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Mitochondrial fusion is essential for organelle function and cardiac homeostasis

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Abstract

Rationale—Mitochondria constitute 30% of myocardial mass. Mitochondrial fusion and fission appear essential for health of most tissues. Mitochondrial fission occurs in neonatal cardiomycyte and is implicated in cardiomyocyte death. Mitochondrial fusion has not been observed in post-mitotic myocytes of adult hearts, and its occurrence and function in this context are controversial.

Objective—Determine the consequences on organelle and organ function of disrupting cardiomyocyte mitochondrial fusion in vivo.

Methods and Results—The murine *mfn1* and *mfn2* genes, encoding mitofusins (Mfn) 1 and 2 that mediate mitochondrial tethering and outer mitochondrial membrane fusion, were interrupted by Cre-mediated excision of essential exons in neonatal (*Nkx2.5*-Cre) and adult (*MYH6* MER-Cre-MER plus tamoxifen or Raloxifene) hearts. Embryonic combined Mfn1/Mfn2 ablation was lethal after e9.5. Conditional combined Mfn1/Mfn2 ablation in adult hearts induced mitochondrial fragmentation, cardiomyocyte and mitochondrial respiratory dysfunction, and rapidly progressive and lethal dilated cardiomyopathy. Before heart failure developed, cardiomyocyte shortening and calcium cycling were unaffected by absence of Mfn1 and Mfn2. Based on the time course over which fusion-defective mitochondrial size decreases, a mitochondrial fusion/fission cycle in adult mouse hearts occurs approximately every 16 days.

Conclusions—Mitochondrial fusion in adult cardiac myocytes is necessary to maintain normal mitochondrial morphology and is essential for normal cardiac respiratory and contractile function. Interruption of mitochondrial fusion causes lethal cardiac failure at a time corresponding to 3 or 4 cycles of unopposed mitochondrial fission.

Keywords

Mitochondrial fusion; mitochondrial fragmentation; dilated cardiomyopathy

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Disclosures
None.

Introduction

Mitochondrial fusion and fission are evolutionarily conserved mechanisms that promote mitochondrial health via exchange of mitochondrial proteins, lipids, and genomes. Dilution of senescent enzymes and mutated mitochondrial DNA is a reparative process for dysfunctional organelles. Subsequent mitochondrial fission restores normal mitochondrial morphology and contributes to mitochondrial health by packaging damaged mitochondrial components into a daughter organelle that is eliminated [1]. Mitochondrial fusion is mediated by several GTPases: mitofusins (Mfn) 1 and 2 on the outer mitochondrial membrane, and optic atrophy 1 (Opa1) on the inner mitochondrial membrane. Loss of function mutations of these mitochondrial fusion proteins produces the degenerative neurological disease Charcot Marie Tooth Syndrome Type 2A (Mfn2 mutations) and autosomal dominant optic atrophy (Opa1 mutations) [2]. Heart disease is not a factor of these conditions [3], suggesting that mitochondrial fusion may be dispensable in the heart. Indeed, whereas mitochondrial fission has been observed and implicated in programmed death or differentiation of cardiac myocytes [4, 5], mitochondrial fusion has not been described in adult cardiac myocytes, and there is controversy over whether it occurs [6]. Cardiac deletion of Mfn2 had little effect on normal hearts, and appeared protective after ischemic damage [7], but Mfn1 can induce mitochondrial fusion in the absence of Mfn2 [8]; without both mitofusins organelle fusion does not occur. Thus, we engineered genetic mouse models in which both mitofusins are ablated in cardiac myocytes of either mouse early embryos or adults.

Methods

Detailed methods are in the Online Supplement.

Results

We ablated murine cardiomyocyte *mfn1* and *mfn2* using floxed allele mice [9] (Figure 1a) crossed onto *Nkx2.5* Cre [10] that promotes gene recombination in the embryonic mouse heart (Figure 1b). Mfn1 and Mfn2 single cardiac null mice were born at expected Mendelian ratios (Figure 1c). In contrast, Mfn1/Mfn2 double cardiac knockout mice (DKO) were not observed at birth, with lethality between e9.5 and e10.5 (Figure 1c). Thus, expression of either Mfn1 or Mfn2 in embryonic hearts is sufficient for viability, but absence of both is incompatible with life.

To avoid embryonic lethality, cardiac Mfn1/Mfn2 DKO mice were generated using a tamoxifen-inducible modified estrogen receptor (MER) cardiac-specific *MYH6*-Cre transgene [11] (Figure 2a). Mfn cardiac DKO mice were born at expected Mendelian ratios and survived normally. Three weeks after tamoxifen induction (8 week old mice), cardiac Mfn1 and Mfn2 immunoreactivities were decreased by >80% (Figure 2b). The inner mitochondrial fusion protein Opa1 was modestly upregulated in Mfn1/Mfn2 DKO hearts, whereas the mitochondrial fission protein DRP1 was unaffected (Figure 2b).

The time course of tamoxifen-mediated cardiac gene ablation was assessed using the ROSA26 lacZ marker gene (Figure 2c). Gene recombination (blue-stained myocardium) was evident 1 day after the first tamoxifen dose, was observed throughout the heart on day 3, and was uniform and intense on day 7.

We determined the consequence of mitofusin deficiency on mitochondrial morphology using flow cytometry of cardiac mitochondria three weeks after tamoxifen-induced *mfn1/mfn2* gene deletion. Forward scattered light measures mitochondrial size and side-scattered light measures complexity of shape. Histograms of forward scatter showed the typical normal distribution of mitochondrial size in control hearts (Figure 2d, **left; black line**), but a

leftward shift and loss of normal size distribution in Mfn cardiac DKO hearts (Figure 2d, **left; red line**). Mitochondrial size was decreased ~40% (Forward scatter; P=0.002) (Figure 2d, **middle**) and shape complexity was decreased ~60% (Side scatter; P=0.001) (Figure 2d, **right**). Mitochondrial protein content of Mfn cardiac DKO hearts was approximately twice that of controls (Figure 2e, P=0.0026), similar to findings in skeletal muscle Mfn DKO mice[16]; the mechanism for "proliferation" of fusion-defective mitochondria is currently unknown.

These results suggest that loss of adult cardiomyocyte Mfn proteins results in a plethora of small, round mitochondria. Ultrastructural studies confirmed this interpretation, and further revealed abnormal or degenerated mitochondrial cristae in Mfn cardiac DKO cardiomyocytes (Figure 2f). This form of mitochondrial dysmorphology is referred to as mitochondrial "fragmentation", although it is actually the result of mitochondrial fission unopposed by normal fusion [9]. Mfn1/Mfn2-deficient cardiomyocytes exhibited diminished ADP-stimulated O_2 consumption and decreased maximal O_2 consumption of uncoupled isolated DKO mitochondria (Figure 2h), revealing impaired respiration (Figure 2g). Thus, mitochondrial fusion mediated by Mfn1 or Mfn2 is essential for normal cardiac mitochondrial morphology and respiratory function.

Our studies were designed to interrupt mitochondrial fusion in adult mouse hearts. Because Mfn2 is implicated in tethering mitochondria to endoplasmic reticulum and inter-organelle calcium cross-talk [8, 12], we investigated the possibility that altered calcium caused the Mfn cardiac DKO cardiomyopathy. Both contractility (Figure 3a) and cytosolic calcium transients (Figure 3b) were normal in Mfn cardiac DKO cardiomyocytes 1 week after tamoxifen treatment. Thus, combined Mfn1 and Mfn2 ablation does not primarily affect cardiomyocyte excitation-contraction coupling.

Cardiomyocyte mitochondrial dysmorphology, impaired cellular respiration, and the early lethal phenotype of embryonic cardiac Mfn1/Mfn2 ablation suggested that disrupting cardiomyocyte mitochondrial fusion might have pathological consequences, given sufficient time. Therefore, we serially interrogated cardiac structure and function after combined *mfn1/mfn2* gene ablation induced by tamoxifen. Compared to littermate controls (tamoxifen-treated floxed allele mice without Cre), cardiac Mfn DKO hearts were normal 1 week after tamoxifen, but progressively dilated during the subsequent five weeks (Figure 4a). Conditional combined *mfn1* and *mfn2* ablation with Raloxifene [13] resulted in a similar progressive dilated cardiomyopathy, although the time course was slightly delayed (Figure 4b). Signs of overt heart failure (rapid respirations, decreased movement) were observed after seven to eight weeks, and the mice succumbed shortly thereafter.

Discussion

We used conditional cardiac-specific combined ablation of Mfn1 and Mfn2 to demonstrate that mammalian hearts have the same requirement for mitochondrial fusion as *Drosophila* heart tubes and murine brain and skeletal muscle [9, 14–16]. When the mitochondrial fusion apparatus was disrupted in adult hearts, cardiomyocyte mitochondria "fragmented", whole cardiomyocyte and isolated mitochondrial respiration were compromised, and the hearts dilated and failed over a period of weeks. These results prove that mitochondrial morphological and functional integrity requires an intact fusion/fission apparatus. Although mitochondrial fission can be increased in programmed cardiomyocyte death [17], unopposed mitochondrial fission was not associated with increased cardiomyocyte TUNEL positivity or sensitivity of the permeability transition pore to calcium (Online Figure I). Our findings are similar to a recent report of combined Mfn1/Mfn2 deletion in murine skeletal muscle [16]. Indeed, mitochondrial fragmentation, cellular respiratory dysfunction, and myopathy are

features of both the cardiac and skeletal muscle-specific Mfn1/Mfn2 double knockout mice. Thus, although adult cardiac myocytes are post-mitotic and their mitochondria are limited in motility and physically constrained between myofilaments, the heart is not an exception to the general rule that mitochondrial fusion is important to cellular and tissue health.

Because we used inducible double gene ablation, our data permit us to estimate the rate of in vivo mitochondrial fusion in adult cardiac myocytes. This calculation assumes that steady-state mitochondrial fusion and fission are balanced in normal hearts and that the rate of mitochondrial fission does not change after Mfn1/Mfn2 ablation. Unopposed fission will decrease mitochondrial population size over time in a geometric relationship. Cre-mediated gene excision was complete 1 week after tamoxifen, and mitochondrial size decreased by ~40% two weeks thereafter. Thus, a cardiomyocyte mitochondrial fusion/fission cycle takes approximately 16 days. Lethal heart failure observed 7–8 weeks after Mfn1/Mfn2 ablation therefore corresponds to 3–4 cycles of unopposed mitochondrial fission.

Mitofusins are regulated in cardiac disease or cardiomyocyte injury [18–20]. Our results suggest that decreased mitofusin expression or function has the potential to contribute to cardiac pathology by interfering with essential mechanics of mitochondrial fission/fusion.

Novelty and Significance

What is known?

- Myocardium is the most mitochondria-rich tissue.
- In most tissues mitochondria periodically tether, fuse, exchange organelle contents, and divide.
- Mitochondrial fusion has not been observed in vertebrate hearts; its existence in this context is disputed and its role (if any) is unknown.

What new information does this article contribute?

- By combinatorially ablating the genes encoding outer mitochondrial membrane fusion proteins, Mfn1 and Mfn2, in embryonic or adult mouse hearts, we interrupted mitochondrial fusion in cardiomyocytes.
- Mitofuscin-deficient adult heart cells contained unusually small mitochondria with structurally abnormal cristae and exhibited respiratory compromise.
- Interrupting mitochondria fusion in either embryonic or adult hearts proved lethal, in the latter case inducing rapidly progressive cardiac remodeling.

Mitochondria are engines that fuel cardiac contraction and mediators of programmed cardiomyocyte death. In many tissues, mitochondria cyclically tether to each other, fuse, and divide, thereby exchanging constituent proteins and nucleic acids. The highly organized intracellular architecture of cardiomyocytes enforces close mitochondrial approximation, suggesting that tethering may be irrelevant and fusion functionally unimportant. Compound genetic deletion of mitofusins that mediate mitochondrial tethering and fusion in embryonic and adult hearts proved lethal, inducing organelle fragmentation from un-opposed fission, respiratory compromise, and rapidly progressive dilated cardiomyopathy without programmed cardiomyocyte death. Thus, mitochondrial fusion is essential to vertebrate cardiac health.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard abbreviations and acronyms

Mfn	mitofusin
MER	modified estrogen receptor
DKO	double (cardiac) knockout

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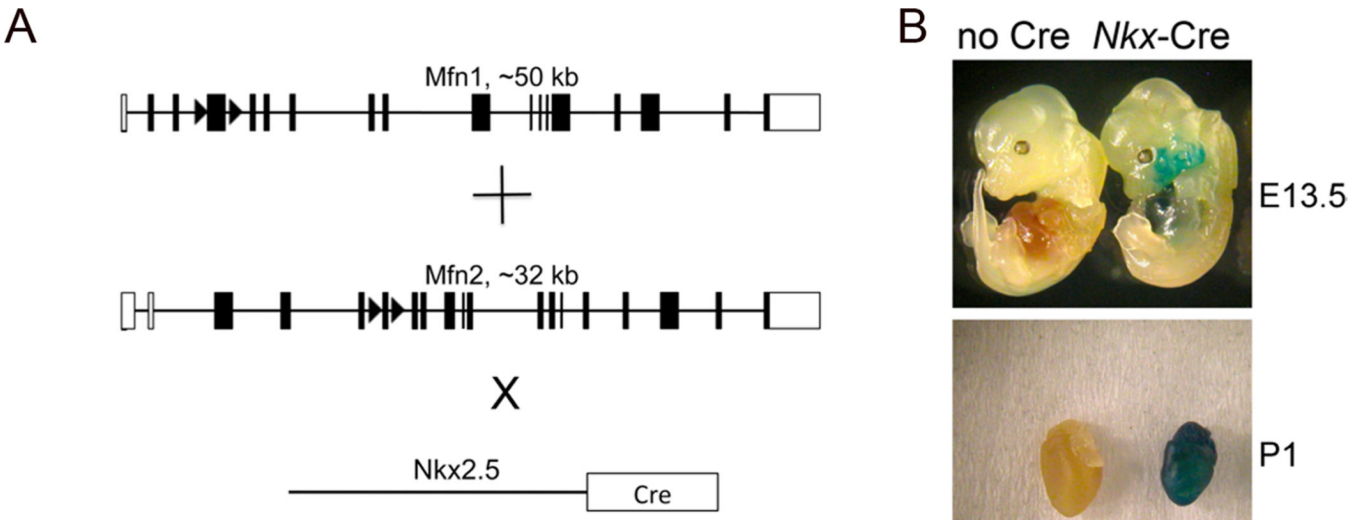

C

Genotype		n	Expected (%)	Expected (n)	Observed (n)
Mfn1 cardiac KO	*P7*	103	50	52	53
Mfn2 cardiac KO	*P7*	298	50	142	138
Mfn1/Mfn2 DCKO	*E8.5*	13	25	3	3
	E9.5	18	25	4-5	6
	E10.5	14	25	3-4	0
Mfn1/Mfn2 DCKO	*P7*	85	25	21	0

Figure 1. Combined ablation of Mfn1 and Mfn2 in the early embryonic heart is lethal

(**A**) Schematic representation of Cre-Lox strategy for combined cardiomyocyte-specific deletion of *mfn1* exon 4 and *mfn2* exon 6 using Nkx2.5-Cre knockin. (**B**) Cardiomyocyte gene recombination by *Nkx-2.5* Cre knock-in crossed to ROSA-26 LacZ reporter line and assayed for recombination (blue staining) at 13.5 days p.c. (E13.5), the second day post birth (P2) and three weeks of age (P21). (**C**) Yield of Nkx2.5-Cre driven cardiac Mfn1, Mfn2, and Mfn1/Mfn2 double cardiac knockout (DCKO) mice.

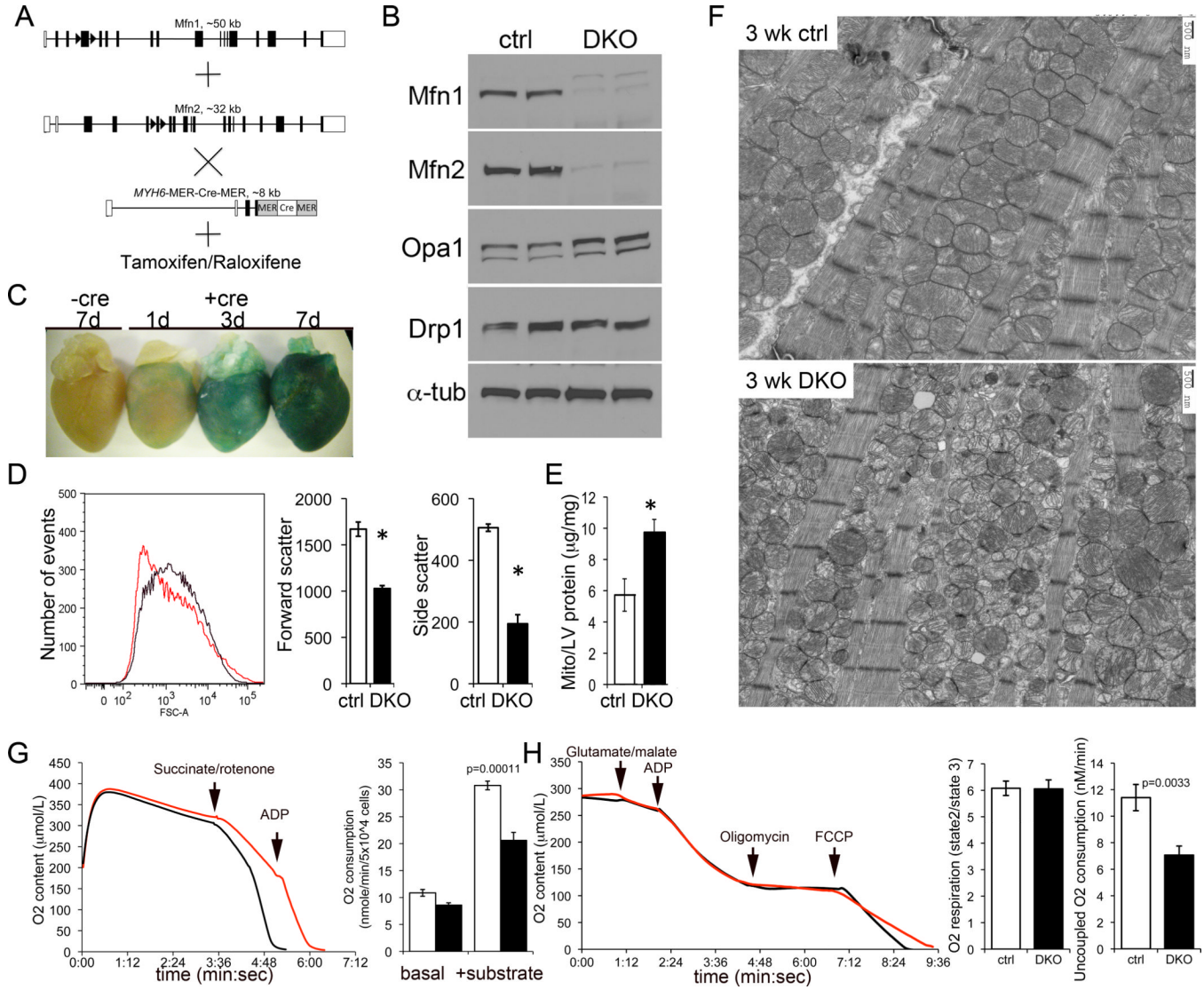

Figure 2. Conditonal combined ablation of Mfn1 and Mfn2 in adult hearts induces mitochondrial fragmentation and cardiomyocyte respiratory dysfunction

(**A**) Schematic representations of conditional Cre-Lox strategy for combined cardiomyocyte-specific deletion of *mfn1 and mfn2* using *MYH6*-driven modified estrogen receptor-Cre and tamoxifen or Raloxifene. (**B**) Immunoblot analysis of mitochondrial fusion and fission proteins 3 weeks after tamoxifen administration. Control (Ctrl) is *mfn1*fl/fl + *mfn2*fl/fl without Cre treated with tamoxifen. Each column is a separate mouse heart. (**C**) Time course of gene recombination induced by tamoxifen, assayed by LacZ staining of ROSA-26 crosses as in Figure 1b. (**D**) Flow cytometric analysis of isolated cardiac mitochondria size (forward scatter; FSC) and sphericity (side scatter) in Mfn1/Mfn2 double cardiac KO mice 3 weeks after tamoxifen. Left graph shows representative mitochondrial size (Forward scatter) distribution from identically treated ctrl (black) and Mfn1/Mfn2-DKO (red) hearts. Group mean data for forward and side scatter are to the right (n=3; *=P<0.05 vs ctrl). (**E**) Mitochondrial mass measured as protein content indexed to heart weight (n=4 ctrl and 3 DKO; * is P=0.0026 vs ctrl). (**F**) Transmission electron microscopic examination of cardiomyocyte mitochondria (5,000x). (**G**) Time-dependent oxygen consumption of digitonin-permeabilized cardiomyocytes from ctrl (black) and Mfn1/Mfn2 DKO (red) mice.

Right panel shows group data; white is ctrl, black is DKO. n=3. (H) Isolated cardiac mitochondrial respiration studies. Data are plotted as in (G). The respiratory control index (state 3 ADP-stimulated/state 2 ADP-limited) is shown immediately to the right, and maximal uncoupled respiration in response to FCCP at the extreme right (n=3 each).

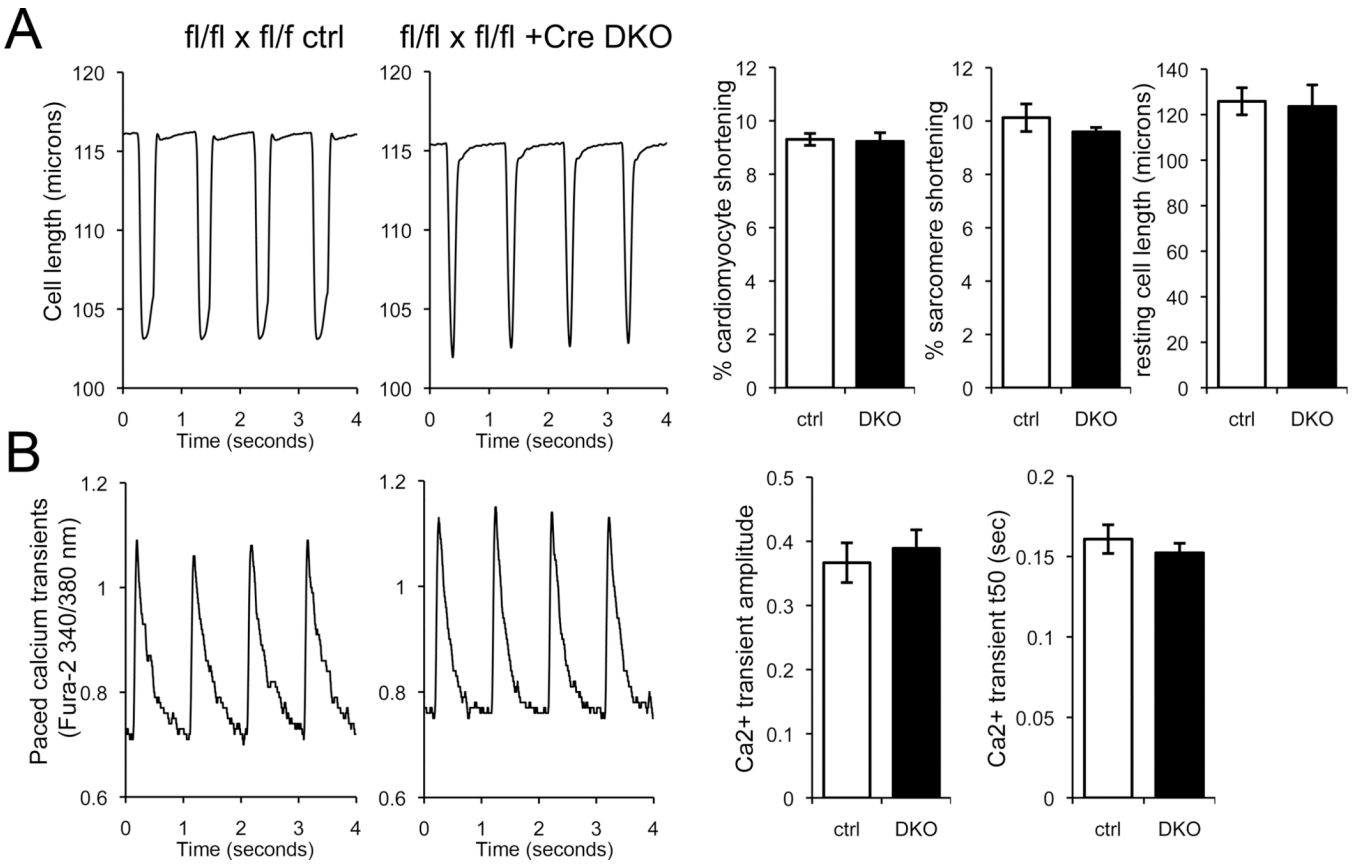

Figure 3. Cardiomyocyte contraction and calcium cycling are normal 1 week after combined cardiac Mfn1 and Mfn2 ablation

(**A**) Unloaded cardiomyocyte shortening with field stimulation at 1Hz. Representative tracings show time-dependent change in cell length. Left tracing is tamoxifen-treated *mfn1*fl/fl, *mfn2*fl/fl without Cre; right tracing is identically treated *mfn1*fl/fl, *mfn2*fl/fl Cre+. Bar graphs show group quantitative data (ctrl is white, DKO is black). (**B**) Phasic calcium transients in Fura-2 loaded cardiomyocytes as above. Group quantitative data for transient amplitude and time for 50% normalization of the transient (t50) are shown to the right. Data are mean ± SEM of n= 3 paired hearts, with each experimenting averaging 8–12 cardiomyocytes myocytes/heart. There were no differences between DKO and ctrl.

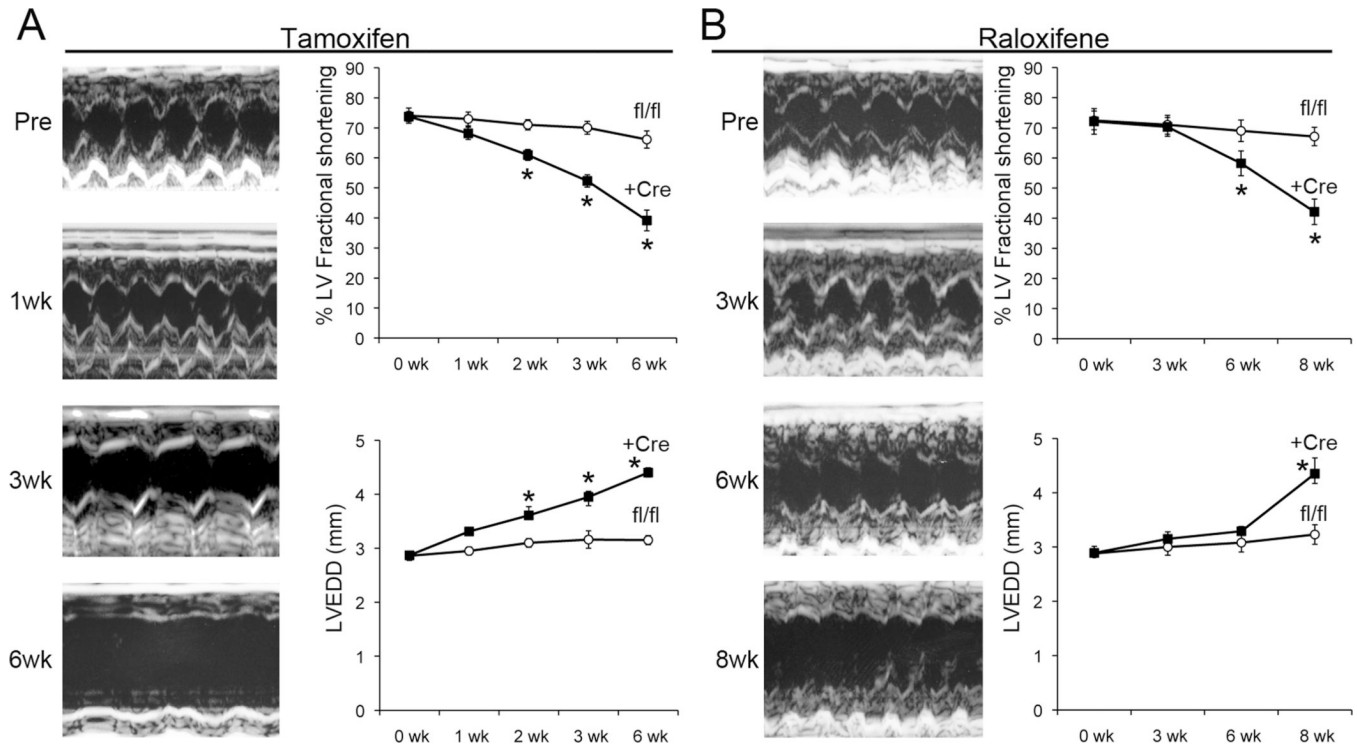

Figure 4. Combined Mfn1 and Mfn2 ablation in adult hearts induces rapidly progressive dilated cardiomyopathy

(**A**) Representative M-mode echocardiograms of un-anesthetized mouse left ventricles before (Pre) and at intervals after conditional ablation of Mfn1 and Mfn2 with tamoxifen. Group data for fractional shortening (top) and LV end diastolic dimension (LVEDD, bottom) are to the right (n = 4–5 per group). (**B**) Same as in (A), but using Raloxifene to activate MER-Cre-MER (n=4 per group).