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Mice lacking *COX10* in skeletal muscle recapitulate the phenotype of progressive mitochondrial myopathies associated with cytochrome *c* oxidase deficiency

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Abstract

We have created a mouse model with an isolated cytochrome *c* oxidase (COX) deficiency by disrupting the *COX10* gene in skeletal muscle. Missense mutations in *COX10* have been previously associated with mitochondrial disorders. Cox10p is a protoheme:heme-*O*-farnesyl transferase required for the synthesis of heme *a*, the prosthetic group of the catalytic center of COX. *COX10* conditional knockout mice were generated by crossing a LoxP-tagged *COX10* mouse with a transgenic mouse expressing cre recombinase under the myosin light chain 1f promoter. The *COX10* knockout mice were healthy until approximately 3 months of age when they started developing a slowly progressive myopathy. Surprisingly, even though COX activity in *COX10* KO muscles was <5% of control muscle at 2.5 months, these muscles were still able to contract at 80–100% of control maximal forces and showed only a 10% increase in fatigability, and no signs of oxidative damage or apoptosis were detected. However, the myopathy worsened with time, particularly in female animals. This *COX10* KO mouse allowed us to correlate the muscle function with residual COX activity, an estimate that can help predict the progression pattern of human mitochondrial myopathies.

INTRODUCTION

Cytochrome *c* oxidase (COX) or complex IV is the terminal enzyme of the respiratory chain. COX catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen, a process that is coupled to the translocation of protons across the inner mitochondrial membrane (1). Defects in COX have been associated with various mitochondrial and neurodegenerative diseases (2,3).

In mammals, COX is a 200 kDa enzyme, active as a dimer (4). Each monomer comprises 13 subunits encoded both by the nuclear and the mitochondrial genomes. The core of the complex is formed by three catalytic subunits that are encoded by the mitochondrial DNA (mtDNA). The remaining subunits are encoded by the nuclear DNA (5,6).

Disease-related mutations have been described in mtDNA-encoded subunits of COX and in nuclear-encoded accessory proteins important in COX biogenesis (reviewed in 7,8). COX biogenesis is a complex and incompletely understood process. More than 30 accessory proteins have been described for COX assembly in yeast and many of these factors have homologs in humans (9). One of these accessory proteins is the product of *COX10*, a protoheme:heme-*O*-farnesyl transferase, involved in the biosynthesis of heme *a*. The farnesyl chain of heme *a* acts as a lipophilic anchor to hold the prosthetic group in the proper position within complex IV (10).

Cox10p is an evolutionarily conserved protein. Cox10p of yeast, mouse and human are almost identical in size and share a high sequence similarity. These proteins have a hydrophilic amino terminal domain (about 143 amino acids) that is absent from the bacterial homologs cyoE and ORF1 in *Escherichia coli* and *Paracoccus denitrificans*, respectively (11,12). The human *COX10* gene contains seven exons (13) and the gene product is located in the inner mitochondrial membrane and contains nine putative transmembrane domains (12). Several missense mutations in the *COX10* gene have been found in patients with different clinical presentations: leukodystrophy and tubulopathy (14); anemia and Leigh syndrome; anemia, sensorineural deafness and fatal infantile hypertrophic cardiomyopathy (15) and Leigh-like syndrome (16). To better understand the molecular mechanisms responsible for COX deficiencies, we used the loxP-Cre system (17) in mice to knock out *COX10* only in skeletal muscle. Mice carrying a floxed *COX10* gene (exon 6) were crossed with transgenic mice carrying cre recombinase under the regulation of the myosin light chain 1f (*mlc1f*) promoter (18). Knockout of *COX10*, especially in skeletal muscle, resulted in COX-deficient mice with a phenotype that resembles mitochondrial myopathies in humans.

RESULTS

Creation of a muscle-specific *COX10* KO mouse

To generate an animal model with COX deficiency, we ablated exon 6 from the *COX10* gene using the Cre-loxP system (17). Exon 6 was selected because its sequence is homologous to the region that codes for part of the active site of the bacterial enzyme (19). We used the targeting vector pflox (20). This vector contains three loxP sites and a neomycin/thymidine kinase selection cassette (Fig. 1A). Three steps were involved in disrupting the *COX10* gene using this construct. First, the loxP sites were introduced into the gene in ES cells by homologous recombination. Secondly, the selection cassette was removed by transient cre recombinase expression to obtain the floxed gene *COX10^{Flox}* (Fig. 1B). ES cells containing the floxed gene were then injected into blastocysts to produce chimeric mice and to obtain germline transmission of the *COX10^{Flox}* gene (Fig. 1D, mice 2, 3 and 4; wild type, mice 1 and 5). Thirdly, exon 6 was excised *in vivo* (*COX10^d*) by crossing *COX10^{Flox}* mice with transgenic animals that expressed cre recombinase specifically in skeletal muscle under the *mlc1f* promoter (18). To obtain the muscle-specific *COX10* KO, we crossed homozygous *COX10^{Flox}* mice with *COX10^{Flox}/COX10^{Flox};mlc1f-cre* mice. The latter animals were healthy and had a normal life span.

Figure 1E shows the Southern blot of the *COX10* gene for one control mouse (C) and two floxed mice, using muscle and heart DNAs. The control mouse is *COX10/COX10^{Flox}*, whereas the knockout mice are *COX10^{Flox}/COX10^{Flox}*. Expression of cre recombinase results in the presence of the deletion allele *COX10^d* only in skeletal muscle, but not in cardiac muscle. Figure 1F shows that *COX10* mRNA levels are highest in mouse heart, liver, kidney and testis.

Brain and skeletal muscle had medium levels of *COX10* mRNA, whereas the lowest levels of *COX10* mRNA were in spleen and lung.

Muscle-specific *COX10* KO mice exhibit severe COX deficiency

The presence of the floxed gene does not have any apparent effect on the mouse phenotype. The *COX10^{Flx}/COX10^{Flx}* mice are healthy, fertile and have a normal life span. These animals and wild-type mice were used as controls. In contrast, the *COX10* muscle knockout mice appear healthy until they reach 3–4 months of age when they start to lose weight progressively and become less active. Mice died between 4–7 months of age.

To determine the effect of the *COX10* deletion on oxidative phosphorylation enzymes, mitochondria were isolated from hind limb muscles of different aged control and KO animals. Enzyme activities were measured spectrophotometrically. Figure 2 shows the enzyme activities for different respiratory complexes in KO mice normalized to data from control animals of the same age (mostly littermates analyzed on the same day). The KO/control pairs were also matched for sex (10 males and 4 females of each). NADH cytochrome *c* oxidoreductase (complex I + III), succinate decylubiquinone DCPIP reductase (complex II) and succinate cytochrome *c* reductase (complex II + III) activities were not altered, whereas citrate synthase activity was slightly increased in young KO mice when compared with control mice (1–4 months, Fig. 2A–2C and 2E). Deletion of *COX10* resulted in a severe decrease in cytochrome oxidase activity as early as 1 month of age. Cytochrome oxidase activity in young KO mice averaged ($\pm$ SD) $12.9 \pm 5.7\%$ of control levels (Fig. 2D). We did not observe any difference between the COX activities in males or females, which were markedly decreased. Surprisingly, these muscles were still functional, and no overt myopathic phenotype could be observed until ~3 months of age. These already low levels of complex IV activity progressively decreased to $2.2 \pm 0.9\%$ of control as the KO mice aged (6–7 months old). There was a small but significant decrease of CII and CII + III activities in older KO mice when compared with controls (6–7 months).

The complex IV defect was further characterized by blue native (BN) gel electrophoresis on isolated muscle mitochondria from 1-, 5- and 6-month-old animals. Figure 3A shows that complexes I, V, III and II were fully assembled and present at similar levels in all mice. The band corresponding to complex IV was barely visible in KO mice even at a young age (1 month). *In gel* activity assays were performed in BN gels for complex I, IV and V. Complex IV activity was only detectable in the mitochondria of 1-month-old muscle (Fig. 3C). Low levels of complex IV activity were seen in control mice and this enzyme activity increased with age. One-month-old control animals had ~20–30% less COX than 6-month-old mice (data not shown). *In gel* activity assay of complexes I (Fig. 3B) and V (not shown) showed that control and KO mice had comparable levels of these complexes. However, the levels of the F1 segment, dissociated from the F₁/F₀ complex, also detected by this assay, were increased in KO mice.

The steady-state levels of the fully assembled respiratory complexes were determined by western blot of BN gels using different monoclonal antibodies against subunits of the respiratory complexes (Fig. 3D). BN gel western blots of tissues taken from 1-, 3- and 6-month-old KO mice showed no detectable bands that corresponded to the fully assembled COX, using antibodies against the Cox1p subunit (Fig. 3D) or the Cox4p subunit (not shown). Control and KO mice showed comparable steady-state levels of the other fully assembled respiratory complexes (Fig. 3D).

The oxidative phosphorylation complexes were also analyzed by 2D-BN western blots. Blots were incubated successively with different antibodies, with control and KO samples exposed and analyzed in parallel. Pseudo colors were assigned to each image (blue for complex IV, red

for complex I, yellow for complex III, green for complex V and magenta for complex II). The images obtained for each antibody were superimposed to compose Figure 3E. Western blots for complex IV subunits (Cox1p and Cox4p) showed that fully assembled COX was barely detectable in the 6-month-old KO mouse (Fig. 3E). We did not detect subassembly intermediates of complex IV under these conditions even when overexposing film in either 1D or 2D-BN western blots (not shown). The levels of complex II and V were slightly increased in the 2D BN western blots for the 6-month-old *COX10* KO mouse, whereas complexes I and III looked similar in both KO and control animals.

The steady-state levels of several subunits of complex IV were assessed using antibodies against Cox1p, Cox5bp and Cox4p subunits. Mitochondrial samples from 1-, 5- and 6-month-old mice were separated by SDS-PAGE. Figure 3F shows no detectable levels of Cox1p subunit and barely detectable levels of Cox4p in the *COX10* KO mice of different ages. Overexposure of these western blots showed the presence of very low levels of Cox1p and Cox4p (data not shown). The Cox5bp subunit was also decreased markedly in the KO mice (Fig. 3F). The steady-state levels of cytochrome *c*, NDUFS9 (complex I subunit), UQCRC2 (core 2 subunit of complex III), ATPase β (complex IV subunit) and SDH (complex II 70 kDa subunit) were similar for *COX10* KO and control mice (Fig. 3F).

Cytochrome oxidase-deficient fibers, ragged red fibers and abnormal mitochondrial size in muscle tissue of *COX10* KO mice

Serial sections of skeletal muscle from hind limbs (triceps surae) of control and KO mice (1-, 3- and 6-month-old) were evaluated histochemically. Figure 4 shows the typical mosaic pattern of muscle fibers, where more oxidative fibers stain stronger for cytochrome oxidase or SDH. In control mice, COX and SDH stains were stronger in older animals. We have also seen this trend in spectrophotometric assays of enzyme activities (data not shown). In contrast, in KO mice, COX activity progressively weakened with age. At 6 months, the majority of the muscle fibers in the KO animals were COX negative. The double-stain COX/SDH revealed increased SDH staining in COX-deficient fibers in the *COX10* KO mice, an activity stain that is otherwise masked by the COX stain in normal mice. The increased SDH reaction in the KO animals suggests mitochondrial proliferation. With the Gomori trichrome stain, we could not observe mitochondrial proliferation in young KO animals, but with age, mitochondria proliferation and scattered ragged red fibers (RRF) became more apparent. COX deficiency was also monitored by immunohistochemistry using monoclonal antibodies against Cox1p. The immunohistochemical pattern of Cox1p correlated with the COX activity stain. In young control mice, the Cox1p signal was weak, but it increased with age. In comparison, *COX10* KO mice showed a progressive decrease in Cox1p staining as they aged.

Fibers in muscles of *COX10* KO mice were more heterogeneous in size than fibers in control muscles. Scattered atrophic fibers were also present in muscles from KO mice, but there were no signs of fibrosis, inflammation (assessed from hematoxylin and eosin stains) or apoptosis (determined by tunnel assay; data not shown). We also did not detect signs of neurogenic alterations, such as fiber-type grouping or angular atrophic fibers (determined by myosin ATPase staining; data not shown). Using western blots, we detected a slight decrease in the levels of free ubiquitin in 5- to 6-month-old KO mice (Fig. 3F), but no significant difference in the levels of ubiquitin-conjugated proteins between control and KO mice (not shown). Tests for levels of free radical scavenger enzymes (SOD1 and SOD2), as well as oxidative damage to proteins (oxyblot) in mitochondria and muscle homogenates, did not show any significant differences between control and KO mice at different ages (data not shown).

Analysis of skeletal muscle by electron microscopy showed areas of mitochondrial proliferation and markedly enlarged and swollen mitochondria in *COX10* KO muscle (Fig. 5). These mitochondria were bigger than 1 μ m in diameter and contained abnormal cristae. We

did not find evidence of paracrystalline inclusions, nor did we observe irregularities in the ultrastructure of the muscle other than abnormal mitochondria.

Muscle-specific *COX10* KO females are more intolerant to exercise and had a shorter life span than KO males

KO mice appeared healthy until 3–4 months of age when they started showing visible changes in phenotype (Fig. 6A). At this age, they started to lose weight, had severe reductions in muscle mass, developed kyphosis and showed less spontaneous activity. Growth curves for the animals, normalized to body weight at 1 month of age, showed that male KO mice and control littermates had the same weight until about 3 months of age. The weight of the male KO mice then started to decline progressively (Fig. 6B, for control and KO mice: open and closed squares). Some of the animals showed phases of weight recovery but eventually their weight kept declining. In contrast, female KO mice weighed less than their control littermates at an early age, and they barely reached 20 g even at 3 months of age (Fig. 6B, closed and open circles).

Mice were also subjected to a running test on a treadmill equipped with a motivational grid discharging a weak electric shock. The number of times that control and KO mice fell into the grid when running on the treadmill was recorded. Figure 6C shows that 2-month-old KO mice, both females and males, fell into the grid more frequently than the control mice. The performance of the female KO mice on the treadmill was also significantly poorer than that of the KO males ($P = 0.01$). After this exercise test, many of the KO mice sweated profusely, seemed extremely fatigued and took a relatively long time to recover from the exercise. As KO animals of either sex became older (3–4 months) and the disease progressed, they were unable to run on the treadmill even for a few seconds at the slowest setting of the machine.

The *COX10* knockout mice have a shorter life span when compared with their littermate controls (Fig. 6D). Females were more affected by the *COX10* deficiency and died younger than males. Fifty percent of the KO females died by ~4 months of age, whereas it took 7 months for 50% of the KO males to die. There were exceptions, however. Three KO females out of 15 lived for more than 15 months.

***COX10* KO muscles become markedly weaker with age and slightly more fatigable**

Figure 7A shows examples of the maximal force evoked in medial gastrocnemius muscles of three different aged control mice and three KO mice. Maximal force was similar in control and KO mice up to 70 days of age. Thereafter, maximal force increased significantly with age in control mice, but decreased significantly for KO mice (Fig. 7B). Unbiased measurement of muscle fiber areas in 3-month-old KO and control mice showed that the cross-sectional area of fibers from the KO mice was, on average, 19% smaller than the control (KO mean $\pm$ SD: $1705 \pm 831 \mu\text{m}^2$; control: $2098 \pm 721 \mu\text{m}^2$; $P < 0.0001$). Because average fiber size provides an accurate estimate of the contractile apparatus mass, these results suggest that the reduction in muscle size may contribute, but cannot fully account for the reduction in force (~50% decrease in animals older than 80 days; Fig. 7B).

The medial gastrocnemius muscles of control and KO mice were fatigued by intermittent stimulation at 40 Hz for 2 min. Figure 7C shows the declines in muscle force (fatigue) seen in mice every 30 s throughout the test. With repeated stimulation, the force of each muscle declined progressively, particularly during the first minute, but the relative force decline was greater in the muscle from the KO mouse. Stimulation at 40 Hz initially evoked an average ($\pm$ SD) of $75 \pm 4\%$ and $73 \pm 6\%$ of the maximal force (at 100 Hz) recorded for control and KO mice, respectively ($P = 0.62$). However, with repeated stimulation, the muscles from KO mice were more fatigable than control muscles (Fig. 7D). There was a small (~10%, on average) but

significant difference in the mean relative force declines for control and KO mice from 40 to 120 s. Twitch and tetanic forces were reduced similarly by the fatigue protocol in KO mice ($22 \pm 10\%$ and $19 \pm 7\%$ initial, respectively), but in control muscles, there were significantly greater reductions in tetanic forces than in twitch forces ($29 \pm 4\%$ and $41 \pm 11\%$ initial, respectively, $P = 0.025$).

DISCUSSION

The present study describes the development of a mouse model with a muscle-specific *COX10* knockout that results in a severe COX deficiency and myopathy. The mice presented with a phenotype that closely resembles the one in mitochondrial myopathies associated with COX deficiency in humans. As described in what follows, there are some unique aspects to these mice when compared with the few previously described models of mitochondrial myopathies. The muscle conditional knockout for mitochondrial transcription factor A (Tfam) (21) also recapitulates features of mitochondrial diseases, but in this case, all OXPHOS complexes were altered because of an mtDNA depletion (22). The adenine nucleotide translocator 1 (ANT-1) knockout mice (23) developed a muscle disease and exercise intolerance, but a hypertrophic cardiomyopathy was also present in these mice as ANT-1 is expressed in both heart and skeletal muscle. The same applies to the mice knockout for the subunit VIa-H of COX, which developed heart pathology (24). The mice described here have an isolated myopathy associated with a defect in a single OXPHOS complex.

Studies with the bacterial homolog cyoE (heme-*O*-synthase) showed that the mouse exon 6 forms part of the catalytic site of the enzyme and is necessary for function (19). We could not test directly the effect of exon 6 deletion on Cox10p levels. Our efforts to develop Cox10p-specific antibodies by different methods (peptide immunization, fusion protein expression and genetic immunization) failed to produce antisera that could detect a specific band in immunoblots, possibly due to the very low expression levels of this protein. Nevertheless, because heme *a* is necessary for Cox1p maturation and stability, we could use Cox1p as a marker of Cox10p function. As observed in yeast, deletion of *COX10* caused COX deficiency in mouse as well. The *mlc1f Cre* transgenic mouse expresses the recombinase in development and the enzyme is active at embryonic day 10. It has been estimated that 45% of the nuclei within skeletal muscle tissue is within muscle cells, as many other tissue types are also present (e.g. adipocytes, fibroblasts, Schwann cells, etc.) (18). We obtained similar levels of *COX10^{Flx}* and *COX10^Δ* alleles in Southern blots. As mentioned earlier, this result can be explained by the presence of additional cell types in muscle homogenates, as well as incomplete deletion in muscle.

Young mice appeared normal even though they had very low levels of COX activity at 1 month of age (87% deficient when compared with control littermates). These levels decreased even further as animals became older (reaching 98% deficiency at 6–7 months of age; Fig. 2D). Surprisingly, no overt phenotype was observed until 3 months, even though COX activity was less than 5% of normal. This observation reflects a remarkable capacity of skeletal muscle to function using alternative mechanisms for ATP production (most likely glycogenolysis/glycolysis). The levels of COX deficiency achieved in our *COX10* KO mice parallel the low levels of complex IV activity reported in human pediatric patients with *COX10* missense mutations. Complex IV activities in these patients were between 5 and 16% of control in muscle biopsies (15,16). An important factor contributing to the residual muscle function in our model is the mosaic and non-synchronous deletion of floxed *COX10* alleles. This mosaic pattern of defect, shown in Figure 4, resembles defects associated with heteroplasmic mtDNA mutations (25). At older ages, the defect is more generalized, resembling more nuclear-coded gene defects (7,8).

Steady-state levels of complex IV subunits were decreased in fibroblasts of patients with *COX10* defects (14,26). Studies performed in human fibroblasts treated with *N*-methylprotoporphyrin (inhibitor of ferrochelatase) to cause heme deficiency also revealed decreases in the steady-state levels of COX subunits (27). Likewise, steady-state levels of COX subunits in *COX10* KO mice were affected when compared with controls. Cox1p was barely detectable and Cox4p and Cox5bp were markedly decreased.

Heme *a* insertion occurs after the formation of the first subassembly of Cox1p but before the formation of Cox1p, Cox4p and Cox5bp subassemblies (26). Analysis of COX assembly in the *COX10* KO mouse by BN gels did not reveal the presence of subassembly intermediates. Even when samples were resolved using 2D-BN electrophoresis, a more sensitive way to detect subcomplexes than 1D analysis, we were unable to detect any intermediates. These results are in agreement with studies that examined tissues from patients with *COX10* mutations (15,16,26).

Histochemical examination of muscle from *COX10* knockout mice showed that many fibers with increased SDH staining were deficient in COX stain, but this was not always the case. These results are similar to those seen in a mouse myopathy model where the mitochondrial transcription factor A was deleted specifically in muscle (22). Increases in mitochondrial mass were also observed in *COX10* mice, probably a compensatory mechanism for the enzymatic deficiency to improve energy production in the affected tissues.

The few animals that reached old age (8 months or more) had severe muscle wasting. The molecular mechanisms of muscle wasting are not completely understood but involve reduced protein synthesis, increased proteolysis (calpain, cathepsins and ubiquitin) and oxidative stress (28,29). We examined *COX10* KO muscle for levels of ubiquitin by immunoblot and observed that the levels of free ubiquitin were reduced in 5-to 6-month-old animals. A reduction in the levels of free ubiquitin could imply an increase in the levels of protein-bound ubiquitin, but we were unable to detect significant differences in the levels of ubiquitin conjugates. No evidence for apoptosis or oxidative stress was obtained. Therefore, the muscle wasting observed in the *COX10* KO mouse may be the sole consequence of an energy shortage for biosynthetic and metabolic pathways. This suggestion is consistent with KO animals showing a reduction in spontaneous activity with age and exercise intolerance.

The force decline seen in gastrocnemius (mostly a type II fiber-containing muscle) of both KO and control animals with repeated stimulation probably relates to ongoing metabolic changes that impair both calcium dynamics (release, uptake and/or buffering) and the ability of the cross-bridges to produce force because of the unavailability of ATP (30). Tetanic forces were reduced more than twitch forces in controls, whereas both forces had similar reductions in the *COX10* KO, suggesting problems of calcium release (31). The protocol used here may also have revealed increased fatigability in *COX10* KO mice when compared with the Tfam KO mice (22). The relative intensity of the initial contractions was similar for our KO and control animals. However, muscles from Tfam KO animals were activated at lower initial intensities than control muscles, relative to maximal muscle force (22), a factor that potentially could mask small differences in fatigability due to the knockout. In addition, the different muscle groups and different techniques used in these studies could account for these small differences. In any case, the increased fatigability in the *COX10* KO was small when compared with the marked decrease in force, a trend compatible with the results described for the Tfam muscle KO (22).

Exercise tests performed with younger *COX10* KO animals (2 month of age) showed a gender difference: males performed better. The larger muscle mass usually observed in males (32), triggered by testosterone, may account for this difference (33). In terms of life span or survival,

we also observed a gender bias. On average, ~50% of the females died at ~4 month of age, whereas 50% of the males died at ~7 month of age. One of the most common mitochondrial~genetic diseases, Leber hereditary optic neuropathy (LHON), presents with an incomplete penetrance and affects males more frequently than females, implying that there might be additional genetic or environmental factors that regulate the phenotypic expression of the disease (34).

In conclusion, we have created and characterized in detail a skeletal muscle-specific *COX10* KO mouse with a phenotype that recapitulates the progressive mitochondrial myopathies associated with COX deficiency. Our studies showed that COX activity can be reduced by more than 90% in muscle before an overt myopathy is observed and also showed a gender bias for clinical manifestations. This novel mouse model will be instrumental for the development of new therapeutic approaches to treat mitochondrial myopathies.

MATERIALS AND METHODS

Generation of *COX10* conditional knockout mice

We amplified exon 6 of the *COX10* gene from mouse skeletal muscle RNA by RT-PCR. The product was cloned into the pCDNAII vector and sequenced to confirm *COX10* identity. The purified fragment was sent to Incyte Genomics (Palo Alto, CA, USA) for isolation of 129svj BAC clones that contained the *COX10* gene. We received three positive clones that were mapped with different restriction digestion enzymes. Different regions of a 12 kb *Kpn* I digestion fragment that contained exon 6, but not exon 5 or exon 7, were cloned into the pFlox vector (20) to obtain the targeting construct. The cloning strategy of the targeting construct is illustrated in Figure 1A. At the 5' recombination arm, a 0.6 kb *Kpn* I-*Nco* I fragment was cloned into the *Bst*XI-*Xho* I cloning site of the vector. The fragment *Nco* I-*Hind*III (1.3 kb) that contained exon 6 was introduced into the *Bam* HI. Finally, at the 3' recombination arm, the *Bam* HI-*Kpn* I fragment (8.9 kb) was cloned into the *Sal* I site of the vector. The restriction sites that were required to clone the genomic sequence into the vector were added by PCR using specific primers containing the necessary restriction sites. The fragments obtained were either sequenced or analyzed by restriction digestion to confirm their identity. The final construct was linearized with *Not* I and electroporated into 129svj mouse embryonic stem (ES) cells (Incyte Genomics). Cells were grown under 300 µg/ml G418 selection. Non-differentiated clones were picked on days 6 and 7. Clones were screened for homologous recombination by PCR. From 360 clones, we obtained only three homologous recombinant clones. From these three clones, only one clone had all three loxP sites. This clone was amplified and electroporated with low concentrations of pCre-Hygro plasmid (35) to delete the selection cassette (Fig. 1B). After electroporation, cells were grown in 0.2 µM [2'-fluoro-2'-deoxy-5-iodo-1-beta-D-arabinofuranosyluracil (FIAU), TK negative selection] and screened by PCR. We obtained 14 clones in which exon 6 was retained, but the selection cassette was deleted. Some of these clones were injected into C57Bl/6 blastocysts at the transgenic core facility at the University of Miami. Three chimeric mice were obtained. These animals were crossed with C57Bl/6 mice to obtain germline transmission of the floxed allele. Figure 1D shows the genotype of those animals that were heterozygous or homozygous for the floxed allele. The control and *COX10* knockout mice had a mixed genetic background (129svj and C57Bl/6).

Animal husbandry

The mice were kept in a condition of 12 h light/dark cycle at room temperature. Food and water were administered *ad libitum*.

PCR and Southern and northern blots

Total DNA was obtained from tissues by phenol–chloroform extraction and isopropanol precipitation. Deletion of exon 6 was detected by PCR and Southern blot. PCR amplification of the deleted allele was performed within the intron sequence flanking exon 6. To detect the deleted allele, as well as the floxed allele by Southern blot, 20 µg of total DNA (from cells, tail or muscle) were digested with *Bam* HI, separated on a 0.7% agarose gel and transferred to a Z-Probe membrane (BIO-RAD, Hercules, CA, USA). The membrane was hybridized with a *COX10* genomic probe (Fig. 1A). Northern blot was performed using a commercially available membrane (BD Biosciences, MTN™ mouse blot, Clontech, Palo Alto, CA, USA) that was hybridized with a 1 probe, stripped, and then hybridized with a β-actin probe. Probes were labeled with [³²P]dCTP using a random Primer Labeling Kit (Roche Diagnostics, Indianapolis, IN, USA).

Mitochondria isolation and determination of enzyme activity of respiratory complexes

Mitochondria were obtained by homogenization and differential centrifugation of muscle tissue taken from the hind limbs of different aged animals. Muscle homogenates were prepared in 10 mM Hepes, 0.5 mM EDTA, 0.5 mM EGTA and 250 mM sucrose (pH 7.4) that contained a complete protease inhibitor cocktail (Roche Diagnostics), using a motor-driven teflonpestle homogenizer. Samples were centrifuged at 2000g for 3 min. The supernatant was saved. The pellet containing unbroken cells was homogenized and centrifuged again. Both supernatants were pooled and centrifuged at 12 000g for 10 min. The mitochondrial-enriched pellet was washed once and resuspended in the above buffer. The mitochondrial fraction was frozen in liquid nitrogen and stored at −80°C until needed. The activity of NADH cytochrome *c* oxidoreductase or complex I + III, succinate dehydrogenase (SDH) or complex II activity, succinate cytochrome *c* reductase or complex II + III activity, COX or complex IV activity and citrate synthase activity were determined spectrophotometrically in mitochondrial preparations as described previously (36). Protein concentration was determined by the method of Bradford (37) using BSA as a standard.

Blue native gel electrophoresis and western blots

Mitochondria (200 µg) isolated from muscle were resuspended in 1.5 M amino-caproic acid, and 50 mM Bis-Tris (pH 7.0), extracted with 20 µl of 10% lauryl maltoside for 45 min on ice, and then centrifuged for 30 min. A 10 µl of 5% Serva blue were added to the solubilized protein fraction. Samples (25–40 µg) were separated by BN-PAGE in a 4–13% acrylamide gradient as described previously (38). For the 2D electrophoresis (SDS-PAGE), lanes from the native gel were cut with a razor blade and first incubated with 1% SDS and 1% β-mercaptoethanol for 30 min and then incubated with 1% SDS for another 30 min. The second dimension gel was cast around the original gel strip using a 10% acryl-amide-separating gel and a 4% acrylamide-stacking gel. After BN- or SDS-PAGE, proteins were transferred to a PVDF membrane and immuno blots were performed with a series of monoclonal antibodies against subunits of the respiratory complexes (Molecular Probes, Eugene, OR, USA). For *in gel* activity assays, the blue cathode buffer was replaced with colorless buffers after it had run for 3 h at 30 V. The gel was then run until the dye at the front left the gel. To assess complex I activity, the gel was incubated with 0.14 mM NADH and 1 mg/ml nitro blue tetrazolium in 100 mM Tris–HCl (pH 7.4) at 37°C until the color developed (39). Complex IV *in gel* activity was performed as described (40).

Western blots were performed after the mitochondrial proteins (25 µg) were separated by SDS-PAGE in 4–20% acryl-amide gradient gels and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk in 0.1% Tween 20 in PBS and then incubated with specific antibodies. Horseradish peroxidase-labeled secondary antibody was used and the enzymatic reactions were developed by chemiluminescence using the Superwest™ signal reagent (Pierce,

Rockford, IL, USA). Antibodies against different subunits of oxidative phosphorylation complexes were obtained from Molecular Probes and an antibody against ubiquitin was obtained from Chemicon International (Temecula, CA, USA).

Histological, immunohistochemical and electron microscopy analysis

For histological studies, muscle tissue was frozen in isopentane cooled in liquid nitrogen. Cross-sections (8 μm thick) of frozen muscle were stained for SDH and cytochrome oxidase activities. Muscle sections were also stained with hematoxylin–eosin and Gomori trichrome to assess muscle fiber area and general morphology (25). For immunohistochemistry, muscle sections were treated with 0.5% triton x-100 in PBS for 30 min and washed in PBS for 30 min prior to the addition of an anti-COX1p-Alexa fluor 488-conjugated monoclonal antibody (Molecular Probes) diluted to a concentration of 2 $\mu\text{g}/\text{ml}$ in 2% BSA in PBS.

For electron microscopy analysis, muscle tissue was fixed overnight in 2.5% glutaraldehyde, and 2% paraformaldehyde in 100 mM sodium phosphate (pH 7.4). Tissue was rinsed with phosphate buffer, incubated with 1% osmium tetroxide and then processed for transmission electron microscopy as described previously (41). Images were captured using a JEOL CX 100 system at the EM core facility of the University of Miami.

Treadmill test

Mice ($n=5$ for each of the four groups) were run on a treadmill (Columbus Instruments, Columbus, OH, USA) set at 7 m/min for 2 min. The back of the treadmill was equipped with a grid that discharged a mild current, a stimulus designed to motivate the animal to keep running on the treadmill. Performance was measured by the number of times a mouse failed to stay on the running belt and fell into the stimulus grid. Running time was limited to 2 min because the majority of KO mice could not run any longer.

Measurements of muscle strength and fatigue

Control and KO animals of different ages were anesthetized with 1–2% halothane and nitrous oxide until areflexic. The sciatic nerve was exposed in one leg and denervated except for the branch to the medial gastrocnemius muscle. The medial gastrocnemius muscle and tendon were dissected from surrounding muscles.

During physiological recordings, each mouse lay prone on a heating blanket so as to maintain body temperature close to 37°C. Muscle temperature was maintained at 35–37°C by radiant heat. The sciatic nerve was laid across two silver electrodes for stimulation. The medial gastrocnemius tendon was tied to a transducer (Grass FT03, Grass Astro-Med, Inc., West Warwick, RI, USA) to measure isometric force at optimal muscle length. Force was sampled online at 400 Hz using an SC/Zoom system. Data were analyzed offline using Zoom software (Physiology Section, Umeå University, Sweden).

Stimulus pulses (50 μs duration; S88 stimulator, Grass Astro-Med, Inc.) of increasing intensity (1 or 0.1 V steps) were delivered to the sciatic nerve at 1 Hz to determine the threshold for muscle activation. All subsequent stimuli were supramaximal (>3 times threshold). Pulses were delivered at 1 Hz to evoke muscle twitches and then at 100 Hz for 0.5 s to evoke maximal muscle force. These stimuli were repeated immediately after the fatigue protocol. Maximal muscle force was normalized to muscle weight as an estimate of the intrinsic force-generating capacity of the muscle fibers. Each muscle was fatigued by delivering 13 pulses every second at 40 Hz for 2 min (42). The absolute force evoked by the first train of pulses at 40 Hz was normalized to the maximal muscle force to evaluate the initial contraction intensity. The relative force decline after 2 min of stimulation at 40 Hz (final force/initial force) was used as a measure of muscle fatigue.

Maximal muscle forces for different aged control and KO animals were analyzed by least squares linear regression. *t*-tests were used to assess differences in normalized maximal forces for KO and control animals and changes in twitch forces before and after fatigue. Fatigue was analyzed by repeated measures ANOVA with time and animal genotype as factors. Significance was set at $P < 0.05$.

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Figure 1.

Creation of muscle-specific *COX10* knockout mice using the Cre-loxP system. **(A)** A *Kpn* I fragment from a BAC clone containing only exon 6 from *COX10* was cloned into a pFloxed vector. The construct was linearized and electroporated into ES cells. Homologous recombinant clones were transiently transfected with a plasmid expressing Cre recombinase to delete the selection cassette neomycin-thymidine kinase and to obtain the floxed *COX10*^{Flx} allele. **(B)** Cells with the floxed allele were injected into blastocysts to produce chimeric mice. **(C)** The deletion allele (*COX10*^Δ) was obtained after the floxed allele was exposed to cre recombinase. **(D)** *COX10* Southern blot [using probe indicated as a black bar in (A)] of F1 mice from the chimera were crossed with C57Bl/6 mice: lanes 1–5 represent DNA from different mice. Mice 1 and 5 are homozygous for the wild-type allele of *COX10*. Mice 2–4 are heterozygous, carrying the wild-type allele and the *COX10* floxed allele. **(E)** Southern blot showing a tissue-specific deletion of exon 6: lane C, control heterozygous mouse, *COX10*/*COX10*^{Flx}. Lanes M1 and M2, homozygous knockout mice, *COX10*^{Flx}/*COX10*^{Flx}, expressing cre recombinase in skeletal muscle. The deletion allele is present only in skeletal muscle tissue, but not in heart tissue from the same mice. **(F)** Northern blot with RNA from different tissues of wild-type mice probed for *COX10* and β-actin.

Figure 2.

Respiratory complexes activities of control and *COX10* KO mice. Mitochondrial enzyme activities in hind limb muscles of different aged control and KO mice were determined spectrophotometrically (as described in 9). (A) NADH-cytochrome *c* oxidoreductase activity or complex I + III activity. (B) Succinate decylubiquinone DCP/IP reductase or complex II activity. (C) Succinate cytochrome *c* reductase or complex II + III activity. (D) COX or complex IV activity. (E) Citrate synthase activity. Enzyme activities for tissue from each KO mouse were expressed as a percentage of mean values obtained from control animals performed on the same days. Each symbol corresponds to one KO mouse. Ten male and 4 female KO mice (and respective age- and gender-matched controls) were used in this experiment.

Figure 3.

Steady-state levels of respiratory complexes in control and *COX10* KO mice. Respiratory complexes in muscle mitochondria (40 μ g) from control and KO mice were separated by BN-PAGE. **(A)** Coomassie-stained BN gel. Lane M, high molecular weight markers for native electrophoresis; C, control; K, KO mice of 1, 5 and 6 months of age. **(B)** Complex I *in gel* activity assay of BN gel samples used in (A). **(C)** Complex IV *in gel* activity assay of samples used in (A). **(D)** Immunoblots of BN gel of muscle mitochondria (40 μ g) taken from 1-, 3- and 6-month-old mice using antibodies against NDUFA9 (subunit of complex I), ATPase β (subunit of complex V), UQCRC2 (subunit of complex III), Cox1p (subunit of complex IV) and SDH-Fp (subunit of complex II). The same blot was incubated successively with these antibodies without stripping the membrane. **(E)** Mitochondria (40 μ g) from 6-month-old control and KO mice were analyzed by BN-PAGE and 2D-SDS-PAGE immunoblots. The same blots were incubated successively with different antibodies. Control and KO blots were treated and

exposed in parallel. The images were colored and superimposed to facilitate comparison. Complex I is represented in red, complex II in magenta, complex III in yellow, complex IV in blue (two antibodies used against CoxIp and CoxIVp) and complex V in green. (F) Mitochondrial proteins (25 µg) from 1-, 5- and 6-month-old control and KO mice were separated by SDS-PAGE. Membranes were blotted with several antibodies against subunits of complex IV, cytochrome *c*, complex I, III, V, II and ubiquitin.

Figure 4.

Histopathology of muscle from *COX10* KO mice at different ages. Triceps surae muscle from 1-, 3- and 6-month-old mice was snap frozen in isopentane cooled in liquid nitrogen. Serial cross-sections of 8 μ m thickness were stained for COX activity, combined COX and succinate dehydrogenase activity and SDH activity. They were stained with Gomori trichrome and immunostained with Alexa-conjugated Cox1p monoclonal antibody to show the progressive COX deficiency and mitochondrial proliferation in muscles of the *COX10* knockout mice.

Figure 5.

Electron microscopy of *COX10* KO skeletal muscle. Skeletal muscle (triceps surae) from 3-month-old control and KO mice was fixed in 2.5% glutaraldehyde, and 2% paraformaldehyde in phosphate buffer, and then analyzed by transmission electron microscopy. The micrographs show accumulation of enlarged, abnormal mitochondria in tissue from a KO mouse (right panel) when compared with a control (left panel). Scale bar: 2 µm.

Figure 6.

Phenotype of *COX10* KO Mice. **(A)** Comparison of body size for 5-month-old control and KO littermates. At a young age, control and KO mice were indistinguishable, but after 3–4 months, KO mice start to lose muscle mass, develop kyphosis and show less spontaneous activity. These symptoms deteriorated progressively. **(B)** Growth curve of control and *COX10* KO mice [open squares, control male mice ($n = 9$); closed squares, KO male mice ($n = 7$); open circles, control female mice ($n = 6$); closed circles, KO female mice ($n = 6$)]. **(C)** Treadmill performance test: 2-month-old male and female control and KO mice were exercised on a treadmill at 7 m/min. The number of times they fell onto the electrified grid was recorded. Bars represent mean and standard deviation of two tests performed ($n = 5$ mice for each group). KO mice had difficulty in performing the task. Females fell onto the grid more frequently than males. Older KO animals could not perform this task. They were unable to run. **(D)** Survival curve for female ($n = 9$) and male ($n = 11$) KO mice. Fifty percent of the female population died at ~4 months of age, whereas 50% of the male population died at ~7 month of age. Triangles represent data for control mice.

Figure 7.

Physiological characterization of medial gastrocnemius muscle. **(A)** Maximal forces evoked in medial gastrocnemius muscles of three different aged control and KO male mice in response to supramaximal stimulation of the sciatic nerve at 100 Hz for 0.5 s. **(B)** Relationship between maximal muscle force and age for control and KO animals (control: open squares, $y = 0.025x - 0.9911$, $r^2 = 0.9811$, $P = 0.002$; KO: closed squares, $y = -0.0064x + 1.0887$, $r^2 = 0.534$, $P = 0.039$). **(C)** Forces evoked in medial gastrocnemius muscles of a control and KO animal (both 73 days old) in response to 13 pulses every second at 40 Hz for 2 min. Traces shown were recorded at 0, 30, 60, 90 and 120 s (top to bottom). **(D)** Mean ($\pm$ SD) relative force declines for control and KO mice ($n=5$ animals/group) every 5 s during the 2 min of stimulation at 40 Hz.