copper levels and CTR1 abundance were both significantly lower in the liver of Sco1hr/hrmice (Fig. 1G and I), there was no significant change in hepatic COX activity (Supplementary Material, Fig. S1E) or COX IV subunit abundance (Supplementary Material, Fig. S1F). The copper content of plasma and of other peripheral tissues of Sco1hr/hrmice was also comparable to wild-type littermates (Supplementary Material, Fig. S1G).

**Striated-muscle specific Sco1 knockout mice also develop a severe, diluted cardiomyopathy**

The sudden perinatal lethality of the Sco1hr/hrmice precluded any meaningful investigation of disease progression owing to the loss of SCO1 function in the heart of this animal model. We therefore used a different Cre driver line in conjunction with the breeding strategy described above to generate striated muscle-specific Sco1 knockout mice, hereafter referred to as Sco1stm/stm mice. Sco1stm/stm mice were born at the expected Mendelian frequency (data not shown), and Cre mediated excision at the Sco1 locus was restricted to the striated muscle (Supplementary Material, Fig. S2A). Sco1stm/stm mice had a median lifespan of 100 days (Fig. 2A) without an observable change in their body weight (Supplementary Material, Fig. S2B). Unlike the hearts of Sco1hr/hrmice, SCO1 protein remained readily detectable in Sco1stm/stm hearts during the early postnatal period (Fig. 2B). Hearts from Sco1stm/stm mice were significantly larger at postnatal day 90 (P90), and weighed roughly 2-fold more than control hearts when normalized to body weight (Fig. 2C and D). P90 Sco1stm/stm cardiomyocytes were also markedly enlarged, vacuolated and displayed pronounced fibrosis (Fig. 2E), and expressed elevated transcript levels of markers of fetal reprogramming (Fig. 2F). To examine the impact of the observed histological and genetic changes on cardiac function, we next conducted non-invasive echocardiography imaging studies. Consistent with the observed dilated cardiomyopathy, P90 Sco1stm/stm hearts had a significantly increased left ventricular end-systolic diameter, and a corresponding reduction in ejection fraction and fractional shortening (Fig. 2G and H).

**Dysfunction in Sco1stm/stm hearts is due to a combined COX and copper deficiency that worsens with age**

To further investigate the underlying cause of the ventricular dilation in Sco1stm/stm mice, we characterized P90 hearts at a biochemical, molecular and elemental level. Sco1stm/stm hearts had a significant reduction in COX activity and COX IV protein levels when compared with the hearts of wild-type littermates (Fig. 3A and D, Supplementary Material, Fig. S2C). Sco1stm/stm hearts also exhibited a severe defect in total SOD activity (Fig. 3A) that was specific to the copper-dependent SOD1 form of the enzyme (Supplementary Material, Fig. S2D). Like Sco1hr/hrmice, the COX deficiency in Sco1stm/stm hearts was not caused by changes in mitochondrial content or remodelling, as the activity of citrate synthase (Fig. 3A) and the abundance of other OXPHOS complexes (Fig. 3B) remained unchanged. Sco1stm/stm hearts had significantly lower copper levels than wild-type hearts, in the absence of changes in the total abundance of iron and zinc (Fig. 3C). None of the other somatic tissues of Sco1stm/stm mice we investigated exhibited altered copper, iron or zinc levels (Supplementary Material, Fig. S2E). Immunoblot analysis revealed that the severe copper deficiency in the P90 Sco1stm/stm heart was not attributable to a reduction in CTR1 abundance (Fig. 3D).

To more thoroughly characterize disease progression and the underlying cause of the copper deficiency in the Sco1stm/stm heart, we next investigated wild-type and Sco1stm/stm hearts at three earlier time points. At P18, Sco1stm/stm hearts exhibited a mild COX deficiency in the absence of a detectable change in total copper content (Fig. 4A and B). Sco1stm/stm hearts were both COX and copper deficient at P30, with the combined deficiency worsening significantly by P60 (Fig. 4A and B). Immunoblot analysis of wild-type and Sco1stm/stm hearts demonstrated that there was no difference in the total abundance of ATP7A (Fig. 4C), suggesting that the observed increase in ATP7A at P90 (Fig. 3D) occurs well after the initial onset of the copper deficiency. The abundance of CCS, a known biomarker of cytoplasmic copper status (39), was also unchanged. Interestingly, steady-state levels of CTR1 were elevated in response to Sco1 deletion, even at P18 when total cardiac copper content is comparable across genotypes (Fig. 4B and C). These data collectively suggest that the copper deficiency in Sco1stm/stm hearts is caused by loss of CTR1 copper import activity rather than its degradation.

**Copper deficiency in Sco1stm/stm and Sco1G115S/G115S knockin hearts is caused by mislocalisation of CTR1 to endosomes**

We directly addressed the possibility that the cellular CTR1 pool was inappropriately distributed due to loss of SCO1 function by analysing wild-type and Sco1stm/stm hearts from P60 animals by indirect immunofluorescence. We found that in the wild-type heart CTR1 was primarily localized to the plasma membrane, with intense fluorescence at the intercalated discs, a pattern that was coincident with the plasma membrane marker protein Na+/K+ ATPase (Fig. 5). In contrast, CTR1 immunofluorescence was more diffuse in its distribution and less coincident with the Na+/K+ ATPase in the Sco1stm/stm heart (Fig. 5).

To compare and contrast the tissue-specific knockouut with the human condition, we created a homozygous Sco1G115S/G115S knockin mouse to model the SCO1 patient who presented neonatally with a fatal hypertrophic cardiomyopathy (36). Sco1G115S/G115S hearts exhibited a severe, combined COX and copper deficiency, like the knockout mouse model (Fig. 6A and B). The copper deficiency was not attributable to changes in the steady-state levels of CTR1 or ATP7A, and was not accompanied by changes in CCS abundance (Fig. 6B). Further mirroring the Sco1stm/stm heart, CTR1 was predominantly observed within the cytoplasm of the Sco1G115S/G115S heart, rather than at the plasma membrane and intercalated disks (Fig. 6C).

These data strongly suggest that perturbation of cardiac SCO1 function causes a copper deficiency by impairing CTR1 trafficking to, or maintenance at, the plasma membrane. To distinguish between these two possibilities, we co-stained wild-type and Sco1stm/stm hearts from P60 animals with antibodies raised against CTR1 and Rab5, an early endosomal marker. We found that internalized CTR1 convincingly co-localized with Rab5 in the Sco1stm/stm heart (Supplementary Material, Fig. S3A). However, follow-up imaging experiments failed to detect significant co-localization of CTR1 with either EEA1, a Rab5 effector.
and early endosomal marker, or LAMP1, a late endosomal/lysosomal marker (Supplementary Material, Fig. S3B). These data argue that loss of SCO1 function in the heart leads to CTR1 endocytosis from the plasma membrane.

Discussion

One of the puzzling hallmarks of disease in SCO1 patients is the tissue-specific clinical presentation and, in particular, the differential susceptibility of the liver, brain or heart among SCO1 pedigrees (30,36,37). SCO1 is an essential, ubiquitously expressed protein whose function couples the metallation of the CuA site of COX to the regulation of cellular copper homeostasis (28,34,36,40). How mitochondrial signaling in individual tissues is integrated into the regulatory framework that controls copper homeostasis remains unclear. To further explore this issue, and to build on our earlier work on a Sco1 mouse model of the human disease caused by liver failure (38), we generated two mouse models that lacked Sco1 expression in the heart.

Figure 2. Sco1<sup>stm/stm</sup> mice also develop a severe, dilated cardiomyopathy. (A) Kaplan-Meier survival curve of wild-type (black) and Sco1<sup>stm/stm</sup> (grey) mice (Controls, n = 22; Sco1<sup>stm/stm</sup>, n = 18). (B) Western Blot analysis of SCO1 and COX IV abundance in wild-type and Sco1<sup>stm/stm</sup> hearts at E17, P1, P3 and P9. GAPDH was used as a loading control. (C) 2X magnification of wild-type (left) and Sco1<sup>stm/stm</sup> (right) hearts at P90. Scale bar represents 1 cm. (D) Heart to body weight ratio for wild-type and Sco1<sup>stm/stm</sup> mice (P = 0.0006; n = 7 per genotype). (E) 20X magnification of P90 hearts from wild-type (left) and Sco1<sup>stm/stm</sup> (right) mice stained with hematoxylin and eosin (top) and Masson’s trichrome stain (bottom). Scale bar represents 50 μm. (F) Semi-quantitative RT-PCR analysis of ANP, BNP, α-SERCA2a and MT1 transcript levels. GAPDH was used as a loading control. (G) Representative echocardiogram comparing left ventricular function in P90 control (top) and Sco1<sup>stm/stm</sup> (bottom) mice. Scale bar represents 0.1 s. (H) Left ventricular internal diameter end systolic (LVIDs, P = 0.05), left ventricular internal diameter end diastolic (LVIDd, P = 0.13), fractional shortening (P = 0.002) and ejection fraction (P = 0.006) in P90 control and Sco1<sup>stm/stm</sup> hearts (n = 4 per genotype). No differences in these parameters were observed between wild-type and Sco1<sup>stm/stm</sup> hearts at P60 (data not shown).
demonstrating the importance of SCO1 function to the developing and adult myocardium.

A dilated cardiomyopathy owing to a combined COX and copper deficiency was manifest in both mouse models. SCO1 abundance was exceedingly low in the hearts of Sco1hrt/hrt mice as early as E15, and continued to drop postnatally. In contrast, SCO1 was readily detectable in the hearts of Sco1stm/stm mice up to P3. It is therefore likely that in this model there was sufficient residual SCO1 to support the development and rapid growth of the heart during this early perinatal window. Consistent with this idea, P18 Sco1stm/stm hearts only exhibited a mild COX deficiency relative to wild-type littermates. We cannot, however, discount the possibility that changes in the skeletal muscle also contributed to the longer lifespan in Sco1stm/stm mice, as a previous study elegantly demonstrated that simultaneous deletion of an unrelated mitochondrial gene in the heart and skeletal muscle contributed to a sparing effect on the heart itself (41). Hearts from both Sco1hrt/hrt and Sco1stm/stm mice were comprised of enlarged and vacuolated cardiomyocytes, and Sco1stm/stm hearts exhibited profound fibrosis and changes in the expression of multiple markers for fetal cardiac remodeling, presumably owing to their longer lifespan. These data combined with the significant increase in heart to body weight ratio and the dilation of the left ventricle observed by echocardiography suggest a dilated cardiomyopathy with mild cardiac hypertrophy in the P90 Sco1stm/stm heart.

Multiple lines of evidence strongly suggest that the primary factor for the copper deficiency in the Sco1stm/stm and Sco1G115S/G115S hearts is the mislocalisation of CTR1. In the wild-type heart, CTR1 is localized primarily to the plasma membrane with particularly intense staining at the intercalated discs.
These data corroborate the findings of an earlier study in the heart (42), and suggest that CTR1 facilitates copper transport between cardiomyocytes at these cell-cell junctions, similar to other ions and metabolites (43–45). In contrast, CTR1 abundance at the intercalated disc was greatly attenuated in the Sco1stm/stm heart, where the protein was primarily localized to punctate intracellular structures found within the cytosol. CTR1-positive intracellular structures co-localized significantly with Rab5, but not with EEA1 or LAMP1. There is emerging evidence of an alternative endocytic pathway where relatively long-lived, Rab5-positive early endosomes contain the effectors APPL1 and APPL2 rather than EEA1 (46,47). Though these endosomes can be discriminated from the canonical EEA1-positive, early endosomes and are known to play important roles in storage, trafficking and signal transduction, their biological properties remain largely unknown (46,47). Our results raise the intriguing possibility that CTR1 is internalized in APPL-positive vesicles, although future studies will be required to confirm that loss of cardiac SCO1 function promotes CTR1 internalization via this alternate pathway. Similar intracellular redistribution of the total CTR1 pool away from the plasma membrane and intercalated disks was also observed in a heart homozygous for the SCO1 G115S substitution, the same allelic variant that in humans results in a hypertrophic cardiomyopathy (36). These data collectively argue that loss of SCO1 function in the heart causes a copper deficiency by impairing CTR1 localisation to the plasma membrane and therefore copper uptake into the cardiomyocyte.

Yet another intriguing finding of the present study is that perturbing the function of SCO1 in cardiomyocytes has a fundamentally different effect on CTR1 than it does in other cell types. Deletion of Sco1 in both murine liver and embryonic fibroblasts promotes the degradation of CTR1, leading to a significant reduction in its steady-state levels (38). In contrast, Sco1stm/stm and Sco1G115S/G115S hearts have wild-type CTR1 abundance but fail to maintain adequate levels of the protein at the plasma membrane and intercalated disks to support copper import as evidenced by cardiac copper deficiency in these animals. The glycine to serine substitution in both mouse (unpublished data) and human SCO1 (36,37) does not completely abolish protein function, and allows for a longer lifespan in the Sco1G115S/G115S mice. Nonetheless, CTR1 is still mislocalized in the Sco1G115S/G115S heart, arguing that the effects we observe on CTR1 in the Sco1stm/stm heart are not simply a consequence of Sco1 ablation and the resultant reduction in COX activity. While we do not yet know why perturbing SCO1-dependent mitochondrial signaling has distinct effects on CTR1 in the heart and liver, it may reflect different copper requirements in these tissues. Indeed, tissue-specific deletion of Ctr1 in the heart is associated with rapid cardiac hypertrophy and lethality (48,49), suggesting a continuous need for CTR1-mediated copper uptake by cardiomyocytes and, by extension, little storage capacity. Therefore, it may be necessary in the heart to acutely regulate the localization rather than the steady-state levels of CTR1 in the face of Sco1 deletion, to allow for more rapid responses to intrinsic changes in copper demand or extrinsic fluctuations in plasma copper levels. In contrast, the liver is a copper storage organ with higher total copper concentrations than the heart (34,50,51). Thus, degradation of CTR1 rather than recycling in response to Sco1 deletion may reflect the relative abundance of copper in this organ or the existence of alternative pathways of copper acquisition. Consistent with this hypothesis, tissue-specific deletion of Ctr1 in the liver during the postnatal period is associated with very mild hepatic copper deficiency and normal longevity (52).

How the loss of SCO1 function alters CTR1 localization in the heart while promoting CTR1 degradation in the liver remains an open question. Differences in redox balance between these two organs may contribute to the observed tissue-specific consequences of perturbing SCO1-dependent mitochondrial signaling on CTR1 function. The severe COX deficiency in Sco1 null livers
and hearts likely leads to oxidative stress. However, the metabolic poise and redox buffering capacity of each of these tissues is significantly different (53,54), and both of these factors may modulate the rate or nature of the signal that is transduced from the mitochondrion and sensed by other copper handling machinery. Signaling may also involve the release of one or more soluble, tissue-specific factors from the organelle that then acts to regulate CTR1 trafficking or degradation (28,40). Alternatively, loss of SCO1 function may affect signaling mediated by copper fluxes between mitochondria and the cytosol in a cell-type specific manner. Consistent with this idea, perturbing SCO1 function in the liver but not the heart alters the abundance of CCS (38), the only known biomarker of cytosolic copper status (39).

The reduction in hepatic copper levels in our Sco1hrt/hrt mouse model is an equally intriguing observation and points to crosstalk between the two tissues. Indeed, our findings mirror many aspects of those from an earlier study in which the high affinity copper importer Ctr1 was deleted in the heart using the same Cre driver line (55). Because CTR1 is the only high affinity copper importer, we favour the idea that the COX-deficient Ctr1hrt/hrt heart is signaling to the liver in an attempt to correct this metabolic defect. Kim et al. (55) in fact demonstrate that CCS abundance is very high in the Ctr1hrt/hrt heart, suggesting that the organ is appropriately sensing a copper-deficient state in these animals. They then go on to show that there is a soluble factor in the plasma of Ctr1hrt/hrt mice that drives hepatic mobilization of copper into the circulation by increasing the steady-state levels of ATP7A. Mobilization of hepatic copper stores is consistent with one of the known roles of the liver in early perinatal life, which is to distribute copper and other essential metal ions to peripheral tissues to support organogenesis, and in the Ctr1hrt/hrt mouse is presumably occurring at a disproportionate rate in an attempt to rescue the failing and copper-deficient myocardium. In our Sco1hrt/hrt mouse model, however, liver ATP7A abundance and plasma copper levels are normal, and none of the peripheral tissues we examined have increased copper levels. CCS abundance is also unchanged in the Sco1hrt/hrt heart, leading us to speculate

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**Figure 6.** Sco1G115S/G115S knockin mice exhibit a severe copper deficiency that is also caused by the mislocalisation of CTR1. (A) Western blot analysis of ATP7A, CTR1, SCO1, CCS and COX IV abundance in P120 hearts of wild-type and Sco1G115S/G115S mice. GAPDH was used as a loading control. The arrow and the asterisk denote the glycosylated and truncated forms of CTR1, respectively. (B) Total copper levels (µg copper/mg sulphur) in P120 Sco1G115S/G115S and wild-type hearts. (C) Immunofluorescence analysis of CTR1 localisation in P120 wild-type (top) and Sco1G115S/G115S (bottom) hearts. Images were taken at 40X magnification, and the plasma membrane marker Na+/K+ ATPase was used as a positive control. Scale bar represents 20 µm. Inset contains magnified image of selected region of interest (white box).
that deletion of Sco1 in the heart in this mouse model somehow signals a state of copper overload that is communicated to the liver and results in reduced CTR1 levels and increased excretion of copper in the bile.

Our findings emphasize the essential role of SCO1 in the mitochondrial regulation of copper homeostasis in both the developing and adult myocardium. They also strongly suggest that tissue-specific factors impinge upon how organelle signaling is sensed and integrated into the regulatory framework that controls copper homeostasis. Studies to identify the mechanistic basis that underpins the modulation of SCO1-dependent mitochondrial signaling in different tissues are ongoing.

Materials and Methods

Generation of Sco1 mouse models

Conditional Sco1 knockout models were generated by crossing Sco1 floxed mice (Sco1lox/lox) (38) with mice in which Cre recombinase expression is driven by the α-myosin heavy chain (The Jackson Laboratory, strain #018972) or the creatine kinase promoter (The Jackson Laboratory, strain #006475). Cre positive, F1 progeny were then backcrossed to Sco1floxed animals to generate heart-specific (Sco1hrt/hrt) or striated muscle-specific (Sco1stms/stm) Sco1 knockout mice. PCR genotyping of all animals in this breeding scheme was performed as previously described (38).

Sco1G115S/wt knockin mice were generated fee-for-service at the Toronto Centre for Phenogenomics. Briefly, the guide RNA 5’-GCATTACGACCGCTTTTACA-3’, a single-strand oligonucleotide repair template with a silent mutation to disrupt the protospacer adjacent motif sequence, and Cas9 mRNA were co-injected into C57BL/6NCrI zygotes. Resultant pups were screened by sequencing end-point PCR products to identify the point mutation, and bred to C57BL/6NCrI for germline transmission of the knockin allele. Sco1G115S/wt knockin mice were then backcrossed to C57BL/6NCrI animals for 3 successive generations to reduce the likelihood of animals carrying second site, off-target mutations. Sco1G115S/wt knockin mice were intercrossed, and then bred with Sco1G115S/G115S knockin mice to both maintain a breeding colony and generate experimental animals. Tail DNA was prepared with the KAPA Mouse Genotyping Kit (KAPA Biosystems, KK5621), and 2 μl of a 1: 1 dilution in water was used in a 10 μl real-time PCR reaction with PerfeCTaq qPCR ToughMix (VWR, CA97065-966) on the Applied Biosystems Step One Plus Real Time Thermocycler. The PCR reaction mix contained 500 nM of primers (F: TTAGAGCTGGAGAAACAAGCG, R: GCCTTCCATTGTAGTGTCTA) and 250 nM of probes (wild-type HEX probe, CA + TT + g + G + aAA + GCC and G115S knockin FAM probe, CA + TT + a + G + CAA + G + CCT) purchased from Integrated DNA Technologies, and was run for 40 cycles with an annealing temperature of 60°C. All animal-related experiments were approved by the Animal Ethics Review Board at the University of Saskatchewan (AUP #20100091).

Echocardiography

The procedure for the mouse echocardiogram is described in detail elsewhere (56). In brief, cardiac function of wild-type and Sco1stms/stm mice (n = 4) was evaluated at P60 and P90 using a high resolution Vevo 2100 ultrasound system (VisuaSonics, Toronto, ON, Canada) equipped with an MS550 transducer. Mice were anesthetized using 3% isoflurane for 1–2 min, and then maintained at 1.5–2% isoflurane after losing the righting reflex. Mice were laid supine on a heated platform during echocardiography. Left ventricle internal diameter end systole (LVIdse) and diastole (LVIdsd), fractional shortening and ejection fraction were measured from the standard LV short-axis M-mode view. The heart rate, electrocardiogram (ECG), respiratory rate and body temperature of the mouse were monitored throughout the experiment.

Tissue preparation and immunohistochemistry

Hearts from Sco1hrt/hrt mice and wild-type littermates were fixed in situ using 10% formalin for at least 48 h, dissected out of the chest cavity and embedded in paraffin. Hearts from Sco1stms/stm mice and wild-type littermates were dissected from the chest cavity and promptly perfused with HEPES buffered Tyrode’s solution [25 mM HEPES (pH 7.4), 119 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2 and 33 mM glucose] using a modified Langendorff setup. For hematoxylin and eosin and Masson’s Trichrome staining, additional Sco1stms/stm hearts were fixed in 10% formalin for at least 48 h and embedded in paraffin. Hearts from both mouse models were sectioned into six micron slices, and slide mounted for staining. For immunofluorescent imaging, hearts from wild-type, Sco1stms/stm and Sco1G115S/G115S knockout mice were perfused as above, rapidly contact frozen in optical cutting temperature compound with dry ice chilled isobutane and stored at −80°C. Six micron frozen slices were sectioned with a cryostat and slide mounted. Slides were fixed post-sectioning for 10 min in acetone chilled to −20°C, washed twice for 5 min in phosphate buffered saline [PBS (pH 7.4); 2.7 mM KCl, 134 mM NaCl, 1.5 mM KH2PO4, 8 mM K2HPO4] and then once with 25 mM glycine for 10 min. For Rab5 and CTR1 stained slides, a permeabilization step (PBS + 0.1% Triton X-100) was added prior to blocking. Slides were blocked for 1 h in PBS containing 10% goat serum and 6% bovine serum albumin, and incubated overnight in a humidified chamber at 4°C in antibody buffer [1X sodium/citrate buffer (0.15 mM NaCl and 15 mM Na2C6H5O7·2H2O), 2% goat serum, 1% BSA, 0.05% Triton X-100, 0.02% NaN3]. The slides were washed twice more in PBS for 5 min and incubated at room temperature in a humidified chamber for 1 h in secondary antibodies [Abcam: anti-Rab IgG Alexa Fluor 467 (1: 1000); Cell Signaling Technologies: anti-Rabbit IgG Alexa Fluor 488 (1: 1000) and anti-Mouse IgG Alexa Fluor 594 (1: 750)] in antibody dilution buffer. Finally, the slides were washed twice with PBS for 5 min. All imaging was done using a ZEISS LSM-700 confocal microscope with image acquisition performed using ZEISS Zen Black imaging software.

Native and denaturing electrophoresis

Tissues from cervicaly dissected mice were harvested and frozen at −80°C. Blue native polyacrylamide gel electrophoresis (BN-PAGE) analyses were conducted as described in detail elsewhere (57). Briefly, a piece of heart tissue was resuspended and gently homogenized in extraction buffer [20 mM bis-Tris (pH 7.4), 50 mM NaCl, 10% (v/v) glycerol and 0.2% (w/v) lauryl maltoside]. The protein concentration was then quantified and adjusted to 2 mg/ml, and samples were incubated on ice for 30 min. Following a 5-min spin at 18 000g at 4°C and the addition of loading dye, equal amounts of protein (20 μg/lane) were fractionated on a 6–16% acrylamide gradient gel and transferred.
to a nitroc Cellulose membrane under semi-dry conditions. For
denaturing electrophoresis, proteins were extracted from ex-
cised tissues by dounce homogenization in a RIPA extraction
buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium
deoxycholate, 0.1% SDS, 1 mM EDTA and 1% Complete
protease inhibitor cocktail (Roche), adjusted to a final concen-
tration of 5 mg/ml, incubated on ice for 30 min and then cen-
trifuged at 4 °C for 10 min at 12 000 × g. Equal amounts of protein
were separated on precast 4–20% SDS-PAGE gradient gels
(BioRad), transferred to nitrocellulose membrane, blocked for
2 h in Tris buffered saline supplemented with Tween 20 [TBST;
25 mM Tris (pH 7.4), 137 mM NaCl, 2.5 mM KCl and 0.05% Tween
20] containing 5% milk (US Biologicals), and incubated overnight
at 4 °C in primary monoclonal [Abcam, COX I (1: 1000), COX IV
(1: 1000), Core I (1: 1000), ATPase α subunit (1: 1000), NDUF9
(1: 1000), SDHA (1: 1000); Cell Signaling Technologies, GAPDH
(1: 1, 000) and polyclonal |CTR1 (1: 250, GenScript fee-for-
service antibody production with peptide VSIRYNSMVP
GPNTILC); CCS (1: 1, 000, kind gift from Dr. Joe Prohaska,
University of Minnesota); SCO1 (1: 1, 000, in house fee-for-
service antibody production with peptide EIDDIPSIFNL7])
antibodies. Membranes were then washed six
times for 5 min in TBST, incubated for 60 min in the appropriate
horseradish peroxidase conjugated secondary antibody [Biorad,
1: 2500] in TBST containing 5% milk, and washed again in TBST
as above. Membranes were developed using a homemade
luminol-enhanced chemiluminescence solution, and visualized
with X-ray film or the BioRad ChemiDoc™ MP Imaging System.

RT-PCR analysis
Total RNA was isolated from hearts and reverse transcribed into
cDNA, as previously described (38). PCR reactions contained
equal amounts of cDNA and unique primer sets to generate the
following amplicons; ANP (F: GCCATCGCTCTACCTCCTTG,
G: GCCATTCCCTCGACCTTCC), BNP (F: GAGGTCACCTCCTATCC
TGG, G: GCCATTTCCCTCGACTTTTCC), α-S (F: CCNACGC
TACCGGCGAGAG, G: CCAGAAATCCAACAGGTG), SERCA2 (F:
TGGACAAAGCCGGGTAAAAAGGT, G: CACCAGGGCCTAATGAGC
AG), MT1 (F: CTCTCTAAAGCCGTCC ACCAC, G: GAGCAGCTG
GGTCCAT) and GAPDH (F: ATGGTTGAAGGCTGGTCTGAA,
G: GC CCTGACTGAGCTCATACT). PCR conditions were optimized to
ensure the amplification of a single product was captured in the
log phase of the reaction.

Miscellaneous
Elemental analyses, total protein quantitation and enzyme ac-
tivity assays were performed as described in detail elsewhere
(23). Where applicable, data were presented as the mean ±
standard error of the mean, and significant differences between ex-
perimental groups were detected using a two-sample t-test
assuming unequal variance. * denotes P < 0.05, ** denotes P < 0.01, ***
denotes P < 0.001 and NS denotes not significant.

Supplementary Material
Supplementary Material is available at HMG online.

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