Mitofusins 1 and 2 Are Essential for Postnatal Metabolic Remodeling in Heart

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Rationale: At birth, there is a switch from placental to pulmonary circulation and the heart commences its aerobic metabolism. In cardiac myocytes, this transition is marked by increased mitochondrial biogenesis and remodeling of the intracellular architecture. The mechanisms governing the formation of new mitochondria and their expansion within myocytes remain largely unknown. Mitofusins (Mfn-1 and Mfn-2) are known regulators of mitochondrial networks, but their role during perinatal maturation of the heart has yet to be examined.

Objective: The objective of this study was to determine the significance of mitofusins during early postnatal cardiac development.

Methods and Results: We genetically inactivated Mfn-1 and Mfn-2 in midgestational and postnatal cardiac myocytes using a loxP/Myh6-cre approach. At birth, cardiac morphology and function of double-knockout (DKO) mice are normal. At that time, DKO mitochondria increase in numbers, appear to be spherical and heterogeneous in size, but exhibit normal electron density. By postnatal day 7, the mitochondrial numbers in DKO myocytes remain abnormally expanded and many lose matrix components and membrane organization. At this time point, DKO mice have developed cardiomyopathy. This leads to a rapid decline in survival and all DKO mice die before 16 days of age. Gene expression analysis of DKO hearts shows that mitochondria biogenesis genes are downregulated, the mitochondrial DNA is reduced, and mitochondrial encoded transcripts and proteins are also reduced. Furthermore, mitochondrial turnover pathways are dysregulated.

Conclusions: Our findings establish that Mfn-1 and Mfn-2 are essential in mediating mitochondrial remodeling during postnatal cardiac development, a time of dramatic transitions in the bioenergetics and growth of the heart. (Circ Res. 2012;111:1012-1026.)

Key Words: biogenesis • cardiac growth cardiomyopathy • mitochondrial fusion • mitochondrial DNA • p62

Remodeling a fetal heart into an adult heart is a complex process and relies on important transitions that are triggered soon after birth. During this early postnatal stage, the heart undergoes a switch in substrate utilization to catabolize fatty acids, and carbohydrates become a secondary source of energy.1-3 Failure to enact this metabolic switch in mice is marked by cardiac defects and a lifespan of up to 2 weeks.3,4 Apart from the metabolic switch, the early postnatal stage is also marked by a switch in the mode of cardiac growth. Cardiac myocytes largely cease proliferation at approximately postnatal day (P) 4 (P04) and undergo hypertrophic growth, leading to an increase in diameter and mass.5 In mice, physiological myocyte hypertrophy between P05 and P14 results in a nearly three-fold increase in heart weight.6 Furthermore, mitochondrial density doubles in cardiac myocytes during early postnatal development, and the small, round, and tubular mitochondria that are found in fetal hearts are re-formed into large ovoid and rectangular mitochondria.7,8 This change in mitochondrial shape is paralleled by reorientation of mitochondria such that there is larger contact surface and closer proximity between mitochondria and the myofibrils and between mitochondria and the sarcoplasmic reticulum, a transition that is noted to take place at approximately P07 in mice.9 This histological evidence suggests that mitochondrial remodeling is important for the passage of the heart from a fetal state to an adult state.

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Genetic and functional studies firmly established mitofusins as key regulators of mitochondrial morphology in a wide spectrum of organisms ranging from yeast (single gene, Fco1p) to mammals (two genes, mitofusin [Mfn]-1 and Mfn-2).10-11 Murine Mfn1 and Mfn2 have approximately 65%
identity at the amino acid level, and in several contexts they exhibit functional overlap or complementation in mediating outer mitochondrial membrane fusion.\textsuperscript{13–16} The molecular characterization of mitofusins has been instrumental in elucidating the significance of mitochondrial dynamics, an enigmatic process that involves the flow of mitochondrial matter through merging, splitting, and transposition of mitochondria.\textsuperscript{17,18} Mitochondrial dynamics have implications in many aspects of mitochondrial function\textsuperscript{19} and appear to be particularly important in mitochondrial quality control, the pathway that selectively separates out defective mitochondria for subsequent elimination.\textsuperscript{20,21} Importantly, the dynamic behavior of mitochondria is not the same in all cell types and is strongly influenced by the intracellular motility and the cytoskeleton dynamics.\textsuperscript{22–24} Along these lines, the striated contractile cells of the adult heart and skeletal muscle have a very rigid cytoskeleton and their mitochondria are stationary.\textsuperscript{25,26} Nevertheless, the expression of Mfn-1 and Mfn-2 is very robust in these cells with immotile mitochondria, indicating that mitofusins are still biologically important.

To address the functional significance of mitofusins in striated tissues, several laboratories have pursued the conditional inactivation of Mfn-1 and Mfn-2 in the skeletal and cardiac muscle of mice. So far, it has been found that both Mfn-1 and Mfn-2 are necessary for the maintenance of the mitochondrial genome in skeletal muscle.\textsuperscript{27} Furthermore, Mfn-2 in skeletal muscle is important in preserving insulin sensitivity and repressing oxidant stress.\textsuperscript{28} Regarding the heart, both Mfn-1 and Mfn-2 are shown to be important during early embryonic cardiac development, and in adulthood they protect against long-term cardiac dysfunction.\textsuperscript{29} Our studies with cardiomyocyte-restricted mitofusin knockouts have focused on the identification of common boundaries and cause organelle dysfunction by triggering mitochondrial permeability transition.\textsuperscript{31} The resistance to mitochondrial permeability transition was detectable in both Mfn-1- or Mfn-2-deficient mitochondria, suggesting a direct relationship between mitochondrial membrane fusion and permeabilization.\textsuperscript{30,31,33} Finally, these studies showed that the baseline heart function is not severely impacted by the deletion of either Mfn-1 or Mfn-2 in adult animals.\textsuperscript{30,31}

In this report, we present work regarding the dual deletion of Mfn-1 and Mfn-2 in cardiac myocytes. We show that permanent inactivation of mitofusins in cardiomyocytes during midgestation has profound effects on the function of the early postnatal heart. Our findings also show that cardiac Mfn-1 and Mfn-2 have important roles for survival postnatum and provide evidence for the significance of mitochondrial remodeling during this particular developmental stage.

### Methods

Detailed methods are provided in the Online Data Supplement.

### Genetically Engineered Mice

Mice had a mixed background containing 129S, Black-Swiss, and C57Bl/6. Littermates with the appropriate genotypes were used in all experiments. The cre-mediated recombination of loxP sites excises exons encoding key residues of the G1 motif of the GTPase domain of Mfn-1 (exon 4) or Mfn-2 (exon 6).\textsuperscript{34} Cardiac myocyte-specific cre expression was driven by a constitutive Myh6 promoter (active during embryogenesis) or a modified estrogen receptor that was inducible (active on injection with raloxifene). For studies with adult animals, male littermates were used. Animal euthanasia and tissue collection were performed according to protocols approved by the Institutional Animal Care and Use Committee of Boston University School of Medicine.

### Physiological Studies

Adult animals were lightly anesthetized before undergoing echocardiography or hemodynamic analysis, as previously described.\textsuperscript{35} Echo-cardiography of neonates was performed during the conscious state. The neonatal ECGs were obtained as previously described\textsuperscript{35} with the aid of light isoflurane anesthesia delivered through a mouth/nose cone.

### Results

#### Mitofusins Have Redundant Functions in Cardiomyocytes That Are Important for Survival Postnatum

We have previously described mice with conditional deletions of Mfn-1 or Mfn-2 in cardiomyocytes and found that single-knockout mice are viable and fertile and display a normal basal heart function as young adults.\textsuperscript{30,31,36} Given the high similarity between Mfn-1 and Mfn-2, we asked whether they could operate interchangeably in cardiac myocytes, and therefore attempted to generate double-knockout (DKO) animals in both the adult and embryonic hearts. Previously, it has been shown that depletion of mitofusins by the tamoxifen-activated protein modified estrogen receptor (Mer) CreMer leads to progressive decreases in fractional shortening (FS) in adult mice.\textsuperscript{37} In agreement with this study, we found that mitofusin depletion in mice (herein referred to as Mer-DKO) leads to cardiac dysfunction evident at 8 weeks after raloxifene administration and death ensues 3 weeks later (Online Figure I). These observations confirm the notion that Mfn-1 and Mfn-2 operate redundantly and are essential for cardiac function and survival in adulthood.

To examine the roles of Mfn-1 and Mfn-2 in cardiomyocyte specifically during late gestation/early postnatal development of the heart, we used the Myh6-cre transgenic line to selectively disrupt the mfn-1 and mfn-2 loci of mice (genotype: Mfn-1\textsuperscript{pp}; Mfn-2\textsuperscript{pp}; Myh6-cre\textsuperscript{−/−}, herein DKO). Genotyping of 104 animals from multiple litters at their weaning (P21) did not detect any mice with the desired genotype (Online Table I),

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<tr>
<td>PGC-1</td>
<td>peroxisome proliferator-activated receptor γ coactivator 1</td>
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<tr>
<td>Myh</td>
<td>myosin heavy chain</td>
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<tr>
<td>DCM</td>
<td>dilated cardiomyopathy</td>
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<tr>
<td>Mfn</td>
<td>mitofusin</td>
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<tr>
<td>PGC-1</td>
<td>peroxisome proliferator-activated receptor γ coactivator 1</td>
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<tr>
<td>PGCo</td>
<td>coactivator 1</td>
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<td>mtDNA</td>
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\textsuperscript{1} Crucial Role of Mitofusins in Early Postnatal Life
suggesting that DKO mice were not viable and that all four mitofusin alleles were needed for survival either during midgestation or during the first 3 weeks of life. In this analysis, it was found that even one functional allele (regardless of whether this is from Mfn-1 or Mfn-2) is sufficient to compensate for the absence of the other three alleles. Mice harboring one functional mitofusin allele (Mfn-1<sup>F/F</sup>; Mfn-2<sup>+/+</sup>; Myh6-cre<sup>−/−</sup> or Mfn-1<sup>-/−</sup>; Mfn-2<sup>F/F</sup>; Myh6-cre<sup>−/−</sup>; collectively referred to as monoallelic) survive through adulthood, although their hearts exhibit chamber enlargement, decreased FS, and alterations in their elastic properties (Online Figure II).

To test the possibility that DKO mice die during gestation, we genotyped mice at birth (P0). The analysis of a total of 134 pups obtained from 15 litters indicated that DKO mice were born at the expected Mendelian ratios (Online Table II). This rules out the possibility that DKO mice die during gestation, which could potentially lead to their resorption in utero. Furthermore, of the 32 DKO pups identified, all 32 were alive. Therefore, DKO mice survive through the embryonic and fetal stage and are born alive in the expected Mendelian ratios. However, because none of the DKO animals are recovered at P21, we conclude that death occurs at an early postnatal stage and that even a single mitofusin allele in cardiac myocytes is sufficient for survival through this period.

### Cardiomyocyte-Produced Mitofusin Expression Is Elevated During Postnatal Heart Development
To determine the temporal pattern of mitofusin expression in the developing heart, we examined embryonic and early postnatal hearts (ie, embryonic day [E] 15.5 through P07) for the expression of Mfn-1 and Mfn-2. One finding of the transcriptional analysis is that in wild-type (WT) hearts both Mfn-1 and Mfn-2 transcripts are produced at relatively low amounts during gestation (E15.5 and E18.5; Figure 1A and 1B) but undergo more than two-fold upregulation at approximately the time of birth (P0 and P01). Furthermore, significant transcriptional upregulation of mitofusins also is evident at P07 (Figure 1A and 1B). In contrast, in DKO hearts, the levels of Mfn-1 and Mfn-2 were found to be downregulated as early as E15.5 and persisted to these very low levels throughout the remaining gestational and postnatal period examined (Figure 1A and 1B, open bars). Consistent with the low levels of mitofusin expression already at E15.5 in DKO hearts, previous reports have shown that the Myh6 promoter is activated in early myocytes at E9.5 and that genetic recombination using the Myh6-cre construct can be achieved by E12.5.<sup>17,18</sup> The residual levels of mitofusin transcripts detected in DKO extracts likely arise from nonmyocyte cardiac-resident cell populations that do not express the Myh6-cre transgene; however, this analysis reveals that the bulk of mitofusin expression in the postnatal heart is attributable to the cardiomyocyte population (Figure 1A and 1B). Taken together, the analysis of mitofusin expression in WT and DKO hearts indicates that gene ablation takes place during midgestation and provides evidence for transcriptional activation of mitofusin genes during postnatal heart development.

### Myh6-cre–Mediated Ablation of Mitofusins Spares the Heart From Gestational Developmental Abnormalities and Dysfunction
To test whether mitofusin ablation in this model is potentially associated with developmental abnormalities, we examined WT and DKO hearts from E15.5 through P03. This analysis showed that the expected developmental increases in cardiac size that occur in WT hearts also take place in DKO hearts (Figure 1C). To assess a potentially deleterious effect in heart
function that may have arisen in DKO hearts during midgestation or at the time of birth, we used echocardiographic analysis for newborn mice. The morphometric data were obtained by examining conscious mice on the day of their birth (P0). As shown in Figure 2, the evidence is in agreement with the notion that P0 DKO hearts are functionally equivalent to WT hearts (Figure 2A and 2B). More specifically, we identified close similarities between WT and DKO mice in terms of their heart rate, their FS, and a number of other parameters related to the structure and the relaxation/contraction of the heart, including left ventricular (LV) wall thickness, LV volume, and mitral and pulmonic valve flow velocities (Figure 2C–2J). Taken together, the absence of any evidence for heart dysfunction indicates that midgestational ablation of mitofusins, selectively in cardiomyocytes, spares the heart from significant developmental abnormalities and dysfunction through birth.

Morphological Features of P0 Mitochondria

Recently, we have demonstrated that absence of either Mfn-1 or Mfn-2 in adult cardiac myocytes leads to characteristic but disparate mitochondrial morphologies. 30,31 Although loss of Mfn-2 creates larger mitochondria, loss of Mfn-1 has the opposite phenotype and creates smaller mitochondria. To determine the impact of combined Mfn-1 and Mfn-2 ablation in cardiomyocyte ultrastructure, we examined heart sections from P0 hearts by electron microscopy (Online Figure III). Wild-type P0 cardiomyocytes contain tubular and ovoid mitochondria with a characteristic linear arrangement, while DKO mitochondria are more irregular in shape and size. The electron-dense WT mitochondria are dispersed among myofibrils and the P0 myofibrils also exhibit irregular orientations inside the cytoplasm (Online Figure IIIC).
of fully developed hearts. Lipid droplets were frequently observed in other regions of the cytosol. In P0 DKO cardiomyocytes, the cytoplasm appears to be poorly organized, similar to the WT cytoplasm. However, a large proportion of mitochondria assumes a spherical configuration (Online Figure IIIB). In addition to a transition to a spherical state, DKO mitochondria undergo enlargement and diminution, and big mitochondria are found to be intermixed with small mitochondria (Online Figure IIID and IIIE). Tubular mitochondria were also detected, but less frequently (Online Figure IIID). By analysis of the micrographs, we found that the mitochondrial volume density was significantly elevated in DKO (Online Figure IIIF), indicating the presence of higher numbers of mitochondria in these hearts. Nevertheless, P0 DKO mitochondria do not exhibit significant defects in their internal structure and they maintain an electron-dense appearance. Furthermore, the formation of myofibrillar bundles does not appear to be impaired in DKO hearts at P0. Consistently, the volume density of the myofibrillar compartment in these hearts appears to be normal (Online Figure IIIG). In summary, ultramicroscopy of the P0 DKO cardiac myocytes revealed mitochondrial alterations in terms of the structure and number of mitochondria, but a normal assembly of the contractile elements.

**Loss of Mitofusins From Myocytes Leads to Heart Failure by P07**

Both Mfn-1 and Mfn-2 genes are markedly upregulated in the heart between P0 and P07 (Figure 1A). We therefore investigated the cardiac phenotype of DKO mice at P07. As shown in Figure 3, echocardiographic assessment of conscious WT and DKO mice at P07 revealed that the latter underwent a very rapid and severe dilated cardiomyopathy.

**Figure 3.** The heart function at postnatal day 7 (P07) is impaired in double-knockout (DKO) mice. **A,** Motion of the ventricular walls is monitored by echocardiography in wild-type (WT) and DKO mice. Note the poor movement of the walls in the DKO. **B,** Pattern of the inward flow (above baseline) and outward (below baseline) flow of blood through the mitral valve in WT and DKO hearts. **C**—**G,** Morphometric analysis of WT and DKO hearts at P07. The thresholds of significance are indicated for each pairwise comparison. **H,** Representative electrocardiogram (ECG) patterns (lead II configuration) of WT and DKO mice at P07. A single arrow indicates the QRS complex in WT and a double arrow indicates the QRS complex in DKO mice. † indicates P waves. mV indicates millivolts; P07, postnatal day 7; LVPWs, LV posterior wall thickness in systole; LV Vol.d, LV volume in diastole; FS, fractional shortening.
(DCM). The wall motion in DKO hearts was drastically attenuated and the blood flow in the LV chamber was not properly maintained (Figure 3A and 3B). Consistently, significant alterations in different quantitative parameters were detected at P07. As such, the heart rate was lower in DKO hearts and their FS was markedly decreased (Figure 3C and 3D). Furthermore, the internal diameter of the LV was increased, whereas its walls underwent significant thinning (Figure 3E and 3F). The calculated diastolic volume of the LV underwent a nearly three-fold increase in DKO hearts compared with WT, in agreement with the DCM phenotype (Figure 3G). At the same age, DKO had abnormal ECG patterns, where the QRS complex was evidently altered (Figure 3H). Taken together, these characteristics are indicative of the detrimental remodeling and poor pumping function of the DKO heart. The early temporal manifestation of this phenotype (ie, between P01 and P07) suggests that the overlapping actions of Mfn-1 and Mfn-2 in cardiomyocytes are essential during the developmental remodeling of the early postnatal heart.

Rapid Decline in Survival and Cardiac Defects in Mice Lacking Mitofusins

Based on the genotype frequencies (Online Tables I and II), it is apparent that although DKO mice are born alive, they die before their weaning (P21). To define the profile of this premature lethality in greater detail, we performed genotyping on newborn WT and DKO mice and monitored them until weaning and death, respectively (Figure 4A). From the resulting survival curve, it is evident that DKO mice start to die as early as P06 and none survive beyond P16 (Figure 4A, red trace). The majority of the DKO mice die between P08 and P10. This pattern is consistent with the evidence from echocardiography that detected no signs of heart dysfunction at P0 but identified DCM by P07 (Figures 2 and 3). Thus, DKO mice likely undergo premature death because of DCM and heart failure. DKO mice typically display a decline in body condition that becomes evident at approximately P06 and P07 (Figure 4B). The body weight of DKO mice was found to be significantly lower compared with WT littermates at that age (Figure 4D). On autopsy at P04, some DKO hearts displayed readily evident abnormalities (Figure 4C and 4F). At that age, the DKO heart was flaccid and the ventricular walls appeared readily evident abnormalities (Figure 4C and 4F). At that age, the DKO heart was flaccid and the ventricular walls appeared to fold inwardly (Figure 4C, right panel, arrowhead points to the right ventricular wall). This was in contrast to the round and well-shaped WT ventricular walls. In addition, the left atrium appeared to undergo hypertrophy and retained blood in the form of a clot (Figure 4C, arrow).

P04 DKO hearts displayed increases in the right ventricular and LV cavities, as well as substantial thinning of the walls (Figure 4F, arrowhead pointing to the interventricular septum), an observation consistent with the DCM phenotype detected by echocardiography (Figure 3). Furthermore, patches of collagen deposition could be observed in the DKO myocardium, suggestive of the ongoing cardiac fibrosis (Figure 4F, white arrow). The histological analysis also confirmed the atrial enlargement and congestion (Figure 4F, dark arrow). Despite these adaptations, the total heart weight did not differ significantly between WT and DKO mice that underwent autopsy at P04 (results not shown) and P07 (Figure 4E). Consistent with the onset of cardiopathic stimuli in DKO heart, the transcript levels of myocardial stress indicators Anp and Bnp were markedly elevated, whereas the transcript levels of the different myosin heavy chain isoforms and inhibitory troponin isoforms were uniformly downregulated in P07 DKO hearts (Online Table III). Interestingly, there was no evidence for significant activation of apoptosis in P07 DKO hearts (Online Figure IV).

Postnatal Structural Defects in the Mitochondrial and Myofilibrillar Compartments of the Mfn-1/Mfn2 Double-Deficient Cardiomyocytes

Because the absence of mitofusins from cardiac myocytes led to a dramatic decline in heart function within the first week of postnatal life, we examined the ultrastructure of cardiac sections by electron microscopy at P07 to obtain clues regarding the mechanisms that underlie this dysfunction. Imaging of WT myocardium revealed that myocytes at this stage exhibit extensive features that are reminiscent of adult cardiac myocytes, ie, the actin/myosin banding becomes readily evident and the myofilibrillar bundles increase in diameter and assume a linearized orientation and run parallel to the longitudinal axis of the myocyte (Figure 5A). The WT mitochondria at this stage have mostly rectangular or spherical shapes, whereas the tubular mitochondria are less frequent. Coincident with the linear orientation of myofilibrils, P07 mitochondria are arranged parallel to, and stay in close proximity to, the myofilibrils (Figure 5A, 5C, and 5E). In addition to maintaining close apposition to the contractile elements, P07 mitochondria exhibit tight packing, leaving behind little free cytoplasm. Lipid droplets are also present, appearing as gray or white spots within the mitochondrial conglomerate (Figure 5C and 5E).

In P07 DKO hearts, the myocyte structure exhibits marked deviation from that in WT cells. A profound alteration is the high frequency of spherical mitochondria (Figure 5B), a feature consistent with previous reports on double-mitofusin-null models in striated muscle.27,28 The formation of spherical mitochondria was also observed in P0 DKO cardiomyocytes. Although an increased mitochondrial number was also evident at P0 (Online Figure IIIB, IIID, and IIIE), this did not coincide with major aberrations in the structure and organization of the myofilibrils. In P07 DKO samples, the abnormal increase in mitochondrial numbers is still prevalent, but there are regions of cardiomyocytes where the mitochondria appear to displace the myofilibrils (Figure 5B). Related to this disorganized mitochondrial spreading, the distances between parallel myofilaments increase in DKO sections (compare Figure 5C and 5D, arrowheads). These features were also quantitatively important. Using the grid method to determine how the cell volume is allocated into different compartments, we detected a significant increase in mitochondrial density, which is associated with reduced myofilibrillar density in DKO hearts (Figure 5G and 5H). In addition to the apparent increase in mitochondrial number, a characteristic dysregulation in mitochondrial diameter was observed, whereby unusually large mitochondria coexist with petite mitochondria (Figure 5F, arrow).
The mitofusin-null mitochondria presented varying degrees of cristae defects and, as a consequence, the structure of the inner membrane was modestly (Figure 6B and 6B’) or severely (Figure 6C and 6C’) impaired. This pattern of inner membrane dysregulation, as detected in DKO mitochondria, is in sharp contrast to the dense cristae packing that is observed in WT mitochondria at P07 (Figure 6A) or the WT and DKO mitochondria at P0 (Online Figure III). Furthermore, the loss of cristae and the decondensation of the matrix appeared to occur similarly among the different sizes of mitofusin-null mitochondria, ie, loss of the inner structure happens regardless of whether the mitochondria have small, intermediate, or large diameters (compare Figure 6A with Figure 6C).

The mitochondrial boundaries in WT samples are smooth and their shape is defined by subtle changes in membrane curvature (Figure 6D and 6D’). In DKO samples, however, the boundaries of some mitochondria appear to undergo dramatic changes in curvature and the membranes create finger-like projections (Figure 6E, 6E’, 6G, 6H, and 6I). Furthermore, a single DKO mitochondrion is capable of forming one, two, or three such projections (more typically just one, eg, Figure 6G, asterisk), and these projections appear...
to push into the membranes of an adjacent mitochondrion, creating an invagination. Consequently, short chains of mitochondria are created where one mitofusin-null mitochondrion extends a projection towards its neighbor (Figure 6H). In other situations, the surfaces of adjacent mitochondria undergo even more extreme curvature to form Ω (Ω-like) membrane configurations (Figure 6G, hash). The membrane protrusions appear to preferentially form on DKO mitochondria, which exhibit extensive matrix decondensation (Figure 6E, 6G, 6H, and 6I, asterisks). Nevertheless, not all mitochondria with apparent structural defects form these protrusions (Figure 6F, dagger), indicating that the abnormalities in the internal structure of mitochondria (reflecting mitochondrial dysfunction) precede the formation of these membrane deformities.

Finally, DKO sections contained small, vesicle-like organelles that appeared to be voids surrounded by a single membrane (Figure 6F, arrows). These vacuoles do not represent lipid droplets because they are smaller in size and they do not exhibit the gray coloration that is typically seen inside the droplet.

**Altered Expression of Mitochondrial Biogenesis and Mitophagy Markers in Mitofusin-Deficient Hearts at P07**

Forming mitochondria involves the incorporation of de novo synthesized proteins into preexisting mitochondria that replicate their genome and subsequently segregate into daughter mitochondria. However, formation of mitochondria could be counteracted by whole-organelle elimination through mitochondria-directed autophagy (mitophagy). Thus, we analyzed whether these processes were altered, given the severe mitochondrial abnormalities and the appearance of defective mitochondria in DKO hearts at P07. Biosynthesis of new
mitochondrial components is controlled by a group of transcription factors (nuclear respirator factor-1 [Nrf-1], estrogen receptor-related-α [Err-α], and transcription factor α mitochondrial [Tfam]) and the peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1α). As shown in Online Figure V, the transcript levels of these factors in DKO hearts exhibit little or no changes at P0 or P03. Nevertheless, the majority of these genes become significantly suppressed in P07 DKO hearts (Figure 7A). Next, we examined the transcription of genes important in the autophagy pathway. As shown in Online Figure VI, we analyzed genes such as Ulk-1 and Ulk-2 (encoding upstream kinases regulating the initiation of autophagy), Atg-5 and Atg-12 (effectors of autophagosome formation through the Atg-12 conjugation system), LC3-b (component of the Atg-8/LC3 conjugation system), and Atg-6/Beclin-1 (component of the lipid kinase complex). At P0 and P03, these genes did not change significantly in DKO hearts, but at P07 their expression (as well as that of Bnip-3, a candidate mediator of mitophagy) was clearly dysregulated (Online Figure VIC). Dysregulation also was evident at the level of protein turnover, because the levels of p62 and LC3-II were found to be significantly elevated in P07 DKO hearts (Figure 7B–7E). In contrast, the levels of the lysosomal marker cathepsin-D were unaltered (Figure 7D–7E). Taken together, these data suggest that in the present model of heart failure, the activity of the mitochondrial biosynthetic machinery is decreased and at the same time the mitochondrial elimination pathway appears to be halted.

Loss of mtDNA and an Impaired Expression of Mitochondrially Encoded Genes in the Mitofusin-Deficient Heart

The transcriptional repression of mitochondrial and metabolic genes is frequently observed in the context of heart failure. We examined the transcription of genes encoding essential components of the mitochondrial electron transport chain in P0, P03, and P07 DKO hearts. As shown in Online Figure VII, many of the examined genes displayed a progressive downregulation in DKO hearts. Although few changes were observed in their expression at P0, an intermediate reduction was evident at P03 and more severe repression was detected at P07. Similarly, a number of genes encoding enzymes important in glycolysis and fatty acid oxidation pathways were transcriptionally repressed.
in a progressive manner (Online Figure VIII). Nd-5 and Cyt-b that are encoded by the mitochondrial genome were the only genes to be downregulated already by P0 in DKO hearts (Online Figure VII), thus preceding the onset of severe heart failure. Consistent with these early reductions, Nd-5 and Cyt-b transcripts were robustly downregulated in P07 DKO hearts (Figure 8A and 8B).

Previous work identified mitofusins to be important determinants of mtDNA maintenance in skeletal muscle.27 In light of the early reductions in the expression of Nd-5 and Cyt-b, we quantified the levels mitochondrial (mt) DNA in heart extracts from WT and DKO mice using real-time polymerase chain reaction. Regarding WT samples, these analyses showed that in the normally developing postnatal heart there is a more than two-fold increase in the levels of mtDNA between P03 and P07 hearts, consistent with the ongoing mitochondrial biogenesis (Figure 8C, filled bars). In DKO samples, however, the amount of mtDNA was slightly but significantly decreased at P03 and exhibited substantially more severe reductions by P07 (Figure 8C, empty bars). Thus, the reduction in the mtDNA of postnatal DKO hearts occurs in a progressive manner and generally coincides with or precedes the onset of heart failure. Consistent with mtDNA decrease, the protein levels of subunit 1 of the cytochrome c oxidase (COX-1, which is a mitochondrially encoded polypeptide) are severely reduced at P07 (Figure 8D). However, the protein levels of subunit α, a component of the F$_\alpha$-ATPase, which is encoded by the nuclear genome, displayed little or no reduction in P07 DKO hearts (Figure 8D). Consistent with a reduction in COX-1 protein levels, the in situ enzymatic activity of cytochrome c oxidase was markedly reduced in DKO heart sections, as indicated by the paler staining with DAB in cytochrome c oxidase/succinate dehydrogenase staining assays (Figure 8E).

Mitochondria from WT and DKO hearts at P06 and P07 were isolated and assessed for their capacity to produce ATP. In the course of mitochondrial isolation, we found that the mitochondrial protein yield is significantly decreased in DKO preparations (Online Figure IXA) despite the observed increase in mitochondrial number by electron microscopy (Figure 5G). This indicates that the number of extractable mitochondria is markedly lower in P07 DKO hearts, suggesting that the defective mitochondria observed by EM are structurally unstable or that the protein content per mitochondrion is reduced in the DKO. Given this baseline difference, we adjusted the dilution in the different suspensions to contain equal amounts of mitochondrial protein. Assessment of the citrate synthase activity in the extracted mitochondrial samples showed the mitochondrial content to be equal in the different preparations after this adjustment (Online Figure IXB). Next, mitochondria were assessed for their ability to synthesize ATP in the presence of different substrates and after the addition of exogenous ADP. Surprisingly, the maximum rates of ATP synthesis did not differ significantly between WT and DKO mitochondrial preparations (Online Figure IXC–F). In addition, measuring total ATP content in whole hearts did not detect significant decreases in P07 DKO hearts, suggesting that the pool of cardiac ATP is maintained even in the context of severe cardiac dysfunction (Online Figure IXG). Taken together, these findings indicate that despite a pervasive mitochondrial deficit, the DKO hearts contain sufficient functionally competent mitochondria that allow the heart to maintain normal ATP levels at this time point (P07).

**Discussion**

The present study highlights the importance of mitofusins (Mfn-1 and Mfn-2) in the early postnatal heart. Mitofusins are mechanoenzymes that support the mitochondrial continuum in cells by catalyzing the integration of the outer mitochondrial membrane, and they are abundantly present in human and other mammalian hearts.41–44 The particularly high expression of mitofusins in this very oxidative organ is not surprising because
the heart exhibits an extremely high mitochondrial content, eg, 25% in humans and 38% in mice. Using a cell-specific gene knockout approach, we find that the combined inactivation of Mfn-1 and Mfn-2 from cardiac myocytes results in a rapidly evolving, early postnatal phenotype that is characterized by major abnormalities in mitochondrial structure, DCM, and premature death during the second week of life.

The gross morphology of DKO hearts at prenatal developmental stages (eg, E15.5 and E18.5) does not differ significantly from WT hearts. Furthermore, there are no structural or functional differences between WT and DKO hearts at P0, although changes in mitochondrial morphology are already evident. At P0, DKO mitofusin hearts assume spherical shapes and are heterogeneous in size. Work with single knockouts showed that cardiac myocytes lacking Mfn-1 have mitochondria with diminished diameters, whereas myocytes that lack Mfn-2 have mitochondria with increased diameters. In what appears to be a combination of these phenotypes, we find here that cardiac myocytes lacking Mfn-1 and Mfn-2 contain both enlarged and small mitochondria at P0. Another characteristic of the P0 DKO hearts is the increase in mitochondrial volume density. Increased mitochondrial volume density is also detected in skeletal muscle after dual mitofusin deficiency. These findings indicate that the absence of mitofusins favors the expansion of mitochondrial numbers. Along these lines, the increase in mitochondrial numbers may represent an adaptation of the muscle to compensate for dysfunctional mitochondria. However, it is important to note that there are no apparent alterations in the internal structure of P0 DKO mitochondria and no matrix decondensation is observed at this time point. Additionally, myofibril volume density is unaltered, consistent with the finding that heart function of DKO hearts is normal at P0. Thus, it appears that although P0 DKO hearts have preexisting abnormalities in mitochondrial structure and number, they maintain short-term functionality before being exposed to the postnatal rigors.

In the developmental window between P0 and P07, the transcripts encoding Mfn-1 and Mfn-2 are markedly upregulated.

Figure 8. Defects in the maintenance and expression of mitochondrial DNA (mtDNA) in double-knockout (DKO) hearts. A and B, Transcript levels of genes located in the mtDNA. C, Temporal pattern of mtDNA expansion in wild-type (WT) and DKO hearts. The quantitation was performed in real time according to the \( \Delta \Delta Ct \) method, where the levels of subunit 1 of the cytochrome c oxidase (COX-I) in WT postnatal day 3 (P03) hearts were used as a reference (value=1). D, Western blot analysis assessing the abundance of COX-I and F1, subunit-α. E, Dual COX/SDH staining in situ in freshly cut heart sections. Arrows indicate cardiac myocytes where SDH-specific staining predominates. These fields are magnified ×40 and the hearts were from P09 mice. Nd-5 indicates NADH dehydrogenase subunit-5; Cyt-b, cytochrome-b (subunit of the bc₁ complex).
in WT but not in DKO hearts. At the P07 time point, DKO hearts exhibit hallmarks of DCM, including decrease in FS, increase in LV diameters, thinning of ventricular wall, and abnormal ECG pattern. These features are also accompanied by poor body condition, atrial congestion, focal collagen deposition, upregulation of the stress markers Anp and Bnp, and a decline in survival, and no live DKO animals are recovered beyond P16.

The DKO failing hearts present some striking and unique characteristics in cardiac myocyte and mitochondrial ultrastructure. As mentioned, mitochondria in double-deficient hearts appear to undergo unrestrained expansion and occupy a significantly larger portion of the cellular volume. This is accompanied by defects in the organization of the contractile apparatus and a state of structural disarray specifically in PO7 DKO hearts. In addition to appearing as exceedingly small or large spherical organelles, PO7 DKO mitochondria have further abnormalities, such as matrix decondensation and loss of cristae structure. In PO7 DKO cells, many dilated/swollen mitochondria are evident. They coexist with unusually small round organelles with little or no inner membrane structure. One possibility is that these voids result from petite mitochondria that underwent extreme degeneration and cast off their internal or external membrane, or they could represent remnants of late-stage dysfunctional mitochondria that collapse into multiple fragments. Apart from its important role in biochemical pathways and energy conversion, the inner mitochondrial membrane is an integral and quantitatively important part of the myocyte. Therefore, its extreme degeneration/loss that is detected in many PO7 DKO mitochondria could be, by itself, an important element contributing to cell and organ dysfunction. Another interesting microscopic observation is that the boundaries between adjacent PO7 DKO mitochondria are sometimes found to rearrange into extreme curvatures and form finger-like protrusions. Chains of mitochondria exhibiting these characteristics are uniquely present in DKO myocytes. We currently interpret the aforementioned projections to reflect abortive fusion attempts of mitofusin-null mitochondria. It can be suggested that in the absence of mitofusins, other unknown factor(s) are recruited or become activated as an utmost effort to induce mitochondrial fusion or tethering.

Soon after birth, the heart undergoes rapid increases in mitochondrial content and respiratory function.46,47 These adaptations are necessary for postnatal cardiac growth and for effective support of the pulmonary and systemic circulation. To coordinate these changes, the heart relies on a specialized transcriptional circuit, into which the coactivator Pgc-1 operates as a central switch.48,49 Consistent with the important role of Pgc-1 in postnatal heart growth, mice with total absence of Pgc-1 (ie, Pgc-1<sup>−/−</sup> and Pgc-1<sup>Δ189/190</sup>) are born in the expected Mendelian ratios but die soon after birth.4 Similarly, we demonstrated here that mice lacking Mfn-1 and Mfn-2 in cardiac myocytes survive embryonic development and start to die largely during the second week of life. Moreover, we find that the expression of Mfn-1 and Mfn-2 in WT hearts is induced from birth and onward, a period coinciding with the activation of Pgc-1 in the heart.50 The similarities in the temporal pattern of lethality between the two knockout models (ie, Pgc-1 null versus mitofusin null) may imply a functional relationship between Pgc-1 and mitofusins. Mitofusins are demonstrated to be transcriptionally activated by Pgc-1 in muscle cells.51–53 Thus, it can be suggested that mitofusins are putative downstream effectors in the mitochondrial biogenic program that is activated by Pgc-1 in postnatal cardiac myocytes.

Apart from the early postnatal phenotype, we also provide evidence for a necessary role of mitofusins in adult mice. This is in agreement with an independent study focusing on the role of mitofusins in the heart.29 Using the MerCreMer, the earlier study reported that the disruption of mitofusins in adult hearts leads to mitochondrial fragmentation and gradual deterioration of heart function.29 Consistent with these findings, using the same Cre-expressing strain, we show reductions in FS and progressive lethality in mitofusin double-deficient animals (Online Figure 1). Chen et al29 also reported that cardiac ablation of Mfn-1 and Mfn-2 during embryonic development causes lethality at approximately E10.5. Interestingly, this differs from the early postnatal lethal phenotype that we detect here. A possible explanation for obtaining dissimilar results could be because different promoters were used to induce Cre expression in the 2 studies. The study by Chen et al used Nkx-2.5cre known to be active in cardiac progenitor cells and therefore has the potential to recombine loxP sites in all cardiac cells, including cardiomyocytes and endothelial, smooth muscle, and mesenchymal cells of the heart.54 By contrast, the expression of the Myh6-cre transgene used in the current study is restricted only to cardiac myocytes.55,56 Thus, one possibility is that the embryonic lethality observed by the earlier study is attributable to the inactivation of mitofusins in the nonmyocyte compartment of the heart. In this regard, mitofusins have been shown to be important for vascular endothelial cell differentiation, survival, and function.56 Apart from differences in cell specificity, there are differences in the timing of mitofusin inactivation between the studies. Nkx-2.5cre begins gene excision at E7.5,57 whereas Myh6cre begins excision at E9.5. Thus, an alternative possibility is that early inactivation of mitofusins by Nkx-2.5cre elicits a severe effect in the developing heart because this period encompasses important transitions such as heart tube formation and looping. However, the utilization of Myh6-cre becomes useful in interpreting the role of mitofusins, specifically in the cardiac myocyte, during midgestation and early postnatal development of the heart.

The evidence from this study suggests that mitofusins, and by extension mitochondrial fusion, are important for the formation of functional mitochondria in cardiac myocytes during the period from P0 to P07. Conceptually, mitochondrial fusion is beneficial during this period of unprecedented high energetic demand because it allows the diffusion of metabolites in different compartments of the mitochondrial network and promotes more efficient energy conversion.58 In this regard, it is demonstrated that fusion is activated during mild intracellular stress or nutrient deprivation to enhance mitochondrial ATP production.59,60 Fusion is also suggested to be important for genome exchange between mitochondria and to facilitate mtDNA complementation.61 Consistent with a relationship between mitochondrial fusion and mitochondrial genome maintenance, previous work has established a link between mitofusins and mtDNA copy number in skeletal muscle.27 In agreement, we found that loss of mitofusin activity is accompanied by reductions in mtDNA. This reduction in mtDNA appears to preexist cardiac dysfunction, although it is likely to be further exacerbated as the hearts begin to fail.
It is also possible that mitofusins are important during the P0 to P07 developmental stage through mechanisms that do not involve fusion and content exchange between mitochondria. One possibility is that mitofusins allow actively proliferating mitochondria to establish connections with the cytoskeleton and correctly position themselves into the myofibrillar blueprint during cardiomyocyte postnatal development. Along these lines, molecular defects in the assembly of the cytoskeleton in cardiac myocytes are common causes of DCM in mice and in humans.62–64 We found that the loss of mitofusins is accompanied by rapid and lethal DCM, which may indicate an underlying defect in cytoskeletal function. The possible interaction of mitofusins with the cytoskeleton has been recently addressed in neurons, where Mfn-1 and Mfn-2 were shown to interact with adaptor proteins that tether the outer mitochondrial membrane to microtubules.65 Regarding striated muscle, the interaction of mitochondria with intermediate filaments is noted and genetic defects in the assembly of intermediate filaments are sometimes accompanied by dramatic changes in mitochondrial morphology in hearts.66,67 Therefore, there is emerging evidence to support a potential role of mitofusins at the mitochondrial/cytoskeleton interface and perturbations in this association may have contributed to the developmental abnormalities that we identify here.

In both the P0 and P07 DKO hearts, there is an overpopulation of mitochondria, indicating altered mitochondrial biogenesis. This increase in numbers could reflect a compensatory response to an underlying mitochondrial dysfunction. Nevertheless, there is no significant evidence for the activation of biosynthetic pathways regulating the formation of new mitochondrial components. This is supported by the finding that in DKO hearts the Pgc-1 transcriptional pathway is either unchanged or suppressed, as is the transcription of many genes that encode key mitochondrial proteins. Increased mitochondrial numbers in DKO hearts may originate from a defect in the elimination of excessive mitochondria through mitophagy and recent work indicates that Mfn2 is important for the autophagy pathway.68,69 However, in the present model, we identify significant evidence of altered autophagy that coincides with the onset of heart failure, but not before. This evidence includes aberrant gene expression of key autophagy mediators (ie, Ulk-2, Atg-5, Atg-12, Beclin1, and Bnip-3) and accumulation of p62 and LC3-II proteins at P07. Thus, heart failure at P07 appears to be associated with a diminished ability to synthesize new mitochondrial components as well as a reduction in the ability to remove mitochondria. Given this reciprocal relationship, it could be suggested that these alterations (ie, decreased autophagy and a concomitant decrease in de novo biogenesis) may be part of a global mechanism that operates to maintain or prolong cardiac homeostasis in the stressed heart. It is widely appreciated that the heart has a remarkable ability to maintain stable levels of ATP despite varying workloads.70 Here, we found that despite the drastic mitochondrial deficit of P07 DKO hearts, isolated mitochondria were capable of producing maximum amounts of ATP and the total levels of ATP in these hearts did not change. Thus, it is tempting to speculate that ATP levels are maintained in the face of mitofusin deficiency by mechanisms that globally adjust rates of mitochondrial biogenesis and mitophagy as the heart begins to fail.

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Disclosures

None.

References


What Is Known?

• Mitofusins are novel regulators of mitochondrial structure and function in the heart.
• The hearts of newly born mammals exhibit rapid increases in mitochondrial biomass.
• The surge in mitochondrial biogenesis coupled with mitochondrial maturation is thought to be key for the physiological growth of the postnatal heart.

What New Information Does This Article Contribute?

• Mitofusins are pivotal moderators of acute mitochondrial biogenesis and maturation that are activated in the early postnatal heart.

Birth triggers mitochondrial biogenesis and maturation in heart, but the molecular mechanisms that control this developmental stage are poorly understood. In the present study, we addressed whether mitofusins are important during the acute phase of mitochondrial remodeling that occurs in hearts after birth. We found that inactivating mitofusins profoundly alters the number, morphology, and distribution patterns of mitochondria in cardiac myocytes. Although mitofusin deficiency is benign for heart function up until birth, the mitofusin-deficient mitochondria undergo marked degeneration and hearts exhibit signs of dysfunction as early as postnatal day 4, followed by premature death. This study provides a link between mitofusins and the metabolic reprogramming of the newborn heart and shows that mitofusins function at the interface between deregulated mitochondrial biogenesis and cardiac dysfunction.

Novelty and Significance
Mitofusins 1 and 2 Are Essential for Postnatal Metabolic Remodeling in Heart
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Detailed methods

*Generation of cardiomyocyte specific double-deficient mice for Mfn-1 and Mfn-2*
Mice with germ line insertion of loxP sites flanking exons 4 and 6 of Mfn-1 and Mfn-2 respectively were described \(^1\) (genetic background 129S/Black Swiss). These mice were cross-bred with mice carrying the cardiomyocyte-specific myosin heavy chain 6 (Myh6)-cre transgene \(^2\) (back-crossed in-house to the C57Bl/6 for more than 6 generations) or the cardiomyocyte-specific Myh6 transgene encoding the modified estrogen receptor (Mer)-cre-Mer fusion protein (the Jackson Laboratory, stock number 005650, background C57Bl/6) in the animal facility at Boston University School of Medicine. The genotype of animals was determined by PCR, using DNA extracted from tail biopsies as template, with primers and conditions available at the Mutant Mouse Regional Resource Center (UC Davis) and the Jackson Laboratory. We refer to mice with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{F/F}\);Myh6-cre\(^+/\) as DKO, mice with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{+/+}\);Myh6-cre\(^+/\) and Mfn-1\(^{+/+}\);Mfn-2\(^{F/F}\);Myh6-cre\(^+/\) as monoallelic and mice with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{F/F}\);Mer-cre\(^+/\) as Mer-DKO. Littermates of DKO mice without cre are referred to as WT. Additionally, cre-only mice (i.e. Mfn-1\(^{+/+}\);Mfn-2\(^{+/+}\);Myh6-cre\(^+/\)) were also used as WT. The temporal activation of Mer-Cre-Mer in cardiomyocytes of adult mice was induced by raloxifen (Sigma, catalog number R1402) dissolved in DMSO, diluted 1:10 (v/v) in warm saline and injected intraperitoneally at daily doses of 40 mg/kg for five days. In these experiments, ‘Control’ denotes animals with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{F/F}\);Mer-cre\(^{-/-}\) or Mfn-1\(^{+/+}\);Mfn-2\(^{+/+}\);Mer-cre\(^{+/+}\) treated with the same dose of raloxifen. Animal handling and tissue collection were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Boston University School of Medicine.

*Timed pregnancies and analysis of embryos*
Female mice with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{F/F}\);Myh6-cre\(^{-/-}\) were housed with males with the genotype Mfn-1\(^{F/F}\);Mfn-2\(^{+/+}\);Myh6-cre\(^{-/-}\) and vaginal plugs were checked every morning. The day at which a plug was observed was termed E0.5 and pregnant females were sacrificed at E15.5 and E18.5 to collect embryos. The embryonic hearts were dissected in PBS and photographed with a stereomicroscope (Olympus SZX16). Embryo tail DNA was used for standard genotyping. Frozen embryonic hearts were homogenized with Tissue Lyzer in RLT buffer (Qiagen), total RNA was purified and 850 ng were reverse-transcribed into cDNA. These procedures were performed with columns and reagents from Qiagen (RNaseasy and Quantitect reverse transcription kits).

*Echocardiographic analysis of P0 and P07 hearts*
Breeding pairs where parents had the genotypes described in the previous section were monitored daily for newborn pups. The day of birth was considered as postnatal (P)0. At that day, conscious pups were subjected to echocardiography using the Vevo770 Imaging system (Visualsonics) equipped with scan head RMV 704, 40 MHz The ambient temperature was maintained with a heating pad and lamps to avoid decreases in body temperature and bradycardia. Parasternal short axis views were used to obtain M-mode echocardiograms (display window 4000 ms). We performed this imaging in triplicates to measure heart rates and left
ventricle (LV) dimensions of the anterior wall, the posterior wall and the internal diameter (LVAW, LVPW and LVID respectively) in diastole and systole. The fractional shortening (FS) and the diastolic ventricular volume (LV Vol. d) were calculated from M-mode measurements with Visualsonics built-in formulas. Additionally, the Doppler transducer was positioned in the ascending aorta to measure aortic valve peak velocity, distally to the pulmonic valve to measure the pulmonic artery flow velocity and in the left ventricle (B-mode, apical view) to measure mitral valve E and A velocities and the E/A ratio. This procedure was carried out essentially in the same way to perform echocardiographic analysis in a different group of P07 pups.

Cardiac physiology in adult animals
Echocardiography on isoflurane anesthetized adult male mice weighting 25-40 g (mean 31.8 ± 3.8) was performed as previously described. The FS was calculated from M-mode echocardiograms and the LV Vol. d was calculated according to Simpson’s equation using measurements from one long axis view and four short axis views taken at different levels between the base and the apex. Imaging, measurements and calculations were performed in triplicates for each heart. Measurements of mitral valve velocities were taken using waveforms created by placing the Doppler transducer in the LV near the valve, while imaging at the four-chamber apical view. Preparation of adult mice weighting 32-40 g (mean 34.9 ± 2.6 g) for cardiac hemodynamic assessment with a 1.4 french (F) Mikro-Tip catheter transducer (model SPR839, Millar Instruments) was performed as previously described. Following insertion of the catheter tip into the LV and stabilization for about 15 minutes, pressure-volume (PV) loops were recorded at steady-state and at different preloads created by occluding the inferior vena cava. The ambient temperature was kept constant and the temperature of animals was closely monitored (body temperature 36.7 ± 0.6 ºC). From steady-state loops the cardiac output (CO) and relaxation time constant (tau, determined by the Glantz) method were calculated using PVAN software (Millar Instruments). The preload-independent index of cardiac muscle contractility \( E_{es} \) (end systolic elastance) was calculated from occlusion loops by fitting end systolic pressure and volume points into a linear fit and measuring the slope. The volume recordings were corrected for parallel conductance with a hypertonic saline (15% w/v NaCl) bolus injection at the end of the experiments. Corrected conductance units were converted to \( \mu l \) using a standard curve created with a blood volume calibration cuvette.

Determination of total ATP levels in postnatal hearts
Hearts were rapidly collected from P07 WT and DKO isoflurane-anesthetized pups, rinsed and directly placed into liquid nitrogen to freeze immediately. The frozen heart was then placed in 300 \( \mu l \) ice-cold perchloric acid (0.4 M) and was disrupted/homogenized with a mill mixer at 4 ºC. The homogenate was kept on ice for 30 minutes and subsequently spun at 18,000×g at 4 ºC for 10 min. The supernatant was collected and neutralized by the addition of 4 M \( K_2 CO_3 \) and the solution was stored at -80 ºC for 2 hours. The remaining tissue pellet was flash frozen and its mass was determined with a microbalance. The tubes recovered from -80 ºC were centrifuged from 18,000×g at 4 ºC for 10 min and samples from the supernatant were diluted in ATP-free water and used to determine the ATP content using the Enliten assay system (Promega). The ATP content was measured in a luminometer (Lumat, Berthold) and an integration time of 10 seconds. A standard curve with known concentrations of ATP was constructed in parallel reactions and used convert relative light units (RLU) into [ATP] in the sample.
Mitochondrial isolation, assessment of mitochondrial ATP production and citrate synthase activity

All chemicals were purchased from Sigma unless otherwise noted and the entire extraction procedure was performed at 4 °C using homogenization buffer (HB) which contained (in mM): 50 Sucrose, 200 Mannitol, 5 KH$_2$PO$_4$, 5 MOPS, 2 Taurine, 1 EGTA and 1 % (w/v) fatty acid free BSA. The final pH was adjusted to 7.4 with 1 M KOH. A suspension buffer (SB) that lacked EGTA and BSA was prepared and stored on ice. Hearts of P06-P07 pups were collected in ice-cold HB, the atria were removed and the ventricles were finely minced. All pieces resulting from ventricles of three or four mice in the same genotype group were combined and disrupted in HB in a glass homogenizer by hand. The crude homogenate was pelleted by centrifugation at 500×g at 4 °C for 5 min and the resulting supernatant was collected and subjected to a second centrifugation at 10000×g at 4 °C for 5 min. The mitochondria-rich pellet was washed once with 1 ml SB and was finally suspended in a small volume of SB. Protein concentration in the resulting mitochondrial suspension was determined with the bicinchoninic assay (Pierce).

The assay for ATP synthesis was performed as previously described 5. The reaction buffer (A) had pH 7.4 and contained (in mM): 150 KCl, 25 Tris-HCl, 10 KH$_2$PO$_4$, 2 EDTA, 0.1 M MgCl$_2$ and 0.1% (w/v) BSA. Each reaction was setup in buffer A by the addition of 1 mM pyruvate, 1 mM malate, 5 % (v/v) luciferase reagent (Promega), 0.15 mM di-adenosine pentaphosphate (inhibits adenylate cyclase) and 30 μg mitochondria. In other reactions, 5 mM succinate was used as substrate and 40 μM rotenone were used for complex I inhibition. ADP was added at a final concentration of 0.1 mM to initiate ATP synthesis and the luminescence signal was recorded every 15 seconds (Lumat, Berthold) for a total duration of three minutes. Subsequently, FCCP was added in the reaction vial (final concentration 12.5 μM) and luminescence was monitored for another 90 seconds. In separate vials, 2 μg/ml oligomycin was added to determine non-mitochondrial rates of ATP synthesis in the same conditions. Luminescence recordings (relative light units, RLU) were plotted against time and the linear part of the curve was used to calculate the maximum rate of ATP synthesis. The rate of ATP synthesis attributable to mitochondria was calculated by subtracting the rate of ATP synthesis in the presence of oligomycin.

Citrate synthase (CS) activity was determined in isolated mitochondria using a commercially available kit (Sigma) according to manufacturer’s specifications. Briefly, isolated mitochondria were lysed to liberate CS and 20 μl of the lysate were used in 1 ml reactions containing 300 μM acetyl-CoA, 500 μM oxaloacetate 100 μM dithiobis nitrobenzoic acid (DTNB). The conversion of DTNB into thionitrobenzoic acid (TNB) reflects the formation of the thioester bond and was monitored spectrophotometrically at 412 nm (UV-1601PC, Shimadzu) for 90 seconds. The slope of the curve (Δabsorbance) was used to calculate the CS activity in the reaction mixture using an extinction coefficient of 13.6 for TNB.

Tissue processing for electron microscopy

Rod-shaped pieces of ventricle myocardium were collected from hearts of P0 or P07 pups in ice-cold phosphate buffer (0.15 M, pH 7.4) and were placed in fixative overnight at 4 °C. Fixative components were purchased from Electron Microscopy Sciences (catalog number 15720) and their final concentrations were (in w/v): 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH adjusted to 7.4. Rods were washed from fixative, dehydrated embedded in epon plastic, semi- and ultra-thin sectioned and stained as previously described 3. Myocardial micrographs were collected using Philips CM12 or Tecnai G$^2$ transmission electron microscopes.
at 80 kV and at magnifications ×2900-3000 or ×6300-6800. The mitochondrial and myofibrillar volume densities were calculated by superimposing a grid of 9×11 points on digital micrographs using ImageJ software.

**Quantification of mtDNA by real-time PCR**
Total nucleic acids were extracted from P03 and P07 hearts using the Tissue Lyzer disruption system. The homogenate was treated with 1.6 mg/ml RNase A (Qiagen) for 10 minutes at room temperature and then with proteinase K for 10 minutes at 55 °C. The digests were centrifuged at 10000×g at room temperature for 3 minutes and the cleared supernatant was mixed with ethanol and loaded on DNeasy columns to purify total heart DNA according to manufacturer’s protocol (Qiagen). The DNA concentration was quantified spectrophotometrically (Nanodrop). Twenty ng of DNA were used as template in Taqman-based quantitative real-time PCR. The gene-specific assay for mtDNA was Mm04225243_g1 for murine cytochrome-c oxidase subunit 1 (COX-I) and Mm99999915_g1 for glyceraldehyde phosphate dehydrogenase (GAPDH, nuclear gene), from Life Technologies. The amounts of COX-I relatively to GAPDH were calculated according to the ΔCt method and were further normalized (ΔΔCt) to P03 WT values to obtain a measure of mtDNA in the different samples.

**Quantification of RNA by real-time PCR**
Total RNA from P0, P01, P03 and P07 hearts was isolated according to procedures described earlier for embryonic hearts. The RNA content was quantified in the different samples and adjusted so that 850 ng RNA were present as template in reverse-transcription reactions with Quantitect (Qiagen). Additionally, control reactions were performed where the reverse transcriptase was omitted. cDNA was diluted 60-fold and used in Taqman-based real time PCR. Gene-specific assays for mouse Mfn-1 and Mfn-2 were purchased from Life Technologies (Mm00612599_m1 and Mm00500120_m1, respectively). Additionally, Rpl-30 (subunit L30 of the 60S ribosome) was used as a house-keeping gene as its expression was tightly similar among the different developmental stages. The amount of Mfn-1 and Mfn-2 cDNA in the different samples was expressed relatively to Rpl-30 and relatively to E15.5 WT hearts according to the ΔΔCt method. In a different group of samples (P0, P03 and P07 hearts) quantitative real time PCR was performed using the SYBR green reagent as previously described. Primers were designed using Primer3 and evaluated for gene specificity using basic local alignment search tool (BLAST).

**In situ analysis of COX and SDH activities and other histology**
Eight-micron-thick, freshly-cut frozen sections were collected from P07 hearts, placed on glass slides and stained to determine cytochrome c oxidase (COX) and succinate dehydrogenase (SDH) activities as described with modifications. Briefly, the COX staining buffer had pH 7.4 and contained the following: 45 mM Phosphate buffer, 1 mg/ml cytochrome c, 1 mg/ml diaminobenzidine (DAB) and 0.2 mg/ml catalase from Aspergillus niger. The SDH staining buffer had pH 7.4 and contained the following: 45 mM Tris-HCl, 1 mg/ml nitro blue tetrazolium (NBT), 0.02 mg/ml phenazine methosulfate (PMS) and 1 mg/ml sodium succinate. All chemicals were from Sigma except PMS which was purchased from MP Biomedicals and NBT was from Fisher. COX staining buffer was applied to sections for 3 minutes at room temperature, followed by rinses in PBS. SDH buffer was applied for 5 minutes at room temperature to the same section which was rinsed with PBS and mounted in aqueous mounting medium (Dako). The specificity
of the COX reaction was confirmed by adding 0.01 M KCN in the buffer before applying on sections. Slides were photographed at magnification ×400. Masson trichrome staining of paraffin embedded heart sections used reagents from Sigma (HT15).

**Western blotting and caspase-3/7 activity**

Protein extraction, quantification, electrophoretic separation (20 μg protein/lane) and electrophoretic separation and electroblotting onto PVDF membranes were performed according to standard procedures as previously described 3, 7. For the present studies the following primary antibodies were used: anti-Mfn-1 (NeuroMabs, clone N111/24), anti-Mfn-2 (N-terminus-directed, Sigma, M6319), anti-Drp-1 (BD Transduction, 611112), anti-F1-ATPase subunit α (Invitrogen, clone 7H10), GAPDH (Cell Signaling, clone 14C10), anti-COX-I (Invitrogen, clone 1D6E1A8), anti-p62 (Cell Signaling, 5114), anti-LC3 (Cell signaling, 2775), anti-Cathepsin-D (SCBT, sc-6494), anti-Bax (Cell signaling, 2772), anti-Bcl-2 (BD Transduction, 610539), anti-Bcl-XL (Cell signaling, clone 54H6). To determine Caspase-3/7 activities at P07, hearts were homogenized in a hypotonic buffer containing (in mM): 25 HEPES, 5 MgCl₂, 1 EGTA, 1 PMSF with the pH adjusted to 7.5. A mix of protease inhibitors (Sigma, P8340) was also added to the buffer. Whole hearts were disturbed using the TissueLyzer system and protein concentration in the homogenate was quantified. Samples of cardiac protein (10 μg/ml) were incubated with the Caspase-Glo reagent (Promega, G8091) in 50 μl reactions for 1 hour at room-temperature. The caspase activity was measured in triplicate reactions for each sample using a luminometer set for an integration time of 2 sec. In some reactions, the recombinant caspase-3 (Enzo Life Sciences, BML-SE169) was added as a positive control.

**Statistical analysis**

Storing and presentation of the data were performed using SPSS software. Bars represent means ± standard error (multiplier:1). To compare means between two groups (i.e. WT vs. DKO), the independent-samples two-tailed t-test was used (nonpaired). To identify significant differences in survival between groups, curves were constructed according to the Kaplan-Meier method and used the log-rank (Mandel-Cox) criterion for statistical significance. To identify significant deviations in birth frequencies from the expected Mendelian frequencies in the different genotypes we used chi-square test.

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Online Figure I. Temporal deletion of mitofusins in adult cardiomyocytes results in late-onset cardiomyopathy. A) Western blot analysis showing reductions in Mfn-1 and Mfn-2 proteins in hearts of Mer-DKO compared to control. Both groups of animals received five intraperitoneal injections of raloxifen (40 mg/kg). B) Representative M-mode echocardiographs of adult male mice showing increased chamber dimension in the Mer-DKO group. Mice were 6 weeks old at the time of raloxifene injection and 14 weeks old at echocardiography. C-D) Morphometric analysis of Control and Mer-DKO hearts 8 weeks after raloxifen injections. FS; fractional shortening, HW; heart weight, BW; body weight. Filled bars represent means and error bars represent standard deviation (n=2 per group). E) Kaplan-Meier survival curve of Control and Mer-DKO mice. The study was started with raloxifen treatment (shown as red box) and mice (age: 13-20 weeks old) were followed-up for the next 100 days. The difference in survival between Control and Mer-DKO is significant for a $P=0.020$ (Log Rank). Median survival time of Mer-DKO mice after raloxifen treatment is 73 days.
Online Figure II. Targeting three of the four mitofusin alleles in cardiac myocytes leads to cardiac dysfunction in young adult mice. A) Western blot analysis showing reductions in Mfn-1 and Mfn-2 proteins in hearts of monoallelic mice compared to WT. The two samples shown here in the monoallelic group had the genotype Mfn-1+/F;Mfn-2F/F;Myh6cre+/-. Drp-1; dynamin related protein-1. B) Representative M-mode echocardiographs of adult male mice showing increased chamber dimension in the monoallelic group. C-E) Morphometric analysis of WT and monoallelic hearts by echocardiography. The monoallelic group (n=9) contained 7 Mfn-1+/F;Mfn-2F/F;Myh6cre+/+ and 2 Mfn-1F/F;Mfn-2+/F;Myh6cre+/+ mice. F) Representative pressure-volume loops recorded during vena cava occlusions. The arrow in the monoallelic panel shows the transposition of the loops to higher volume values, indicating ventricular chamber enlargement. G-I) Hemodynamic analysis of WT and monoallelic hearts showing alterations in the relaxation/elastic properties of the monoallelic heart. ms; milliseconds, Ees; elastance, end-systolic. The monoallelic group contained 3 Mfn-1+/F;Mfn-2F/F;Myh6cre+/+ mice.
Online Figure III. Mitochondrial morphology at birth in DKO hearts. A) Ultrastructure of cardiac myocyte from newborn WT heart. Arrows show tubular or exceedingly elongated mitochondria. B) Ultrastructure of cardiac myocyte in DKO heart. Double arrows indicate spherical mitochondria. C) Myofibril and mitochondrial structure in WT hearts. D-E) Myofibrillar/mitochondrial structure in DKO hearts. L; lipid droplet, N; nucleus, Z; z-band of sarcomeres. Single arrows indicate tubular mitochondria and double arrows indicate spherical mitochondria. Shown are representative images from two WT and two DKO pups of the same litter. F-G) Quantification of volume density of mitochondria and myofibrils performed by the grid-method.

** P < 0.01

Age: P0

WT (n=20 fields)

DKO (n=20 fields)
Online Figure IV. Evidence for the absence of ongoing apoptosis in DKO hearts at P07. **A)** Western blot analysis of whole heart extract (20 μg protein per lane). The apoptosis-related proteins examined are indicated on the left. **B)** Calculation of the Bax/Bcl-2 ratio as a measurement of apoptotic activation in WT and DKO hearts. **C)** Luminescence assay for the activity of caspase-3/7 in heart extracts (final protein concentration 5 μg/ml). The red bar indicates a positive control where the heart homogenate was supplemented with 0.06 U of purified caspase-3. RLU; relative light units. The y-axis is in logarithmic scale.
Online Figure V. Gene expression in DKO hearts. Transcription factors/coactivators of mitochondrial biogenesis. The relative abundance of target mRNAs is shown here for three time points: P0 (A) and P03 (B). Results on transcriptional regulation at P07 can be found in Figure 7. Quantitations were performed based on serial-dilution standard curves and all values were normalized according to the average of the WT for each gene. Abbreviations: Nrf-1; Nuclear respirator factor-1, Err-α; Estrogen receptor related-α, Pgc-1α; PPARγ coactivator-1α, Tfam; Transcription factor α mitochondrial.
Online Figure VI. Altered gene expression in DKO hearts. Autophagy markers.
The relative abundance of target mRNAs is shown here for three time points: P0 (A), P03 (B) and P07 (C).
Quantitations were performed based on serial-dilution standard curves and all values were normalized according to the average of the WT for each gene.
Abbreviations: 
Ulk-1;Unc51 like kinase-1, Ulk-2;Unc51like kinase-2, Atg-5;Autophagy related-5, Atg-12;Autophagy related-12
Atg-16;Autophagy related-16
LC3-b;(microtubule associated) Light chain 3-b
Beclin-1;Myosin-like bcl-2 interacting protein-1, Bnip-3;Bcl-2 interacting protein-3
Online Figure VII.
Altered gene expression in DKO hearts.

Mitochondrial markers.
The relative abundance of target mRNAs is shown here for three time points: P0 (A), P03 (B) and P07 (C). Quantitations were performed based on serial-dilution standard curves and all values were normalized according to the average of the WT for each gene. Results on Nd-5 and Cyt-b at P07 can be found in Figure 8.

Abbreviations: Opa-1; Optic atrophy-1, Ndufb-5; NADH dehydrogenase (ubiquinone) polypeptide 5, Cyc-S; Cytochrome c (somatic), Cox-5b; Cytochrome c oxidase subunit-5b, Atp-5o; ATP synthase subunit-5o
Online Figure VIII. Altered gene expression in DKO hearts.

Metabolism markers.
The relative abundance of target mRNAs is shown here for three time points: P0 (A), P03 (B) and P07 (C). Quantitations were performed based on serial-dilution standard curves and all values were normalized according to the average of the WT for each gene. Abbreviations: Gapdh; glyceraldehyde phosphate dehydrogenase, Ldh; Lactate dehydrogenase, Pfk; Phosphofructokinase, Pkm2; Pyruvate kinase (muscle isof.) variant 2, Mcad; Medium chain acylCoA dehydrogenase, Cpt-2; Carnitine palmitoyl transferase-2
Online Figure IX. Functional assessment of isolated mitochondria

A) Mitochondria were isolated from minced ventricles pooled from 3-4 hearts per isolation. Mitochondria were resuspended in BSA-free isolation buffer and a small sample was quantified for protein content with the bicinchoninic acid assay using appropriate standards and blanks.

B) Isolated WT or DKO mitochondria were lysed and assessed for citrate synthase activity using a spectrophotometric assay. TNB; thio-nitro-benzoic acid.

C) Time-course of ATP synthesis catalyzed by isolated mitochondria (30 μg protein) in the presence of malate (M) and pyruvate (P). Final concentration of each substrate 1 mM. The reaction buffer contained 10 mM potassium phosphate. RLU; relative light units.

D) The maximum rate of ATP synthesis is determined from the slope of the linear part of the curve (i.e. 0-60 seconds) for WT and DKO mitochondria. The slope of ATP synthesis in the presence of oligomycin during the same period is subtracted.

E) Same reaction setup as in C but now including succinate (S, 5 mM) as substrate and rotenone (R, 40 μM).

F) Calculation of maximum rates of ATP synthesis in WT and DKO mitochondria is performed by measuring the slope of the linear part of the curve (i.e. 0-45 seconds) and subtracting the slope of the curve in the presence of oligomycin during the same period of time. FCCP; carbonyl cyanide trifluoromethoxyphenylhydrazone.

G) Hearts were excised from WT and DKO pups at P07 and snap-frozen in liquid nitrogen. ATP content was determined in neutralized perchlorate extracts using the rLuciferase/luciferin reagent. RLUs were converted to ATP using a standard curve.
Online Table I. Mice with combined deficiency for Mfn-1 and Mfn-2 are absent from weaned (P21) litters

<table>
<thead>
<tr>
<th>Mfn genotype in offspring</th>
<th>Myh6-cre</th>
<th>Mendelian ratios (%)</th>
<th>Expected (from 104)</th>
<th>Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 alleles floxed</td>
<td>-</td>
<td>12.5</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>3 alleles floxed</td>
<td>-</td>
<td>25.0</td>
<td>26</td>
<td>19</td>
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<tr>
<td>4 alleles floxed</td>
<td>-</td>
<td>12.5</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>2 alleles floxed</td>
<td>+</td>
<td>12.5</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>3 alleles floxed</td>
<td>+</td>
<td>25.0</td>
<td>26</td>
<td>35</td>
</tr>
<tr>
<td>4 alleles floxed</td>
<td>+</td>
<td>12.5</td>
<td>13</td>
<td>0†</td>
</tr>
</tbody>
</table>

Each parent had a total of 3 mitofusin alleles floxed and the male was additionally positive for the Myh6-cre transgene

Example of the breeding setup:

Mfn-1^{F/F}; Mfn-2^{+/F}; Myh6-cre^{+/-}     Mfn-1^{F/F}; Mfn-2^{+/F}; Myh6-cre^{-/-}

13 Litters from 5 breeding pairs were analyzed

† The actual number significantly differs from the expected mendelian numbers.
Online Table II. Mice with dual targeting of Mfn-1 and Mfn-2 in cardiac myocytes are born in the expected mendelian ratios

<table>
<thead>
<tr>
<th>Allele combinations</th>
<th>Number of P0 pups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mfn-1</strong></td>
<td><strong>Mfn-2</strong></td>
</tr>
<tr>
<td>F/F</td>
<td>+/-F</td>
</tr>
<tr>
<td>F/F</td>
<td>+/-F</td>
</tr>
<tr>
<td>F/F</td>
<td>F/F</td>
</tr>
<tr>
<td>F/F</td>
<td>F/F</td>
</tr>
</tbody>
</table>

Breeding pairs were Mfn-1^{F/F};Mfn-2^{+/F};Myh6-cre^{+/+} × Mfn-1^{F/F};Mfn-2^{FF};Myh6-cre^{+/−} and a total of 15 litters were analyzed at P0.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Abbreviation</th>
<th>Fold induction/reduction (DKO vs. WT)</th>
<th>P</th>
<th>Functional class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitofusin-1</td>
<td>Mfn-1</td>
<td>0.102</td>
<td>0.000</td>
<td>Mitochondrial fusion/fission</td>
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<tr>
<td>Mitofusin-2</td>
<td>Mfn-2</td>
<td>0.154</td>
<td>0.000</td>
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<tr>
<td>Dynamin related protein-1</td>
<td>Drp-1</td>
<td>0.948</td>
<td>0.463</td>
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<tr>
<td>Mitochondria protein-18</td>
<td>Mtp-18</td>
<td>0.467</td>
<td>0.533</td>
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<tr>
<td>Stomatin like protein-2</td>
<td>Slp-2</td>
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<td>0.061</td>
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<td>Atrial natriuretic peptide</td>
<td>Anp</td>
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<td>0.007</td>
<td>Stress indicators</td>
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<tr>
<td>Brain natriuretic peptide</td>
<td>Bnp</td>
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<td>0.000</td>
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<tr>
<td>Myosin heavy chain-α</td>
<td>α-Mhc</td>
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<td>0.028</td>
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<tr>
<td>Myosin heavy chain-β</td>
<td>β-Mhc</td>
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<td>0.023</td>
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<tr>
<td>Skeletal muscle actin-α</td>
<td>α-Sk.actin</td>
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<tr>
<td>Troponin-I isoform-1</td>
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<td>Contractility</td>
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<tr>
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<td>TnI-3</td>
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<td>0.024</td>
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<tr>
<td>Leucine-zipper EF hand transmembrane protein-1</td>
<td>Letm-1</td>
<td>0.475</td>
<td>0.043</td>
<td>Mitochondrial ion balance</td>
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