Cardiac deficiency of single cytochrome oxidase assembly factor scox induces p53-dependent apoptosis in a Drosophila cardiomyopathy model

Leticia Martínez-Morentin, Lidia Martínez, Sarah Piloto, Hua Yang, Eric A. Schon, Rafael Garesse, Rolf Bodmer, Karen Ocorr, Margarita Cervera, and Juan J. Arredondo

1Departamento de Bioquímica, Facultad de Medicina, Instituto de Investigaciones Biomédicas “Alberto Sols” UAM-CSIC and Centro de Investigación Biomédica en Red (CIBERER), c/ Arzobispo Morcillo s/n, Universidad Autónoma de Madrid, Madrid 28029, Spain, 2Development, Aging and Regeneration Program, Sanford-Burnham Medical Research Institute, 10901 N Torrey Pine Rd, San Diego, CA 92037, USA, 3Department of Neurology and Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, 630 West 168th Street P&S 4-449, New York, NY, USA and 4Instituto de Investigación Sanitaria Hospital 12 de Octubre (i+12), Madrid 28041, Spain

Abstract

The heart is a muscle with high energy demands. Hence, most patients with mitochondrial disease produced by defects in the oxidative phosphorylation (OXPHOS) system are susceptible to cardiac involvement. The presentation of mitochondrial cardiomyopathy includes hypertrophic, dilated and left ventricular noncompaction, but the molecular mechanisms involved in cardiac impairment are unknown. One of the most frequent OXPHOS defects in humans frequently associated with cardiomyopathy is cytochrome c oxidase (COX) deficiency caused by mutations in COX assembly factors such as Sco1 and Sco2. To investigate the molecular mechanisms that underlie the cardiomyopathy associated with Sco deficiency, we have heart specifically interfered scox expression, the single Drosophila Sco orthologue. Cardiac-specific knockdown of scox reduces fly lifespan, and it severely compromises heart function and structure, producing dilated cardiomyopathy. Cardiomyocytes with low levels of scox have a significant reduction in COX activity and they undergo a metabolic switch from OXPHOS to glycolysis, mimicking the clinical features found in patients harbouring Sco mutations. The major cardiac defects observed are produced by a significant increase in apoptosis, which is dp53-dependent. Genetic and molecular evidence strongly suggest that dp53 is directly involved in the development of the cardiomyopathy induced by scox deficiency. Remarkably, apoptosis is enhanced in the muscle and liver of Sco2 knock-out mice, clearly suggesting that cell death is a key feature of the COX deficiencies produced by mutations in Sco genes in humans.
Introduction

Mitochondrial respiratory chain disorders (MRCDs) due to dysfunctions in the oxidative phosphorylation (OXPHOS) system are among the most frequent inborn errors of metabolism, with an incidence of 1:5000 live births (1). MRCDs are multisystemic diseases and therefore, it is very difficult to distinguish systemic and tissue-specific phenotypes. Moreover, MRCDs are associated with a broad spectrum of clinical manifestations, with dilated or hypertrophic cardiomyopathies representing a common feature of these conditions. Neonatal cardiac abnormalities can be either isolated or accompanied by multi-organ involvement and are frequently associated with metabolic crises and lactic acidosis that may produce a fatal outcome (2).

Cytochrome c oxidase (COX) is the terminal component of the mitochondrial respiratory chain (MRC). COX is a multimeric complex comprised of 13 structural subunits whose assembly into a fully functional holoenzyme is a complicated process requiring accessory factors (3). Indeed, COX deficiency due to mutations in COX assembly factors is one of the most frequent causes of MRC defects in humans (4).

SCO1 and SCO2 are paralogue genes that encode metallochaperones, both of which fulfil essential, non-overlapping cooperative roles in complex IV catalytic core assembly (5). In this way, these genes help maintain cellular copper homeostasis (6) and perhaps redox regulation (7). Pathogenic mutations in SCO1 cause fatal infantile hepatocerebral dysplasia (8), although one such case with hypertrophic cardiomyopathy has been reported (9). Mutations in SCO2 cause fatal infantile cardioencephalopathy (8), with all but one of the patients harbouring the E140 K mutation (10). Despite the similar functions of SCO1 and SCO2, their precise role in COX assembly remains unknown. Although SCO1 predominates in blood vessels, both are expressed ubiquitously, but it is intriguing that mutations in the two genes are associated with different tissue-specific COX deficiencies and distinct clinical phenotypes (11).

SCO2 synthesis is transcriptionally activated by p53, which has been shown to modulate the balance between OXPHOS and glycolysis (12). In addition, p53 appears to promote mitochondrial function and regulate metabolic homeostasis through different target genes, including AIF, parkin, TFAM, POLY and PGC1α (13–17). Given the homeostatic relationships among these genes, it would seem likely that a feedback mechanism would exist between mitochondria and p53. In fact, it was recently shown in Drosophila competitive mosaics that p53 is not only induced as an adaptation to regulate mitochondrial respiration, but that it also plays an important role in metabolic homeostasis by enhancing glycolytic flux (18).

Here, we investigated the genetic and molecular mechanisms that underlie cardiomyopathies associated with SCO deficiency in Drosophila. Unlike vertebrates, Drosophila heart function can be significantly compromised without causing immediate death (19). Furthermore, since the genetic network controlling cardiac specification and differentiation are conserved from flies to mammals, as well as many other aspects of heart function, Drosophila has become a powerful genetic model to study cardiomyopathies (20–22).

In Drosophila, there is a single orthologue of mammalian SCO1 and 2, scox, which has been identified and characterized (23). Ubiquitous scox knockdown (KD) or null mutant flies are lethal at larval stages, whereas weaker mutants are associated with motor dysfunction and female sterility. Indeed, such mutants display a strong disruption of Complex IV assembly and a concomitant reduction of COX enzyme activity (23,24). Here we demonstrate that cardiac-specific scox knockdown causes cardiomyopathy, severely compromising heart function and structure. We show that cardiomyocytes undergo a metabolic switch from OXPHOS to glycolysis, probably accompanied by enhanced lactic acid production, mimicking the clinical features of patients with SCO mutations. The major cardiac defects observed appear to be provoked by cell death, which is dp53-dependent. Significantly, we show that loss of p53 or inhibition of apoptosis blocks the Sco-induced cardiomyopathy. Our study shows strong evidence that dp53 is directly involved in the development of cardiomyopathy produced by SCOX partial loss of function.

Results

Cardiac-specific interference of scox causes mitochondrial impairment

To analyse the role played by SCO proteins in cardiomyopathies, we generated a Drosophila model based on heart-specific RNAi-mediated knockdown of scox. We first tested whether ubiquitous scox knockdown produced a phenotype similar to that described for scox homozygous mutants or its ubiquitous interference, as recently described (23,24). Ubiquitous knockdown of scox using the daughterless (Da::Gal4) driver and a UAS-scox RNAi (UAS-scox) resulted in animals developing only to the third-instar stage, never reaching pupal stage, and displaying a Spargel phenotype typical of mutations affecting mitochondrial proteins (Supplementary Material, Fig. S1A) (25,26). Moreover, scox silencing strongly impaired COX activity, whereas Complex I activity remained unaffected (Supplementary Material, Fig. S1B), demonstrating that ubiquitous interference of scox expression phenocopies its loss of function (23).

Cardiac-specific scox knockdown using TinC::Gal4 as the driver also compromised Drosophila survival. TinC::Gal4>scox flies began to die after 2 weeks and had a mean lifespan 12 days shorter than controls (TinC::Gal4/+). Hence, all experiments were carried out on 1- or 2-week-old flies.

We evaluated the extent of scox KD by measuring mRNA expression in fly hearts by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and by histochemical staining of heart complex IV activity. We observed a 50% reduction in scox mRNA in hearts from 1-week-old TinC::Gal4>scox flies compared with the outcrossed driver and UAS-scox controls (Fig. 1A). We used complex IV activity histochemical staining to evaluate the extent of complex IV activity loss as consequence of scox KD (Fig. 1B, top row). There was a clear decrease in COX activity in vivo in scox KD semi-intact hearts from 1-week-old flies when compared with TinC::Gal4- and UAS-scox controls. In order to confirm that the observed staining was in fact a consequence of Complex IV activity, hearts were stained in the presence of the COX inhibitor KCN. Inhibition of COX effectively prevented the staining (Fig. 1B, second row). To further corroborate that scox interference was causing an isolated COX deficiency, we also examined succinate dehydrogenase (SDH, CI) activity by histochemical staining. In this case, we observed no such differences in SDH activity between the scox KD and controls. Again, treatment of the samples with CI-specific inhibitor Malonate confirmed the staining specificity (Fig. 1B, lower rows). These results indicate that a decrease of just 50% in scox mRNA expression is sufficient to compromise COX activity in the Drosophila heart and that this defect is specific to Complex IV.

Mitochondrial dysfunction alters energy metabolism in many forms of heart disease (27) and therefore, we hypothesized that the COX deficiency displayed by cardiac-specific scox KD hearts should provoke a partial blockage of the citric acid cycle and a
compensatory upregulation in glycolysis. We assessed the expression level of key enzymes involved in both glycolysis and citric acid cycle with qRT-PCR. We found that glycolytic enzymes, including glucose-6-phosphate isomerase (gpi), phosphofructokinase (pfk), and phosphoglycerate kinase (pgk1) were all upregulated in TinCΔ4;Ga4>scox KD hearts. Furthermore, expression of the Drosophila orthologue of human lactate dehydrogenase (LDH), iml3 and the mitochondrial matrix enzyme pyruvate dehydrogenase (pdf) were also enhanced (Fig. 1C). LDH converts pyruvate to lactate, the final product of non-respiratory glucose consumption, and its enhanced expression would be expected to result in lactic acidosis. PDF is a key regulator of glucose oxidation, inhibiting pyruvate dehydrogenase (PDFH), thereby blocking entry of pyruvate into the citric acid cycle. Hence, our data strongly suggest that cardiac-specific interference of scox causes mitochondrial dysfunction, with the concomitant metabolic switch from glucose oxidation to glycolysis.

scox RNAi knockdown causes cardiomyopathy in Drosophila melanogaster

To assess how the mitochondrial dysfunction caused by silencing scox affects heart function, we used high-speed optical recording of semi-intact adult preparations of beating hearts and semi-automated analysis software to characterize cardiac physiology (28). Heart function in these flies is shown qualitatively in the M-mode traces from high-speed movies that illustrate heart wall movement over time (Fig. 2A). Hearts from 2-week-old controls showed regular rhythmic contractions, however, TinCΔ4;Ga4>scox hearts showed a distinctive slowing. The heart period (HP) was quantified from movies of beating hearts from 1- and 2-week-old TinCΔ4;Ga4>scox flies (Fig. 2B). scox KD resulted in a significantly increased HP (reduced heart rate, Fig. 2B and Supplementary Material, Fig. S2A). The increase in the heart period was due to a selective increase in the diastolic interval (DI: Fig. 2C), with 20% of the 2-week-old cardiac-specific scox KD flies displaying DIs longer than 1 s (Supplementary Material, Fig. S2B). In addition, in the region posterior to the conical chamber, the diastolic diameters of KD hearts were significantly smaller than controls. There was little effect on the average systolic diameter, consequently resulting in a significant reduction, from 43% in the controls to 33% in the scox KD flies, in heart tube contractility measured as fractional shortening (FS) (Fig. 2D–F). All cardiac parameters assessed in scox RNAi hearts were aggravated with age, and the phenotype observed was also dose-dependent, since animals harbouring just one copy of both driver and UAS-scoxi developed milder phenotypes (Fig. 2A, compare TinCΔ4;Ga4>scox to TinCΔ4;Ga4/+>scox/+). Thus, cardiac-specific scox knockdown results in severe cardiac dysfunction.

scox knockdown alters myofibril structure

The reduced contractility of the posterior heart tubes from scox KD flies led us to hypothesize that their heart structure might
Cardiac-specific scox knockdown causes heart dysfunction. (A) Representative M-Mode traces (10 s) from high-speed movies of semi-intact Drosophila heart preparations. M-Mode traces represent the movements of the heart walls (y-axis) over time (x-axis). One- and two-week-old control flies (TinC4: Gal4 and UAS-scox) present rhythmic heart beating. Cardiac-specific scox KD causes long DIs between contractions (Di, horizontal) in the homozygous and heterozygous lines compared with controls. scox KD hearts exhibit age-dependent deterioration in cardiac function. (B) Heart period, (C) Di, (D) diastolic diameter, (E) systolic diameter and (F) FS were measured for hearts from 1- and 2-week-old controls (TinC4: Gal4 and UAS-scox) and cardiac-specific scox KD (TinC4: Gal4>scox and TinC4:Gal4/+>scox). Note the significant heart period and DI prolongation with scox KD in hearts from 1- and 2-week-old flies (TinC4: Gal4>scox and TinC4:Gal4/+>scox). Interestingly, hearts from double knockdown flies showed a significant decrease in systolic and diastolic diameters (TinC4: Gal4-scox). FS is also significantly decreased due to a decreased systolic diameter and decrease in diastolic diameter. In all measures, the scox knockdown phenotype is more severe in 2-week-old flies. Significance was determined using a one-way ANOVA and Tukey’s multiple comparisons post-hoc test. Differences are relative to the TinC4: Gal4 control. Error bars indicate SEM (*P < 0.05, **P < 0.01 and ***P < 0.001). Sample size was 20–40 flies per genotype.

be altered. To explore the impact of silencing scox on heart structure, we used immunohistochemistry to examine TinC4-Gal4>scox hearts from 1- and 2-week-old flies. Phalloidin staining revealed that although overall heart structure seemed to be fairly normal, scox RNAi animals exhibited myofibrillar disarray and an obvious narrowing of the heart tube from abdominal segment 4 to the end of the heart tube (arrows in Fig. 3A). This phenotype became more noticeable with age (Fig. 3A, compare 1 and 2 weeks). In addition, the size of the conical chamber diameter increased significantly in 1-week-old TinC4-Gal4>scox flies compared with controls (Fig. 3B), suggesting that heart-specific scox interference causes dilated cardiomyopathy in Drosophila.

We then analysed the myofibrillar organization in phalloidin-stained cardiomyocytes from abdominal segments 3 (A3) and 4 (A4) in 1- and 2-week-old flies. This was the region where we observed a significant reduction in contractility in our functional assays. Cardiomyocytes from controls (TinC4: Gal4 and UAS-scox) displayed tightly packed and well-aligned circumferential myofibrils (Fig. 3C and D, 1–2), whereas myofibrils from cardiomyocytes in TinC4-Gal4>scox hearts were loosely packed and poorly organized, being fully disorganized in those hearts displaying the stronger structural phenotypes (Fig. 3C and D, 3–4). Consistent with the overall morphology observed previously (Fig. 3A), fibre disorganization was strongest in the posterior half (compare A3 and A4 segments, Fig. 3C and D). Moreover, hearts from 2-week-old flies displayed more severe myofibrillar disarray than those from 1-week-old flies, with the heart tube often appearing almost collapsed (Fig. 3C 3–4 and D 3–4).

As one case of fetal wastage harbouring SCO2 mutations has been reported in humans (29), we wondered whether the structural defects observed in TinC4-Gal4>scox heart tube were the consequence of a developmental or pupal heart-remodelling defect or whether the degenerative phenotype described was a consequence of the detrimental consequences of mitochondrial dysfunction accumulating over time. To test the former hypothesis, we used immunohistochemistry to examine cardiac-specific scox KD and control hearts from red-eyed pupae. Phalloidin staining revealed that the heart tubes from TinC4-Gal4>scox pupae exhibited normal heart structure when compared with control hearts (Supplementary Material, Fig. S3), ruling out the possibility that a developmental or pupal heart-remodelling defect provoked the myofibril disorganization observed in the adult heart.
Together with data obtained from live beating hearts, these results suggest that scox downregulation compromises heart function and structure in a time-dependent manner that it is not due to developmental defects.

**COX deficiency enhanced the production of reactive oxygen species**

Mitochondrial respiration, mainly at electron transport chain (ETC) complexes I and III, is the main source of reactive oxygen species. COX deficiency enhanced the production of reactive oxygen species.
species (ROS) in most eukaryotic cells (30), and an increase in ROS levels represents a source of cellular stress often associated with mitochondrial dysfunction (31). Interestingly, ROS formation and oxidative DNA damage have been shown to be enhanced in human SCO2⁻/⁻ cells (32). As heart-specific scox knockdown causes mitochondrial dysfunction, we asked whether reduced COX activity augments ROS production. ROS levels were measured in hearts from 1- and 2-week-old control and cardiac-specific scox KD flies using dihydroethidium (DHE), a dye that is accumulated in the nucleus after reaction with superoxide anions, as an indicator. The stronger nuclear staining (arrowheads in Fig. 4A) in hearts from TinCΔ4-Gal4/+>dp53/+ compared with control. Thin arrows indicate DHE staining in the nuclei of scox KD cardiomyocytes.

To complete our analysis of the role of dp53 in scox KD-associated cardiomyopathy, we co-overexpressed dp53 in TinCΔ4-Gal4/+>scoxi KD hearts using a UAS-dp53 line. We first examined the cardiac physiology of 1-week-old TinCΔ4-Gal4/+>scoxi KD/dp53 overexpression (OE) flies to assess the effect of dp53 OE and to determine whether there was any genetic interaction between dp53 and scox. Overexpression of dp53 alone caused a significant slowing of the heart period, reminiscent of scox KD. Surprisingly, we observed no heartbeat in scox KD hearts that were also overexpressing p53 even at relatively young ages (1 week, Fig. 4C). Structural analyses by phalloidin staining of TinCΔ4-Gal4/+>dp53/+ hearts revealed that dp53 OE itself caused cardiac defects comparable to that of scox KD harbouring two copies of both driver and UAS-scox (Fig. 4D). Significantly, hearts from TinCΔ4-Gal4/+>scoxi KD/dp53 OE flies exhibited very strong heart degeneration, with the complete loss of cardiac myofibrils.
and the presence of only a few longitudinal myofibrils from the dorsal longitudinal muscle in the animals displaying the strongest phenotypes. Note that in contrast, the animals that carried just one copy of each driver and UAS-scox, but no UAS-dp53 (TinC4-Gal4/+;scox/+,), displayed a relatively mild phenotype (Supplementary Material, Fig. S4B).

It is possible that the cardiac degeneration observed in response to dp53 OE in scox KD flies might be due to a general stress response triggered by mitochondrial impairment rather than a specific genetic interaction between scox and dp53. To clarify this possibility, we examined the cardiac response to KD of another complex IV assembly factor, Surf1. Knockdown of Drosophila Surf1 gene expression has previously been shown to cause COX deficiency, with nervous system involvement and developmental arrest (37). We tested whether dp53 OE in cardiac-specific Surf1 KD animals caused a similar cardiac degeneration to that observed in TinC4-Gal4/+;scox/dp53 flies. We evaluated the extent of Surf1 KD by measuring mRNA expression levels in fly hearts by qRT-PCR. Hearts from 1-week-old cardiac-specific Surf1 flies showed a 50% reduction in Surf1 mRNA similar to that observed in scox KD hearts (Supplementary Material, Fig. S4A). Next, we asked whether reduced COX activity, a consequence of Surf1 knockdown, augments ROS production as occurred in scox KD. ROS levels were measured in hearts from 2-week-old control and cardiac-specific Surf1 KD flies using DHE. The stronger nuclear staining (arrowheads in Supplementary Material, Fig. S4B) in hearts from TinC4-Gal4;Surf1i exhibited an increase in ROS production in these hearts. Surprisingly, morphological analyses showed that 1-week-old Surf1 KD animals displayed only a very mild, if any, cardiac phenotype. Most remarkably, TinC4-Gal4/+;Surf1i KD/dp53 OE hearts looked entirely normal demonstrating that, unlike for scox KD, Surf1 heart-specific KD not only has minimal effects on heart function, but appears to rescue the dp53 OE heart phenotype (Supplementary Material, Fig. S4C). Furthermore, in contrast to our observations for scox KD hearts, we found no increase in dp53 transcripts in TinC4-Gal4;Surf1i hearts (Supplementary Material, Fig. S4D).

These results demonstrate that dp53 and scox interact genetically, suggesting that dp53 might play an important role in the development of scox KD-induced cardiomyopathy.

Cardiac-specific knockdown of scox induces apoptosis

Given the pro-apoptotic activity of dp53 (36,38), it would appear that scox knockdown might induce dp53-dependent apoptosis in cardiomyocytes. As dp53 controls cell death through the Reaper-Hid-Grim network (39), we assessed their relative expression and we observed a significant increase in Reaper, Hid and Grim mRNA expression in heart tubes from 1- and 2-week-old TinC4-Gal4;scoxi flies (Fig. 5A), reflecting the activation of the apoptotic pathway in scox KD flies. Whether the cardiac defects caused by scox interference might be due to apoptosis activation was further assessed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining in hearts from 1- and 2-week-old scox KD flies. Although no TUNEL labelling was evident in control hearts from 1- or 2-week-old animals (Fig. 5B, 1 and 3), clear labelling was observed in 1-week-old cardiac-specific scox KD hearts (Fig. 5B, 2), which was markedly stronger after 2 weeks (Fig. 5B, 4). To confirm whether apoptosis was responsible for the structural disarray observed in scox KD hearts, we overexpressed the pro-apoptotic gene Reaper in a TinC4-Gal4++;scoxi background. Hearts from these 1-week-old TinC4-Gal4++;scoxi, rpr flies presented strong myofibril disorganization (Supplementary Material, Fig. S5A and B) reminiscent of that observed in 2-week-old cardiac-specific scox KD flies. Together, these data clearly demonstrate that cardiac-specific scox KD activates apoptosis, inducing cell death.

Disruption of dp53 activity rescues scox cardiomyopathy

The fact that scox downregulation triggers dp53-mediated apoptosis, together with the strong degeneration observed in those flies, which is exacerbated by dp53 OE, led us to hypothesize that blocking the dp53 pathway might impede apoptosis and rescue cardiac function in TinC4-Gal4++;scoxi flies. We used a dominant-negative form of dp53 (UAS-dp53DN, dp533N) to abrogate p53 activity in a scox KD background. M-Mode records from TinC4-Gal4/+;scox/dp53DN and TinC4-Gal4/+;dp533N/+ 2-week-old fly hearts show regular rhythmic contractions and an average heart-beat length (Fig. 6A, compare with Fig. 2A). Furthermore, the quantification of the different physiological parameters showed that the DI and heart period of TinC4-Gal4/+;scox/dp53DN hearts were similar to that of controls and significantly shorter than that of scox KD hearts (Fig. 6B). FS, an indicator of heart contractibility, was also rescued by dp53DN expression in scox KD hearts (Fig. 6B), demonstrating that heart activity was fully rescued by disruption of dp53 activity.

When we examined cardiac structure, there were no obvious morphological defects in the hearts of 2-week-old flies overexpressing dp53DN alone, or in the hearts from cardiac-specific scox KD animals that also overexpressed dp53DN. Specifically, the myofibrillar arrangement within the cardiomyocytes of TinC4-Gal4/+;scox/dp53DN or TinC4-Gal4++;scox/dp53DN flies, respectively, carrying one or two driver copies, were circumferentially aligned (Fig. 6C). Under higher magnification, the structure of the A3 and A4 segments demonstrated that cardiac-specific overexpression of dp53DN fully restored the severe morphological defects found in scox KD (Fig. 6D).

It could be argued that the observed rescue might be explained by the presence of a second UAS in TinC4-Gal4/+;scox/dp53DN flies which could result in a weaker interference of scox expression. In order to rule out this possibility, we expressed the unrelated protein GFP (UAS-GFP) in TinC4-Gal4++;scoxi flies. Animals harbouring one copy of TinC4-Gal4 to drive the expression of both dp53 and scox (TinC4-Gal4/+;scox/GFP) still displayed structural defects comparable to those displayed by TinC4-Gal4/+;scox/+ (Fig. 6C and D), indicating that the cardiac dysfunction rescue was not due to a weaker interference but rather to a blockage of the p53 pathway (Fig. 6B).

To further demonstrate the involvement of dp53 in the development of cardiomyopathy, we disrupted scox expression in a dp53 null background. The heart-specific KD of scox in a dp533−/− flies did not provoke myofibrillar disarray or any other defect (Supplementary Material, Fig. S6A), further emphasizing the role of p53 in the development of scox-meditated cardiomyopathy.

We also asked whether inhibiting apoptosis would rescue the structural degeneration observed in cardiac-specific scox KD cardiomyocytes. This was assessed by expressing the baculovirus caspase inhibitor p35 (40) or the Drosophila inhibitor of apoptosis DIAP1 (41) in a cardiac-specific scox KD background. Two-week-old scox KD flies expressing p35 or DIAP1 in a heart-specific manner showed no structural defects (Supplementary Material, Fig. S7A), although under higher magnification, mild disarray was observed when DIAP1 was overexpressed (Supplementary Material, Fig. S7B). Thus, inhibiting apoptosis appears to rescue the myofibrillar degeneration observed in scox KD flies.
Sco2KIKO mice undergo apoptosis

In light of the above data, we wondered whether our results in the Drosophila heart could be extended to mammals. Although there are no Sco1 KO mice currently available, a Sco2KIKO mouse model has been recently developed that harbours a Sco2 knock-out allele and the Sco2 knock-in E129 K allele which corresponds to the E140 K mutation found in almost all human patients (31). As observed in patients with SCO2 deficiency, these Sco2KIKO mice display motor impairments, as well as biochemical and functional defects. It should be noted that, in contrast to humans, the reduction in COX activity in Sco2KIKO mice was less severe in muscle than in other tissues, such as liver, and it was accompanied by an unexpected defect in complex III activity (31). Sco2KIKO mice do not develop overt symptoms of the cardiomyopathy seen in the human disease and no significant differences in cardiac function between WT and Sco2-mutated mice, as measured by transthoracic M-mode and two-dimensional echocardiography (31). Therefore, we decided to analyse whether there was apoptosis in liver and skeletal muscle, the two most...
Lack of dp53 activity rescues scox cardiomyopathy. (A) Representative M-Mode traces (10 s) from high-speed movies of semi-intact flies. Cardiac-specific dp53DN OE in 2-week-old scox KD hearts (TinCΔ4-Gal4/+>scoxi/dp53DN) causes a significant enhancement in cardiac function compared with that seen in response to cardiac-specific scox KD alone (Fig. 2). Cardiac-specific dp53DN OE in 2-week-old hearts (TinCΔ4-Gal4/+>dp53DN/+ ) does not affect heart function. (B) DI, heart period and FS were measured for hearts from 2-week-old controls (TinCΔ4::Gal4 and UAS-scoxi), cardiac-specific scox KD (TinCΔ4-Gal4>scoxi and TinCΔ4-Gal4/+>scoxi/+) and dp53DN OE (TinCΔ4-Gal4/+>dp53DN/+ and TinCΔ4-Gal4/+>scoxi, dp53DN). In all measures, cardiac-specific dp53DN OE rescues the scox knockdown phenotype in 2-week-old flies. Significance was determined using a one-way ANOVA and Tukey’s multiple comparisons post-hoc test. Differences are relative to the TinCΔ4::Gal4 control. Error bars indicate SEM (**P < 0.01 and ***P < 0.001). Sample size was 20–40 flies per genotype. (C) Confocal images of 2-week-old adult hearts stained with Alexa Fluor 594-phalloidin to identify actin filaments at 10× magnification. Hearts from control (TinCΔ4::Gal4), cardiac-specific scox KD (TinCΔ4-Gal4>scoxi and TinCΔ4-Gal4/+>scoxi/+) and cardiac-specific dp53DN OE (TinCΔ4-Gal4/+>dp53DN, TinCΔ4-Gal4/+>dp53DN/, TinCΔ4-Gal4/+>dp53DN, TinCΔ4-Gal4/+>dp53DN/) and TinCΔ4-Gal4/+>scoxi, dp53DN) are shown. Cardiac-specific dp53DN OE (third and fourth panels on the right) rescues the scox KD structural phenotype (first and second panels on the right and second panel on the left). Controls expressing dp53DN (TinCΔ4-Gal4/+>dp53DN/ and TinCΔ4-Gal4/+>dp53DN/) are shown in third and fourth panels on the left. (D) Representative confocal images of third and fourth abdominal segments (A3 and A4) of the dorsal vessel from 2-week-old flies at 25× optical magnification (2× zoom). Adult hearts are stained with Alexa Fluor 594-phalloidin to identify actin filaments. Cardiac-specific dp53DN OE rescues myofibrillar disorganization caused by scox KD. Cardiomyocytes from cardiac-specific dp53DN OE (TinCΔ4-Gal4/+>scoxi, dp53DN and TinCΔ4-Gal4/+>scoxi, dp53DN) exhibit regular circumferential myofibrillar arrangement (second line panels, second and fourth lines).
affected tissues. TUNEL staining of liver and skeletal muscle from Sco2<sup>KI/KO</sup> mice revealed that there was extensive apoptosis in both tissues in Sco2<sup>KI/KO</sup> mice but not in WT animals (Fig. 7). Furthermore, the liver of Sco2<sup>KI/KO</sup> mice seemed to have more apoptotic cells than the muscle tissue, in agreement with previous observations that the liver has the lowest complex IV activity compared with other tissues in these mice (31). Hence, we conclude that, partial loss of Sco2 function in mice induces apoptosis, as we have observed in Drosophila.

**Discussion**

Cardiomyopathies are a collection of myocardial disorders in which the heart muscle is structurally and functionally abnormal. In the past decade, it has becomes clear that an important proportion of cases of hypertrophic and dilated cardiomyopathies are caused by mutations in genes encoding sarcomeric or desmosomal proteins. In addition, cardiomyopathies (both hypertrophic and dilated) are frequently associated to syndromic and non-syndromic mitochondrial diseases. The importance of oxidative metabolism for cardiac function is supported by the fact that 25–35% of the myocardial volume is taken by mitochondria. The current view of mitochondrial involvement in cardiomyopathy assumes that ETC malfunction results in an increased ROS production, triggering a “ROS-induced ROS release” vicious circle which in turn perpetuates ETC dysfunction via damage in mtDNA and proteins involved in electron transport. Under this view, accumulated mitochondrial damage would eventually trigger apoptosis through mitochondrial permeability transition pore (mPTP) opening other mechanisms (42). Under

![Figure 7. Sco2<sup>KI/KO</sup> mice undergo apoptosis in liver and skeletal muscle. Confocal images of control and Sco2<sup>KI/KO</sup> 6-month-old mice at 25× optical magnification. Skeletal muscle and liver were stained for TUNEL (red) and DAPI (blue). Sco2<sup>KI/KO</sup> mice show high levels of TUNEL-positive fluorescent red nuclei staining (second and fourth line panels). n = 3 per genotype.](image-url)
normal circumstances, damaged mitochondria would be elimi-
nated through mitophagy. Excessive oxidative damage is sup-
posed to overcome the mitophagic pathway resulting in
apoptosis (43). Nevertheless, although several potential mechan-
isms have been suggested, including apoptosis deregulation, oxi-
dative stress, disturbed calcium homeostasis or impaired iron
metabolism, the molecular basis of the pathogenesis of mito-
ochondrial cardiomyopathy is virtually unknown.

Pathogenic mutations in human SCO1 and SCO2 have been re-
ported to cause hypertrophic cardiomyopathy, among other clin-
ic symptoms (8,44). However, the molecular mechanisms underlining this cardiac dysfunction have yet to be elucidated.
We present here the first cardiac-specific animal model to
study human SCO1/2-mediated cardiomyopathy. Cardiac-specific
scsx KD provokes a severe dilated cardiomyopathy, as reflected
by a significant increase in the conical chamber size, due to mito-
chondrial dysfunction. It presents a concomitant metabolic
switch from glucose oxidation to glycolysis and an increase in
chondrial dysfunction. It presents a concomitant metabolic
switch from glucose oxidation to glycolysis and an increase in
ROS levels, leading to p53-dependent cell death. Interestingly,
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ROS levels, leading to p53-dependent cell death. Interestingly,

It has recently been shown that SCO2 OE induces p53-
mediated apoptosis in tumour xenografts and cancer cells (55).
Furthermore, SCO2 KD sensitizes glioma cells to hypoxia-induced
apoptosis in a p53-dependent manner and induces necrosis in
tumours expressing WT p53 (56), further linking the SCO2/p53
axis to cell death. In Drosophila, there is a dp53-mediated upregu-
lation of Reaper, Hid and Grim in response to scsx KD. This, coupled
with the observation that Reaper overexpression in the adult
heart enhances the structural defects caused by cardiac-specific
scsx KD, suggests that scsx normally prevents the triggering of
dp53-mediated cell death in cardiomyocytes in stress response.
Indeed, we found that there is massive cell death in the skeletal
muscle and liver of Sco2−/−/− mice, supporting the hypothesis that
Sco proteins might play this role also in mammals.

We provide evidence that scsx KD hearts exhibit partial loss of
COX activity, with cardiomyocytes undergoing apoptosis. There
is evidence from vertebrate and invertebrate models that partial
inhibition of mitochondrial respiration promotes longevity and
metabolic health due to hormesis (57,58). In fact, it was recently
shown that mild interference of the OXPHOS system in Drosophila
IFMs preserves mitochondrial function, improves muscle
performance and increases lifespan through the activation of
the mitochondrial unfolded pathway response and IGF-like sig-
nalling pathways (59). We speculate that cell death, rather than
mitochondrial dysfunction itself, is likely to be the main reason
for the profound heart degeneration observed in Tnci-14-
Gale-scxxo1 flies. Expression of dominant negative dp53 in scsx
KD hearts rescues dysfunction and cardiac degeneration, and,
most importantly, scsx KD in dp53+/− animals caused no apparent
heart defects, leading us to attribute the rescue observed to
blockade of the p53 pathway. Indeed, inhibiting apoptosis by
p35 or Diap1 OE almost completely rescued the morphological
scsx KD phenotype. As scsx KD in the absence of dp53 causes
no symptoms of heart disease, coupled with the inability of p35
and Diap1 to completely rescue the morphological phenotype,
suggests that, in addition to inducing apoptosis, dp53 plays a
key role in the development of cardiomyopathy.

The fact that heart-specific Surf1 KD neither upregulates p53
nor induces apoptosis supports the idea that the partial loss of
COX function itself triggers dp53 upregulation and apoptosis,
rather than it being a side effect of COX dysfunction and the loss
of cellular homeostasis. In this context, it is noteworthy that
SCO2 interference in mammalian cells induces p53 re-localiza-
tion from mitochondria to the nucleus (60). It is therefore tempt-
ing to hypothesize that scsx might play another role independent
of its function as a COX assembly factor, perhaps in redox regula-
tion as suggested previously (7) and that it may act in conjunction
with dp53 to fulfil this role. Another issue deserves further atten-
tion, the possibility of this interaction being a tissue-specific re-
sponse. It may be possible that the threshold of COX deficiency
tolerated by the heart might be lower than in other tissues,
thus the scsx/dp53 genetic interaction may be a tissue-dependent
phenomenon or the consequence of a tissue-specific role of scsx.
In fact, it was recently shown that mitochondrial dysfunction in
mice is sensed independently from respiratory chain deficiency,
leading to tissue-specific activation of cellular stress responses
(61). Thus, more work is necessary to test these hypotheses and
try to understand how the partial lack of scsx induces cell death
through dp53.

Although the role of mitochondria in Drosophila apoptosis re-
mains unclear, there is strong evidence that, as in mammals,
mitochondria play an important role in cell death in flies. The lo-
calization of Rpr, Hid and Grim in the mitochondria is essential to
promote cell death, and fly mitochondria undergo Rpr-, Hid- and
Drp1-dependent morphological changes and disruption following apoptotic stimulus. Moreover, the participation of the mitochondrial fission protein Drp1 in cell death is conserved in worms and mammals (62). It was recently proposed that p53 plays a role in the opening of the mPTP that induces necrotic cell death (63). According to this model, p53 translocates to the mitochondrial matrix upon ROS stimulation, where it binds cyclophilin D (CypD) to induce mPTP opening independent of proapoptotic Bcl-2 family members Bax and Bak, and in contrast to traditional concepts, independent of Ca2+ (reviewed in Ref. 64).

Apoptotic and necrotic pathways have a number of common steps and regulatory factors, including mPTP opening that is thought to provoke mitochondrial swelling and posterior delivery of necrotic factors (65), although Drosophila mPTP activation is not accompanied by mitochondrial swelling (66). Interestingly, although the p53 protein triggers mitochondrial outer membrane permeabilization (MOMP) in response to cellular stress in mammals, releasing mitochondrial death factors (67), MOMP in Drosophila is more likely a consequence rather than cause of caspase activation (68) and the release of mitochondrial factors does not appear to play a role in apoptosis (69). Thus, in cardiac-specific scox KD flies, dp53 might induce mPTP opening to trigger cell death, which in the absence of mitochondrial swelling would result in apoptosis instead of necrosis, as occurs in mammals. Drosophila mPTP has been shown to be cyclosporine A (CsA)-insensitive in vitro (66), although it was recently shown that CsA administration ameliorates the mitochondrial dysfunction with a severely attenuated ATP and enhanced ROS production displayed by collagen XV/XVIII mutants (70).

In summary, we have generated the first animal model to study human Sco-mediated cardiomyopathy in D. melanogaster. We demonstrate that cardiac-specific knockdown of scox leads to cardiomyocyte cell death in a p53-dependent manner in response to the loss of cell homeostasis and that dp53 genetically interacts with scox and fulfills a key role in the development of cardiomyopathy. Significantly, we show that loss of p53 or inhibition of apoptosis blocks the SCO-induced cardiomyopathy. Moreover, partial loss of SCO2 function also induces apoptosis in liver and skeletal muscle in a Sco2G150D mouse model. Therefore, our finding of p53-dependent pathologies due to SCO deficiency appears to be critical for several organ systems in addition to the heart. This information greatly advances our understanding of the mechanisms and consequences involved in SCO deficiency and will likely have a significant impact on our understanding of human metabolic diseases, such as OXPHOS diseases.

Materials and Methods

Fly stocks
UAS-RNAi transgenic fly lines for scox (CG8885; 7861) and Surf1 (CG9943; 10071), referred in the main text and figures as UAS-scox and UAS-Surf1, were obtained from the Vienna Drosophila RNAi Centre (VDRC, 72). The cardiac-tissue-specific TinCa4::Gal4 was a kind gift from M. Frasch. The UAS-p53Ins, UAS-p53 and p535(5A-1)4 were obtained from Bloomington, and the UAS-p35, UAS-Diap1 and UAS-rpr lines were kind gifts from M. Calleja.

Drosophila cardiac function and morphology
To assess cardiac function, heartbeat recording of semi-intact hearts was performed as described previously. Videos were analysed using a Semi-automatic Optical Heartbeat Analysis software (sohasoftware.com) to quantify the heart periods, systolic and DI, systolic and diastolic diameters as well as FS reflected in the M-mode recordings (28).

Immunohistochemical staining and respiratory complex activity
Briefly, semi-intact hearts were prepared as described above and phalloidin or SDH and COX activity stained.

ROS and TUNEL staining
Oxidative stress was detected over 1 h with DHE (3 mM final concentration in PBS) and the TUNEL reaction was performed following the manufacturer’s instructions (In Situ Cell Death Detection Kit, TMR Red, Roche, Germany).

Real-time PCR
RNA was extracted from 8 to 10 hearts from female flies of each genotype, and it was reverse-transcribed (RT) prior to performing quantitative RT-PCR with the Fast SYBR Green Cells-to-CT™ KIT (Ambion, Applied Biosystems).

Supplementary Material
Supplementary Material is available at HMG online.

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