The submitochondrial distribution of ubiquinone affects respiration in long-lived Mclk1<sup>+/−</sup> mice

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Mclk1 (also known as Coq7) and Coq3 code for mitochondrial enzymes implicated in the biosynthetic pathway of ubiquinone (coenzyme Q or UQ). Mclk1<sup>+/−</sup> mice are long-lived but have dysfunctional mitochondria. This phenotype remains unexplained, as no changes in UQ content were observed in these mutants. By producing highly purified submitochondrial fractions, we report here that Mclk1<sup>+/−</sup> mice present a unique mitochondrial UQ profile that was characterized by decreased UQ levels in the inner membrane coupled with increased UQ in the outer membrane. Dietary-supplemented UQ<sub>10</sub> was actively incorporated in both mitochondrial membranes, and this was sufficient to reverse mutant mitochondrial phenotypes. Further, although homozygous Coq3 mutants die as embryos like Mclk1 homozygous null mice, Coq3<sup>+/−</sup> mice had a normal lifespan and were free of detectable defects in mitochondrial function or ubiquinone distribution. These findings indicate that MCLK1 regulates both UQ synthesis and distribution within mitochondrial membranes.

Introduction

Mutations in the mitochondrial hydroxylase CLK-1/MCLK1 lead to a variety of phenotypes, including increased longevity, in both Caenorhabditis elegans and mice (Ewbank et al., 1997; Liu et al., 2005; Lapointe et al., 2009; Wang et al., 2010). CLK-1/MCLK1 is necessary for the biosynthesis of ubiquinone (UQ). UQ is a benzoquinone with a head group capable of exchanging electrons and a side chain with a species-specific number of isoprene subunits (Bentinger et al., 2010). UQ<sub>9</sub> is the predominant form in C. elegans and in mice, with some UQ<sub>10</sub> in mice as well. UQ is an electron carrier in the mitochondrial respiratory chain, in addition to many other functions (Green and Tzagoloff, 1966; Bentinger et al., 2010), such as the ability to function as an antioxidant (Sohal, 2004; Bentinger et al., 2007). All organs biosynthesize functionally sufficient amounts of UQ, which is found in every membrane of all cells (Dallner and Sindelar, 2000).

Complete loss of Mclk1 is lethal in mice (Levavasseur et al., 2001; Nakai et al., 2001), but heterozygous Mclk1<sup>+/−</sup> animals are healthy and long-lived (Liu et al., 2005; Lapointe et al., 2009). Purified mitochondria from these mutants display impaired electron transport from complex I to III and from complex II to III as well as several additional related phenotypes, in particular altered reactive oxygen species (ROS) metabolism (Lapointe and Hekimi, 2008; Lapointe et al., 2009).

These observations are fully consistent with the observation that mouse cells that completely lack MCLK1 are unable to sustain UQ biosynthesis and only accumulate the UQ precursor demethoxy-ubiquinone (DMQ; Levavasseur et al., 2001; Nakai et al., 2001). However, no DMQ and normal levels of UQ were detected in Mclk1<sup>−/−</sup> mutants in spite of significantly reduced MCLK1 protein levels (Lapointe and Hekimi, 2008; Lapointe et al., 2009).

Previous quantifications of mitochondrial UQ in Mclk1<sup>+/−</sup> mice have been performed on standard extracts, which are considered to be contaminated by other organelles such as peroxisomes, lysosomes, and the endoplasmic reticulum (Graham, 2001c). Some of those organelles contain measurable amounts of UQ, which might have been sufficient to hide a small, but functionally significant, decrease of UQ in Mclk1<sup>−/−</sup> mitochondria. (Kalén et al., 1990; Tekle et al., 2002; Turunen et al., 2002).

The Coq3 gene codes for an O-methyltransferase responsible for the first O-methyltransferase step in UQ biosynthesis, producing 3-methoxy-4-hydroxy-5-hexaprenylbenzoic acid, as well as for the final one, converting demethyl-UQH<sub>2</sub> into UQH<sub>2</sub> (Hsu et al., 1996). The phenotype resulting from the loss of this enzyme has never been reported in mice.

UQ, in the form of UQ<sub>10</sub>, is extensively used as a nutritional supplement because of its antioxidant properties, principally...
The levels of UQ₉ and UQ₁₀ are unaffected in highly purified mitochondrial, peroxisomal, and plasma membrane fractions from Mclkl⁺/− livers

Because UQ is ubiquitously present in all cellular membranes and participates in specific functions at precise sites, its total cellular content might not be a good indicator of an excess or deficiency at a specific location. To obtain high yields of very pure mitochondria free of contaminating organelles, we used HistoDenz-based density gradients to produce mitochondrial preparations from Mclkl⁺/+ and Mclkl⁺/− mice and analyzed both UQ₉ and UQ₁₀ contents (Fig. 1). As expected, no contamination of the mitochondrial fractions by peroxisomes was detected with the peroxisomal marker Catalase (Fig. 1A). HPLC analysis of these highly pure fractions confirmed that there is no difference between Mclkl⁺/+ and Mclkl⁺/− mice in mitochondrial UQ₉ (Fig. 1, B and C) and UQ₁₀ content (Fig. S1).

Because peroxisomes might be involved in UQ biosynthesis (Tekle et al., 2002), we have also quantified UQ in peroxisomal fractions. The purity of the fractions was confirmed by the absence of the specific mitochondrial markers Porin and cytochrome c oxidase subunit IV (COXIV), as well as by the high level of peroxisomal Catalase (Fig. 1A). Similar amounts of UQ₉ were detected in the fractions from both genotypes (Fig. 1D), whereas UQ₁₀ was undetectable (not depicted).

We also determined the UQ content in plasma membrane fractions whose purity and enrichment were assessed by using Pan-Cadherin as a specific plasma membrane marker (Fig. 1E). No differences in UQ₉ or UQ₁₀ content were observed between the two genotypes (Fig. 1F).

The UQ distribution within mitochondria is altered in Mclkl⁺/− mutants

An additional approach based on sub mitochondrial fractionation was used to quantify UQ₉ and UQ₁₀ in individual mitochondrial membranes as well as in the soluble fraction. Specific protein markers were used for each mitochondrial compartment to verify enrichment, purity, and determine potential differences between genotypes (Fig. 2A). The Western blot results confirm that specific fractions were successfully obtained for all compartments and that markers are expressed similarly in Mclkl⁺/+ and Mclkl⁺/− mitochondria. Indeed, monoamine oxidase (MAO) expression was only detected in the outer membrane (OM) fraction, and COXIV was strongly expressed in the inner membrane (IM) fraction, with only traces in the OM, whereas the second mitochondria-derived activator of caspases (SMAC) was mainly detected in the soluble fraction, as expected (Fig. 2A). Quantification of UQ in these fractions revealed significantly elevated UQ₉ and UQ₁₀ in the OM of Mclkl⁺/− mutants compared with control littermates (Fig. 2B and Fig. S1). In contrast, the UQ₉ content of the IM of Mclkl⁺/− mutants was significantly decreased in comparison to Mclkl⁺/+ control littermates (Fig. 2C). Consequently, the OM/IM ratio for UQ₉ content is significantly different between genotypes (Fig. 2D). A trend toward a decrease was also observed for UQ₁₀ in the same fractions, but the difference didn’t reach significance (Fig. S1). In the soluble fraction, which includes components of the inter-membrane and matrix compartments, little UQ₉ was detected and no difference between genotypes was observed (Fig. 2E). No UQ₁₀ was detected in the soluble fractions (unpublished data).

Our observations suggest that in Mclkl⁺/− mutants there is more UQ in the OM and less UQ in the IM than in the wild type. To confirm that protein compositions of both mitochondrial membranes were not affected by reduced MCLK1 levels,
Functional significance of changes in UQ distribution

The lower level of UQ in the IM could be the cause of several reported Mclk1\(^+/−\) mice phenotypes, including: (a) the decline in electron transport between complex I and III as well as between complex II and III; (b) the decreased oxygen consumption of isolated mitochondria with complex I or complex II substrates; and (c) the reduction in mitochondrial ATP levels (Lapointe and Hekimi, 2008; Lapointe et al., 2009). The electron carrier function of UQ from complex I and II to complex III is the most well-established function of UQ, as is the relation between the electron transport rate and UQ concentration (Schneider et al., 1982). It has been reported that endogenous UQ levels are not saturating for NADH oxidation by complex I and, possibly, for succinate oxidation by complex II (Norling et al., 1974; Lenaz et al., 1997). This suggests that variations in physiological UQ levels should impact electron transport in the mitochondrial respiratory chain (Fato et al., 1997). Although UQ is present in mitochondria at a much higher concentration than other constituents of the ETC, it is believed that lowering UQ levels could affect mitochondrial function because of its slow rate of oxidation reduction, whether or not the traditional random collision mechanism or the more recent model involving supercomplex organization is considered (Green and Tzagoloff, 1966; Lenaz and Genova, 2009). Together, these observations suggest that the reduction in MCLK1 levels in Mclk1\(^+/−\) mutants leads to a reduction of the UQ content of the IM, which falls below that required for maintaining physiologically normal levels of electron transport.

The UQ deficiency in the IM could also be the cause of the oxidative stress observed in Mclk1\(^+/−\) mitochondria. The mitochondrial ETC is believed to be a major site of cellular ROS production, and lower respiratory rates resulting from inhibition of electron transport are often accompanied by enhanced ROS release. Moreover, respiratory dysfunction and oxidative stress resulting from UQ deficiency have been reported in various experimental conditions and specific pathologies (Geromel et al., 2002; DiMauro et al., 2007). For example, human skin fibroblasts carrying a homozygous mutation in the COQ2 gene, which encodes a protein implicated in the UQ biogenesis pathway, have significantly less cellular UQ and exhibit a mitochondrial phenotype highly similar to that of Mclk1\(^+/−\) mice (López-Martín et al., 2007). In these fibroblasts, UQ deficiency is also linked to a partial defect in electron transport and ATP synthesis, as well as significantly increased ROS production and oxidation of lipids and proteins (López-Martín et al., 2007; Quinzii et al., 2008, 2010).

The increase in UQ levels in the OM is an unexpected outcome of the partial loss of a UQ biosynthetic enzyme. One possibility is that this phenotype is linked to the antioxidant...
indicating that **Coq3** is essential for embryonic survival. Of 211 live pups from heterozygous mating, 83 were wild type and 128 were hemizygous. This ratio is close to the 1:2 ratio typical for an embryonic lethal gene. As expected, we observed that **Coq3** transcription is decreased in **Coq3**+/− heterozygotes (Fig. 4 A). We raised an antiserum against mouse COQ3 and could show that **Coq3**+/− mice display low COQ3 protein levels (Fig. 4, B and C). Similarly to **Mclk1**+/−, no changes in UQ levels were observed in pure mitochondria isolated from 3-mo-old **Coq3**+/− livers (Fig. 4 D). However, submitochondrial fractionation did not reveal any significant difference in UQ content within mitochondrial compartments (Fig. 4, E and F). As expected from the above results, mitochondrial function assessed by in vitro oxygen consumption experiments as well as mitochondrial oxidative status, which was analyzed by measuring aconitase activity, were not affected by the reduced COQ3 levels (Fig. S2, A and B). Finally, we examined the age-dependent survival of both male and female **Coq3**+/− mice and found that both sexes exhibited normal life spans (Fig. S2, A and B). These results indicate that COQ3, in contrast to MCLK1, is not limiting for UQ biosynthesis, in spite of acting twice in the UQ biosynthetic pathway. This observation does not support the idea that the complex UQ phenotype of **Mclk1**+/− mice is related to a supramolecular complex of UQ biosynthetic enzymes.

functions of UQ. As discussed above, it is likely that the low level of UQ in the IM of **Mclk1**+/− mutants increases oxidative stress by partially inhibiting electron transport. It is well known that under oxidative stress, cells will take protective measures to ensure their survival, such as increasing the levels of an antioxidant such as UQ. In fact, overall tissue levels of UQ have been found to be elevated by conditions that increase oxidative stress and damage (Guan et al., 1996; Navarro et al., 1998). Thus elevation of UQ levels in the OM of **Mclk1**+/− mutants might be a protective response to reduce oxidative damage to the OM and/or reduce ROS leakage from the mitochondria to the cytoplasm. The mechanism by which the mitochondria increase UQ levels in the OM is unknown but could be based on slower turnover or slower export of UQ.

Reduction of COQ3 levels in **Coq3**+/− heterozygotes does not result in a **Mclk1**+/−-like phenotype

We wondered whether the appearance of a mitochondrial UQ imbalance in heterozygotes is specific to the **Mclk1** locus or is a more general characteristic of genes that code for UQ biosynthetic enzymes, which, at least in yeast, function in a supramolecular complex (Turunen et al., 2004). We thus created a targeted inactivation of the mouse **Coq3** gene. No **Coq3** homozygous mice were obtained from heterozygous crosses, indicating that **Coq3** is essential for embryonic survival. Of 211 live pups from heterozygous mating, 83 were wild type and 128 were hemizygous. This ratio is close to the 1:2 ratio typical for an embryonic lethal gene. As expected, we observed that **Coq3** transcription is decreased in **Coq3**+/− heterozygotes (Fig. 4 A). We raised an antiserum against mouse COQ3 and could show that **Coq3**+/− mice display low COQ3 protein levels (Fig. 4, B and C). Similarly to **Mclk1**+/−, no changes in UQ levels were observed in pure mitochondria isolated from 3-mo-old **Coq3**+/− livers (Fig. 4 D). However, submitochondrial fractionation did not reveal any significant difference in UQ content within mitochondrial compartments (Fig. 4, E and F). As expected from the above results, mitochondrial function assessed by in vitro oxygen consumption experiments as well as mitochondrial oxidative status, which was analyzed by measuring aconitase activity, were not affected by the reduced COQ3 levels (Fig. S2, A and B). Finally, we examined the age-dependent survival of both male and female **Coq3**+/− mice and found that both sexes exhibited normal life spans (Fig. S2, A and B). These results indicate that COQ3, in contrast to MCLK1, is not limiting for UQ biosynthesis, in spite of acting twice in the UQ biosynthetic pathway. This observation does not support the idea that the complex UQ phenotype of **Mclk1**+/− mice is related to a supramolecular complex of UQ biosynthetic enzymes.
Supplemented dietary UQ₁₀ induces changes in intramitochondrial UQ distribution

UQ level increases resulting from dietary UQ supplementation have been found to be greater in mitochondria than in whole tissue homogenates (Kamzalov et al., 2003; Fernández-Ayala et al., 2005; Saito et al., 2009), and orally supplemented UQ is able to restore mitochondrial dysfunction and decrease oxidative damage resulting from UQ deficiency (Geromel et al., 2002; Lópeze et al., 2010; Dai et al., 2011). Surprisingly, in spite of this, nothing appears to be known about the intramitochondrial fate of exogenous UQ.

To evaluate the cellular fate of exogenous UQ₁₀, we used a formulation (Q-Gel from Tishcon) that results in consistent UQ uptake (Kamzalov et al., 2003; Preuss et al., 2010). Significant accumulation of UQ₁₀ was detected in whole liver and liver mitochondria of UQ₁₀-supplemented mice (Fig. S3, A and C). Interestingly, we observed a greater accumulation of UQ₁₀ in both Mclk1⁻/⁻ livers (Fig. S3 A), and purified Mclk1⁻/⁻ liver mitochondria, where the trend was significant; that is, Mclk1⁻/⁻ mitochondria accumulated more exogenous UQ₁₀ than their Mclk1⁺/⁺ counterparts (Fig. S3 C). It is thought that UQ uptake by tissues could be enhanced if the endogenous UQ levels have fallen below a critical threshold (Sohal and Forster, 2007). The effect could also be the result of a reaction to the greater oxidative stress due to the intrinsic UQ deficiency. However, endogenous total UQ levels were not affected by UQ₁₀ treatment (Fig. S3, B and D).

Supplemented UQ₁₀ was also followed in submitochondrial fractions. We report here, for the first time to our knowledge, that exogenous UQ₁₀ is successfully incorporated in both the OM and the IM (Fig. 5, A and C). Although we observed increased UQ₁₀ incorporation in whole Mclk1⁻/⁻ mitochondria (Fig. S3 C), this was not observed when membranes preparations were used (Fig. 5, A and C). Possibly, some of the exogenous UQ is more loosely bound to membranes than endogenous UQ and is lost during membrane preparation.

We further observed that UQ₁₀ supplementation reduced UQ content in the OM of Mclk1⁻/⁻ mutants to wild-type levels (Fig. 5 B). It is possible that the antioxidant properties of the exogenous UQ₁₀ decreased the need for more UQs in the Mclk1⁻/⁻ OM, and/or saturating concentrations for UQ of any side-chain length are being generated in the OM by the UQ₁₀ supplementation (Kwong et al., 2002). However, a UQ deficit was still observed in the Mclk1⁻/⁻ IM after UQ₁₀ administration, and this even while UQ₁₀ content was dramatically increased (Fig. 5, C and D). Orally supplemented UQ₁₀ has been shown to increase tissue concentrations of endogenous UQs by an unknown mechanism, but this was obtained with a much longer duration of treatment (Kamzalov et al., 2003).

Rescue of Mclk1⁻/⁻ mitochondrial defects by exogenous UQ

Rescue of Mclk1⁻/⁻ mitochondrial defects in vitro was not attempted previously because no UQ deficit had been observed. The activity of complex II is measured by following the decrease in absorbance caused by the coupled reduction of 2,6-dichlorophenolindophenol at 600 nm with 750 nm as the reference wavelength (see Materials and methods). Under these native conditions, there is a clear decrease in mutant complex II activity in mitochondria isolated from both liver and kidneys (Fig. 5 F and Fig. S3 E). However, no difference in complex II activity between the wild type and Mclk1⁻/⁻ mutants could be observed when exogenous UQ₁₀ was added to the reaction (Fig. 5 F and S3 E). This suggests that the reduction in endogenous UQ, in the IM is sufficiently severe to impact ETC functions, in this case electron transport from complex II to UQ.
Exogenous supplemented UQ_{10} is able to increase mitochondrial electron transport in either normal or UQ-deficient conditions (Fernández-Ayala et al., 2005; López-Martin et al., 2007). A recent publication also suggests that UQ_{10} supplementation might restore several of the phenotypes of *C. elegans* clk-1 mutants (Takahashi et al., 2012). To assess whether the accumulation of supplemented UQ_{10} in the IM is functional, we tested whether it could rescue one of the reported electron transport chain dysfunction of the *Mclk1^{+/−}* mice. We analyzed electron transport between mitochondrial complex I and III, a defect that can reasonably be attributed to the UQ_{9} deficiency observed in the *Mclk1^{+/−}* mitochondrial IM. Although, as expected, a clear difference between *Mclk1^{+/+}* and *Mclk1^{+/−}* mice was observed in the control mice, electron transport rates in the two genotypes were indistinguishable after UQ_{10} treatment (Fig. 5 E). The rate of electron transport in the wild-type UQ_{10}-treated mice appeared somewhat lower than in untreated wild-type mice, but the difference was not significant. This result suggests that the exogenous UQ_{10} was indeed capable of rescuing the endogenous UQ_{9} deficiency. Although it is possible in principle that the UQ_{10} specifically depressed the rate in the wild type but was without effect in the mutant, this appears unlikely.

The findings presented here, that there is an IM-specific UQ deficiency in *Mclk1^{+/−}* mutants and that at least some of the mitochondrial phenotypes of these mutants can be rescued in vitro and in vivo by exogenous UQ, should prompt further study to determine if all *Mclk1^{+/−}* phenotypes can be rescued by exogenous UQ, including whole animal phenotypes such as aging, immune activation, and resistance to cerebral ischemia/reperfusion injury (Liu et al., 2005; Wang et al., 2010; Zheng et al., 2010).
Conclusions
Our study lays emphasis on the importance of carefully analyzing individual mitochondrial compartments when studying membrane-associated mitochondrial proteins or lipids. This approach has helped us to reveal that MCL1K1 activity is limiting for normal UQ content in the mitochondrial IM and that in turn UQ content in this membrane is a limiting factor for electron transport chain activity. In the light of our findings, we also propose that the distribution of UQ in mitochondrial membranes is likely to be an adaptive and dynamic process that can react to changes in UQ synthesis or absorption. Our demonstration that exogenous UQ is able to successfully reach the inner mitochondrial membrane is also important when UQ is used or studied for therapeutic purposes.

Materials and methods

Animals
All the animals were housed in a pathogen-free facility at McGill University and were given a standard rodent diet and water ad libitum. Mcl-1−/− and Mcl-1+/− mice, 3-mo-old males and females from pure BALB/c genetic background, were anesthetized, sacrificed by cervical dislocation, and perfused with phosphate buffer. Tissues were then rapidly removed, rinsed, and placed in ice-cold mitochondrial isolation buffer or immediately frozen in liquid nitrogen. All procedures were approved by the McGill Animal Care and Ethics committees.

Generation of Coq3 gene knockout mice
Coq3 gene knockout mice were generated by inGenious Targeting Laboratories, Inc., using bacterial artificial chromosome (BAC)-mediated recombination. In brief, a mouse genomic Coq3 fragment with 10.1 kb of upstream homology sequence and 2.1 kb of downstream homology sequences was subcloned into a targeting vector. A loxP/FRT-flanked PGK-neomycin resistance cassette was then inserted to replace exons 4 and 5 of the Coq3 gene. NotI-linearized targeting vector was transfected into 129SV/VE embryonic stem cells. Clones were selected in G418, and PCR analysis identified clones that had undergone homologous recombination were microinjected into 129/SvEv blastocysts. The resulting chimeric mice were mated with 129/SvEv mice to generate heterozygous germ-line founders. Coq3 heterozygous mice appear normal and fertile, whereas Coq3-null mice die in utero. For PCR genotyping, DNA was extracted from tail clippings and subjected to PCR using the following primers: KO (5′-GGGGAACCT-CTGACTAGGG-3′) and GR (5′-CCAGTGCTGC-3′) to identify the disrupted allele (522 bp) and wild type (5′-CCAGTGCTGC-TATCGAGGTCGCAAGT-3′) to disrupt the targeted allele (522 bp) and wild type (5′-CCAGTGCTGC-TATCGAGGTCGCAAGT-3′) and GR to identify the wild-type allele (345 bp). PCR conditions were 94°C for 3 min, 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min for 30 cycles followed by 72°C for 10 min. PCR products were resolved on 2% agarose gels.

Isolation of pure mitochondrial fractions
On the day of the experiment, fresh liver was homogenized in 10 vol (wt/vol) of MSHE buffer (210 mM Mannitol, 75 mM sucrose, 10 mM Hepes, pH 7.2, and 1 mM EDTA). The resultant homogenate was centrifuged at 600 g for 10 min at 4°C in an Allegra X-12 bench-top centrifuge (Beckman Coulter) to remove large cellular debris. The 600 g supernatant was centrifuged at 15,000 g for 10 min, and the mitochondria-enriched pellet was further washed with MSHE and centrifuged again at 15,000 g for 10 min in an Avanti J-25 centrifuge (Beckman Coulter). The supernatant corresponded to the cytosolic fraction. The washing pellet was then resuspended in 2 ml of 35% Histodenz (Sigma-Aldrich) to prepare MSHE buffer for further purification using ultracentrifugation through a discontinuous Histodenz gradient. In brief, liver was first processed exactly as it was described earlier for mitochondrial isolation, except that the band at the 35/40% interface, corresponding to the fraction enriched in peroxisomes (Graham, 2001b), was collected and diluted with the same volume of MSHE buffer and then centrifuged at 15,000 g for 10 min. The pellet obtained was resuspended in 2 ml of 35% Histodenz before further purification on Histodenz gradient. This gradient was prepared by carefully toppling the resuspended peroxisomes [in 35% Histodenz] with 2 ml of 25% and 1.5 ml of 15% Histodenz solutions, and centrifuged at 52,000 g for 60 min at 4°C. The purified peroxisomes were then recovered from the pellet, diluted in an equal volume of MSHE buffer, centrifuged at 15,000 g for 10 min, and resuspended again in 35% Histodenz for a second round of purification. Then the mitochondrial pellet was resuspended in MSHE for immunoblot analysis. Fraction purity and possible mitochondrial contamination was assayed via immunoblot analysis of specific markers [peroxisomes, anti-Catalase (EMD Millipore), mitochondria, anti-Porin (EMD Millipore) and anti-COXIV (New England Biolabs, Inc.)].

Isolation of plasma membrane fraction
Plasma membrane fractions were purified according to a published protocol (Song et al., 2006). Mouse livers were homogenized in 10 vol (wt/vol) of MSHE buffer with a Potter homogenizer (Teflon pestle). The homogenates were then centrifuged for 10 min at 1,000 g at 4°C. The pellet was suspended in 20 ml of buffer A (0.3 M sucrose, 50 mM Tris, and 3 mM MgCl₂, pH 7.5) and mixed with 9 volumes of buffer B (1.98 M sucrose, 50 mM Tris, and 1 mM MgCl₂, pH 7.5) to form a 1.8 M [50% wt/vol] sucrose density. After centrifugation at 70,900 g for 90 min, the 0.25 M/1.8 M interface (8.3/50% sucrose) was collected and resuspended in 0.25 M SHE buffer, 10 mM Hepes, pH 7.5, and 1 mM EDTA. The suspension was centrifuged at 1,200 g for 10 min, and the resulting pellet was resuspended using 0.25 M SHE, 2.4 M SHE (10 mM sucrose, 10 mM Hepes, pH 7.5, and 1 mM EDTA) to add the final concentration to 1.45 M. The suspension was then overlaid with 0.25 M SHE to fill the tubes and centrifuged at 68,400 g for 60 min. The pellet was resuspended in 0.25 M SHE, mixed with 2.4 M SHE to bring the final concentration of sucrose to 1.35 M, and centrifuged at 230,000 g for 60 min. The material at the 0.25 M/1.35 M interface (8.3/39% sucrose) was collected and constituted the plasma-membrane fraction. Enrichment and specificity of the plasma membrane fractions were assayed by immunoblot analysis of specific markers anti–Pan-cadherin (Sigma-Aldrich) and anti-Porin [EMD Millipore].

Mitochondrial subfractionation
The purified mitochondria were recovered from the 25/35% interface of the Histodenz gradient as described earlier, diluted in an equal volume of MSHE buffer, centrifuged at 15,000 g for 10 min, and resuspended in MSHE. Fractions enriched in outer mitochondrial membranes were performed by resubmitting the gradient-purified mitochondria in 0.75% digitonin in isolation medium (220 mM Mannitol, 70 mM sucrose, 20 mM HepesNaOH, pH 7.4, and 0.5 mg/ml fatty-acid-free bovine serum albumin) on ice for 20 min with gentle agitation as described previously (Graham, 1993). The resulting mitochondrial suspension was then diluted with 3 vol of isolation medium, mixed carefully by three strokes in a loose fitting Dounce homogenizer, and gently sonicated two times at 15 s at 40 W. Mitoplasts (IM and matrix) were separated from the supernatant (OM and the inter membrane space content) by centrifugation at 15,000 g for 15 min. The OM fraction was obtained by centrifugation of the supernatant at 100,000 g for 60 min. The resulting pellet was resuspended in pure water and sonicated eight times for 15 s at 80 W with 15 s of rest. The IM and matrix fractions were then separated by...
centrifugation at 200,000 g for 45 min. OMA and IM fractions were suspended in MSHE buffer. Fraction purity was assayed by immunoblot analysis of specific fraction markers [OM, anti-monoamine oxidase (Santa Cruz Biotechnology, Inc.)], IM, anti-COXIV (EMD Millipore); soluble fraction, anti-SOD2 (Cedarlane) and anti-SMAC (EMD Millipore) as described previously (Da Cruz et al., 2003). All the fractions were frozen at −80°C for subsequent immunoblot and HPLC analysis.

Relative quantification of mitochondrial membrane proteins
Mitochondria were isolated from the livers of 3-mo-old Mclk1<sup>−/−</sup> mutant and wild-type sibling mice. 20 µg of mitochondrial protein/sample was subjected to Western blot analysis with antibodies against the following proteins: Mitofusion 2, monoamine oxidase A (MOA), and Porin in the OM; respiratory complexes II–V in the IM; and Cyclophilin D located in the matrix of mitochondria. All antibodies were obtained from Abcam. Blots were visualized using ECL plus Western blotting detection system (GE Healthcare) with a Typhoon 9400 scanner (GE Healthcare). Band intensities were analyzed using ImageJ software and normalized to those of Cyclophilin D.

Electron microscopy
3-mo-old mice on the BALB/c genetic background were anesthesized and subjected to intracardiac perfusion with Ringer’s lactate solution and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and 4% sucrose. Livers were removed, cut into small pieces, and immersed in the same fixative overnight at 4°C. After washing with 0.1 M cacodylate buffer, pH 7.4, the sample specimens were postfixed in 1% OsO4 and 1.5% potassium ferrocyanide for 2 h and then routinely processed for transmission electron microscopic observation. In brief, the postfixed specimens were dehydrated through a series of graded acetone solutions, infiltrated with epon-ace tone mixture, and embedded in epon. Polymerization was performed at 60°C for 2 d. Ultrathin sections (90–100 nm) were then mounted on copper grids and counterstained with uranyl acetate and Reynold’s lead citrate. Observations were performed on a Tecnai 12 BioTwin Transmission Electron Microscope (FEI Electron Optics), operated at 120 kV, using a 40-µm objective aperture. Images were digitized with the use of an AMT XR80C (8 megapixel) charge-coupled device camera and Image Capture Engine Software (version 601).

Identification of quinones
Cellular fractions or sub mitochondrial fractions were mixed with an equal volume of 100% ethanol (Thermo Fisher Scientific) and vortexed for 10 s. An equal volume of hexane (Sigma-Aldrich) was then added followed by 2 min of vortexing and 5 min of centrifugation (8,000 g, 4°C). The resulting upper layer was collected and evaporated to dryness using a Speed Vac (Eppendorf Concentrator), after which the residual was resuspended in 100% ethanol and stored at −20°C until HPLC analysis. A Beckman System Gold HPLC with a reversed-phase C18 column (5 µm, 4.6 × 250 mm) was used. The mobile phase was 70% methanol and 30% ethanol at a flow rate of 1.8 ml/min. The UV detector was set at 275 nm. Quinones were identified and quantified using pure UQ standards from Sigma-Aldrich. The total amount of quinone was normalized to the amount of protein (Liu et al., 2005).

Determination of Coq3 mRNA and protein expression
Immunoblot analysis of 3-mo-old female wild-type and Coq3<sup>−/−</sup> mice were quick-frozen in liquid nitrogen and stored at −80°C until use. Quantitative real-time PCR was used to determine the mRNA expression level of Coq3 in liver using the following primers for Coq3 and the housekeeping gene β-actin: Coq3-F, 5′-ACGGTGCTCATCATTAGAG-3′; Coq3-R, 5′-TGAA- GCTGCACTCGCTCCTA-3′; β-actin-F, 5′-CACACGCAGCTCATCGGC-3′; and β-actin-R, 5′-CCACACGACCCACGCTGGCC-3′. Standard curves for each primer set were generated using a dilution series of known amounts of purified PCR products. The Coq3 mRNA levels were normalized to the amount of β-actin mRNA in the same samples, and the final real-time RTPCR results are reported as copy number of Coq3 per 1,000 copies of β-actin. Quantitative real-time PCR was performed using SYBR-Green PCR Master Mix on a CFX96 real-time PCR instrument (Bio-Rad Laboratories), according to the manufacturer’s instructions. The level of COQ3 protein was determined by Western blot analysis with an anti-COQ3 antibody (developed in our laboratory). In brief, 18 µg of mitochondrial protein samples were electrophoresed on 12% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories). The membranes were probed first with rabbit polyclonal anti-COQ3 (dilution 1:1,000) and anti-Porin (dilution 1:1,000; Abcam) antibodies overnight, followed by peroxidase-conjugated secondary anti-rabbit antibody (dilution 1:2,000; Sigma-Aldrich). The blots were developed using ECL-Plus kit (GE Healthcare) and visualized with a Typhoon 9400 scanner (GE Healthcare).

Oxygen consumption by isolated mitochondria
Oxygen consumption was measured polarographically as described by Lapointe and Hekimi (2008). In brief, liver mitochondria were prepared from freshly sacrificed 3-mo-old female mice using Mg<sup>2+</sup>-free buffer containing 0.25 M sucrose, 10 mM Hepes, pH 7.4, and 1 mM EDTA. Oxygen consumption was measured in the air-saturated respiration buffer (250 mM sucrose, 10 mM Hepes, pH 7.2, 20 mM KCl, 5 mM KH<sub>2</sub>PO<sub>4</sub>, and 2 mM MgCl<sub>2</sub>; pH 7.4; 30°C) with a Clark-type oxygen electrode. Mitochondria were suspended at 0.5 mg/ml, and electron transport was initiated by the addition of complex I (5 mM glutamate plus 2.5 mM malate) or complex II (10 mM succinate plus 1.25 mM rotenone) substrates. The rate of maximal coupled (state 3) respiration was then determined after adding 0.6 mM ADP. Mitochondrial functional integrity was assessed by the respiratory control ratio (RCR; ADP-stimulated/unstimulated respiration). In all measurements, the RCR values were >7.0 with glutamate/malate and >4.0 with succinate as electron donors, confirming functional intactness of isolated mitochondria.

Determination of aconitate activity
Mitochondrial aconitate activity was determined spectrophotometrically as described previously (Lapointe and Hekimi, 2008). In brief, citrate was used as the substrate of aconitate and the conversion of citrate into α-ketoglutarate in the presence of NADP+-dependent isocitrate dehydrogenase was monitored by following the formation of NADPH at 340 nm. The reaction mixture of 1 ml contained 50 mM Tris·HCl (pH 7.4), 30 mM sodium citrate, 0.5 mM MnCl<sub>2</sub>, 0.2 mM NADP<sup>+</sup>, and isocitrate dehydrogenase (2 U/ml). The reaction was started by addition of mitochondrial protein, and aconitate activity was calculated as nanomole of NADPH formed per minute per milligram of protein.

UQ<sub>9</sub> supplementation
Female Mclk1<sup>−/−</sup> and Mclk1<sup>+/−</sup> 3-mo-old mice from pure BALB/c background were randomly assigned to be fed either the control diet or UQ<sub>9</sub>-enriched diet (2.81 mg/g) for 3 wk based on previously described protocol (Sumiet et al., 2009). The UQ<sub>9</sub> that was used in diet preparation by Purina Mills Testdiet was provided by Tischon corp. in the form of Q-gel liquid vehicle. Food intake was not affected by UQ<sub>9</sub> diet. Subsequently, mice were sacrificed and the amounts of UQ homologues [UQ<sub>10</sub> and UQ<sub>7</sub>] were measured in homogenates, mitochondria, and mitochondrial subfractions from liver.

Electron transport chain assays
Mitochondrial samples were diluted with 30 mM potassium phosphate, pH 7.4, to reach a concentration of ~1 mg/ml protein concentration and then subjected to three rounds of freezing-thawing. The rate of electron transport between complex I and complex III was assayed by following the reduction of cytochrome c at 550 nm in the reaction mix containing 30 mM potassium phosphate, pH 7.4, 100 µM NADH, and 2 mM KCN. Rotenone insensitive activity was determined by adding 2 µg/ml rotenone in the reaction mixture, and it was subtracted from the total activity (Lapointe and Hekimi, 2008). Complex II activity was measured by following the decrease in absorbance because of the coupled reduction of 2,6-dichlorophenolphendolphosphol in 600 nm with 750 nm as the reference wavelength. The reaction mixture containing 50 mM potassium phosphate, pH 7.4, 20 mM succinate, and 5 mM MgCl<sub>2</sub> was preincubated with 15 µg of mitochondrial protein at 30°C. After 10 min of incubation, 2 µg antimycin, 2 µg rotenone, 2 mM KCN, and 150 µM 2,6-dichlorophenolphendolphosphol were added. 2,6-Dichlorophenolphendolphosphol can accept electrons from the complex II-bound endogenous ubiquinol or exogenous ubiquinol. Thus, the activity was first monitored for 1 min without the addition of exogenous ubiquinone. Subsequently, complex II activity was also measured with the addition of 100 µM ubiquinone (UQ<sub>10</sub>) and was monitored for 2 min. All reactions were initiated by the addition of mitochondrial proteins.

Life span determination
Life span was determined in mouse cohorts comprising heterozygous mutants Coq3<sup>−/−</sup> and their wild-type littermate control. The number of days
that each mouse lived was recorded and graphed by the Kaplan-Meier method. Mice that died of apparent non-natural causes (e.g., cage fight injuries, pan flood accidents) were excluded from the longevity data.

**Statistical analyses**

Group data were presented as mean values ± SEM. Quantitative data were analyzed by GraphPad Prism Version 5.00 for Windows (GraphPad Software, Inc.). Comparisons between Mclk1+/− and Mclk1mice were performed using an unpaired two-tailed Student’s t test. For multiple comparisons, one-way ANOVA followed by Bonferroni’s post hoc analysis was performed. For evaluating survival data, the log-rank (Mandel-Cox) test was performed. For all analyses, a value of P < 0.05 was considered significant.

**Online supplemental material**

Fig. S1 shows that overall mitochondrial UQ_{10} levels are normal in Mclk1+/− mice but display an altered distribution in OMs and IMs similar to that of UQ_{9}. Fig. S2 shows that Coq3−/− mice have no mitochondrial dysfunction, as indicated by normal levels of mitochondrial oxygen consumption and no change in mitochondrial aconitase activity. Both male and female Coq3−/− mice were found to have normal life spans. Fig. S3 shows that UQ_{9} supplementation has no significant effects on the whole liver and liver mitochondrial UQ_{9} content of Mclk1+/− mice, but that accumulation of supplemented exogenous UQ_{9} in mutant Mclk1−/− mice is greater than that in wild-type controls. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201203090/DC1.

We thank Jeannie Mui and the Facility for Electron Microscopy Research (FEMR) at McGill University for technical assistance. S. Hekimi is supported by CIHR grant MOP-97869. S. Hekimi is Campbell Chair of Developmental Biology and Strathcona Chair of Zoology.

Submitted: 16 March 2012
Accepted: 13 September 2012

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