Mitochondrial Cardiomyopathy Caused by Elevated Reactive Oxygen Species and Impaired Cardiomyocyte Proliferation

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Rationale: Although mitochondrial diseases often cause abnormal myocardial development, the mechanisms by which mitochondria influence heart growth and function are poorly understood.

Objective: To investigate these disease mechanisms, we studied a genetic model of mitochondrial dysfunction caused by inactivation of Tfam (transcription factor A, mitochondrial), a nuclear-encoded gene that is essential for mitochondrial gene transcription and mitochondrial DNA replication.

Methods and Results: Tfam inactivation by Nkx2.5Cre caused mitochondrial dysfunction and embryonic lethal myocardial hypoplasia. Tfam inactivation was accompanied by elevated production of reactive oxygen species (ROS) and reduced cardiomyocyte proliferation. Mosaic embryonic Tfam inactivation confirmed that the block to cardiomyocyte proliferation was cell autonomous. Transcriptional profiling by RNA-seq demonstrated the activation of the DNA damage pathway. Pharmacological inhibition of ROS or the DNA damage response pathway restored cardiomyocyte proliferation in cultured fetal cardiomyocytes. Neonatal Tfam inactivation by AAV9-cTnT-Cre caused progressive, lethal dilated cardiomyopathy. Remarkably, postnatal Tfam inactivation and disruption of mitochondrial function did not impair cardiomyocyte maturation. Rather, it elevated ROS production, activated the DNA damage response pathway, and decreased cardiomyocyte proliferation. We identified a transient window during the first postnatal week when inhibition of ROS or the DNA damage response pathway ameliorated the detrimental effect of Tfam inactivation.

Conclusions: Mitochondrial dysfunction caused by Tfam inactivation induced ROS production, activated the DNA damage response, and caused cardiomyocyte cell cycle arrest, ultimately resulting in lethal cardiomyopathy. Normal mitochondrial function was not required for cardiomyocyte maturation. Pharmacological inhibition of ROS or DNA damage response pathways is a potential strategy to prevent cardiac dysfunction caused by some forms of mitochondrial dysfunction. (Circ Res. 2018;122:74-87. DOI: 10.1161/CIRCRESAHA.117.311349.)

Key Words: cardiomyocyte maturation ■ cell cycle ■ mitochondria ■ reactive oxygen species ■ transcription factor

Mitochondria are a central hub of cellular metabolism and energy production. Mutations in genes encoded in both the nuclear and mitochondrial genomes can disrupt mitochondrial function, resulting in diseases with myriad manifestations, including cardiac hypertrophy, noncompaction, and failure.1 Although the need for mitochondria to produce an adequate supply of energy is the most obvious link between mitochondrial diseases and heart failure, other metabolic derangements may be equally if not more important for muscle cell dysfunction.2 Moreover, the mechanisms by which mitochondrial abnormalities cause aberrant cardiac morphogenesis, such as left ventricular noncompaction, are largely unknown. Greater understanding of the role of mitochondria in cardiac morphogenesis and maturation may provide insights into the pathogenesis of mitochondrial cardiomyopathies and identify productive therapeutic avenues to ameliorate the consequences of mitochondrial disease.

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Few studies have examined the role of mitochondria in governing cardiac development and maturation. Hom et al3 implicated mitochondria, and specifically mitochondrial

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Novelty and Significance

What Is Known?

- Mutations in genes essential for mitochondrial function often affect cardiac morphogenesis and postnatal cardiac function.
- Postnatal cardiomyocyte maturation involves striking changes in both morphology and metabolism, which has led to the hypothesis that metabolic maturation is required for morphological maturation.
- The nuclear-encoded mitochondrial protein TFAM (mitochondrial transcription factor A) is required for mitochondrial gene expression and DNA replication.

What New Information Does This Article Contribute?

- Early stage inactivation of a conditional Tfam allele in cardiomyocytes caused lethal defects in cardiac development associated with reactive oxygen species (ROS)-induced cardiomyocyte cell cycle activity.
- Postnatal mitochondrial dysfunction induced by neonatal cardiomyocyte-selective Tfam inactivation likewise reduced neonatal cardiomyocyte proliferation and caused progressive heart failure.
- In this neonatal Tfam cardiomyocyte inactivation model, suppressing ROS or kinases responsible for the G2/M cell cycle checkpoint in the neonatal period delayed the progression of heart failure.
- Mosaic Tfam inactivation in neonatal cardiomyocytes did not have a cell-autonomous effect on their morphological maturation.

The mechanisms that link mitochondrial dysfunction to abnormal cardiac morphogenesis and function are incompletely understood. Here, we study both fetal and neonatal inactivation of Tfam, which is required for mitochondrial gene expression and DNA replication. Tfam inactivation at either stage elevated ROS and activated the DNA damage response, resulting in reduced cardiomyocyte cell cycle activity. Suppressing ROS or blocking WEE kinase, required for the G2/M cell cycle checkpoint, restored cardiomyocyte cell cycle activity. In the neonatal Tfam inactivation model, ROS suppression or WEE kinase inhibition delayed the progression of heart failure. Using a mosaic Tfam inactivation approach, we further demonstrate that mitochondrial dysfunction caused by Tfam deletion did not affect postnatal cardiomyocyte morphological maturation. Together our data demonstrate that reduced cardiomyocyte cell cycle activity is 1 mechanism that links mitochondrial dysfunction to abnormal cardiac morphogenesis and function. In addition, our study points out that the neonatal period may offer a therapeutic window during which intervention can ameliorate later cardiac dysfunction.

Nonstandard Abbreviations and Acronyms

| AAV | adeno-associated virus |
| E  | embryonic day |
| MK | MK-1755 |
| MT | mitoTEMPO |
| P  | postnatal day |
| pH3 | phosphohistone H3 |
| Pparc1α | peroxisome proliferator-activated receptor gamma coactivator 1-α |
| Pparc1β | peroxisome proliferator-activated receptor gamma coactivator 1-β |
| ROS  | reactive oxygen species |
| TFAM | mitochondrial transcription factor A |
| TfamKR | TfamKR inactivated by Nkx2.5 |
| TUNEL | terminal deoxynucleotidyl transferase dUTP nick end labeling |
| T-tubule | transverse tubule |

Gene mutations that impair mitochondrial function often disrupt cardiac morphogenesis and function, but the mechanistic links between mitochondria, cardiac morphogenesis, and heart failure are incompletely understood. Here, we study both fetal and neonatal inactivation of Tfam, which is required for mitochondrial gene expression and DNA replication. Tfam inactivation at either stage elevated ROS and activated the DNA damage response, resulting in reduced cardiomyocyte cell cycle activity. Suppressing ROS or blocking WEE kinase, required for the G2/M cell cycle checkpoint, restored cardiomyocyte cell cycle activity. In the neonatal Tfam inactivation model, ROS suppression or WEE kinase inhibition delayed the progression of heart failure. Using a mosaic Tfam inactivation approach, we further demonstrate that mitochondrial dysfunction caused by Tfam deletion did not affect postnatal cardiomyocyte morphological maturation. Together our data demonstrate that reduced cardiomyocyte cell cycle activity is 1 mechanism that links mitochondrial dysfunction to abnormal cardiac morphogenesis and function. In addition, our study points out that the neonatal period may offer a therapeutic window during which intervention can ameliorate later cardiac dysfunction.

TFAM (mitochondrial transcription factor A) is a nuclear-encoded protein that is required for mitochondrial DNA transcription. Ablation of Tfam prevents expression of the 13 polyptides encoded in the mitochondrial genome, all components of enzyme complexes required for oxidative phosphorylation. TFAM is also required to maintain mitochondrial DNA stability, both by stimulating its replication and by binding it to form nucleoids. Although Tfam inactivation reduced mitochondrial number and mass, in some contexts, it elevated ROS production, likely as a result of electron transport chain impairment. Conditional Tfam inactivation in cardiomyocytes by Myh6 (myosin heavy chain 6)-Cre or MCK-Cre was previously shown to cause lethal dilated cardiomyopathy. Survival to birth was reported to be normal, and most Tfam-Cre neonates died in the first week. However, the effect on cardiac development or cardiomyocyte maturation was not investigated.

Here, we inactivated Tfam in cardiomyocytes to disrupt mitochondrial function and evaluate the consequences on cardiac development, cardiomyocyte maturation, and cardiac function. We found that Tfam inactivation in fetal cardiomyocytes severely impaired cardiomyocyte proliferation, associated with severe myocardial hypoplasia and fetal demise. Impaired cardiomyocyte proliferation was linked to ROS-mediated activation of the DNA damage response pathway. Postnatally, Tfam inactivation impaired neonatal cardiomyocyte proliferation but morphologically did not alter cardiomyocyte maturation. Cardiomyopathy caused by neonatal Tfam inactivation was ameliorated by the inhibition of ROS or the DNA damage response pathway, suggesting a potential therapeutic strategy for mitochondrial cardiomyopathies.

Methods

Detailed Methods are given in the Online Data Supplement.
Animal
All animal procedures were performed following protocols approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital. Tfiamfl/fl, Nkx2.5ires-CreIRES, ROSA26CreERT2, and Rosa26tdTomato mice, and AAV9-TnT-Cre were described previously.3,11–14 Adenoassociated virus (AAV) was injected subcutaneously at postnatal day 1 (P1) or P8 at 4.0×10^10 vg/g (high dose) or 8.0×10^9 vg/g (low dose).

Fetal Cardiomyocyte Culture
Embryonic day 15.5 (E15.5) cardiomyocytes were isolated using the Neomyt Kit (Cellutron, NC-6031). Adenovirus expressing Cre (Ad:Cre) or LacZ (Ad:LacZ) was added on day 1. Mitochondrial function was measured using a Seahorses Biosciences XF96e analyzer.

Histology
Tissues were fixed in 4% paraformaldehyde, and 8 µm cryosections were immunostained using antibodies listed in the detailed Methods (Online Data Supplement). Apoptosis was measured using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Assay Kit (Abcam, ab66108).

Adult Cardiomyocyte Characterization
Adult cardiomyocytes were isolated by Langendorff perfusion as described previously.15 In situ transverse tubule (T-tubule) imaging was performed as described.13 T-tubule organization was quantified using AutoTT.15,16 Contraction of isolated ventricular cardiomyocytes was measured using an IonOptix system. Intracellular Ca2+ recordings were obtained by line scan imaging of cardiomyocytes loaded with Fluo-4 AM (Life Technologies).

RNA-Seq and Gene Expression
Polyadenylated RNA was isolated and converted into stranded RNA-seq libraries using Script-Seq v2 (Illumina). Real-time PCR was performed using primer sequences listed in Online Table I.

Statistics
Unless otherwise specified, bar graphs present data as mean±SD. Significance of intergroup differences was tested using Student t test or the Mann–Whitney test as indicated (SPSS 20.0).

Results
Cardiac-Specific Deletion of Tfiam Causes Embryonic Lethality at E15.5
To disrupt mitochondrial function in fetal cardiomyocytes early in cardiac development, we used Nkx2.5ires-Cre to inactivate a conditional Tfiam allele9 (Tfiam fl/fl; Nkx2.5 IRES-Cre/+ [Tfiam NK]). Tfiamfl/+; Nkx2.5IRES-Cre/+ littermates were used as controls. We analyzed embryos from E13.5 to birth and found that Tfiam NK mutants were present at a normal Mendelian ratio at E13.5 and E15.5, but no viable mutants were recovered at E16.5 or birth (Figure 1A). At E15.5, Tfiam NK mutants had strikingly thin myocardial walls (Figure 1B). E13.5 hearts likewise showed thinner myocardial walls (Online Figure IA). Quantitative RT-PCR demonstrated reduction of Tfiam mRNA in Tfiam NK mutants (Figure 1C), and TFIAM immunostaining confirmed protein depletion (Figure 1D). Immunoblotting also demonstrated the reduction of TFIAM and ATP5B, a nuclear-encoded mitochondrial protein, in Tfiam NK mutants (Online Figure IB). Furthermore, mitochondrial DNA copy number relative to nuclear DNA was reduced by 9.2-fold compared with control (Online Figure IC).

Tfiam Is Essential for Mitochondrial Morphogenesis and Function
To investigate the effect of Tfiam inactivation on mitochondria, we analyzed mitochondrial morphology in E15.5 hearts...
by electron microscopy, TfamNK mitochondria had abnormal morphology and organization (Online Figure IIA). Cristae within mitochondria also appeared more sparse and irregular in TfamNK cardiomyocytes. Quantitative analysis showed that average mitochondrial size was lower in the TfamNK cardiomyocytes (Online Figure IIB). The mitochondrial area fraction was also reduced in TfamNK cardiomyocytes (Online Figure IIC). These data indicate that Tfam inactivation disrupts mitochondrial biogenesis and morphology.

We next assessed the effect of Tfam ablation on mitochondrial function. We cultured cardiomyocytes dissociated from E15.5 Tfam knockout (TfamKO) and Rosa26tdTomato hearts. To deplete Tfam and activate the Cre-dependent tdTomato reporter, we transduced the cultured cardiomyocytes with adenovirus expressing Cre (Ad:Cre), resulting in the activation of tdTomato in >90% of cardiomyocytes. Ad:LacZ transduced cardiomyocytes served as controls. Immunostaining 48 hours after virus treatment showed markedly reduced TFAM immunoreactivity in tdTomato+ Cre-recombined cells, consistent with effective gene inactivation (Online Figure III). Furthermore, staining for mitochondrial marker ATP5B demonstrated decreased mitochondrial abundance (Online Figure III) and perinuclear predominance, in contrast to control cardiomyocytes in which mitochondria were distributed throughout the cytoplasm (Online Figure III).

Having established an efficient system for in vitro Tfam ablation in fetal cardiomyocytes, we next assessed the effect on mitochondrial function using a microfluidic extracellular flux analyzer (Online Figure IVA through IVC). Tfam inactivation increased basal respiration, which was attributable to greater F1F0-ATPase–linked respiration and to a lesser extent on increased proton leak (Online Figure IVA through IVC). Moreover, maximal respiratory rate was significantly lower in Tfam-deleted cardiomyocytes, indicative of reduced maximal electron transport chain activity (Online Figure IVA through IVC). Although Tfam-deficient cardiomyocytes exhibited impaired respiration, their extracellular acidification rate, a measure of glycolytic activity, was elevated (Online Figure IVD and IVE).

Functional mitochondria have hyperpolarized inner mitochondrial membranes. We assessed mitochondrial membrane potential with JC-1, which stains normal, hyperpolarized mitochondria red and impaired, depolarized mitochondria green. Confocal imaging demonstrated increased green and decreased red staining of mitochondria in Tfam-deleted cardiomyocytes treated with Ad:Cre, indicative of mitochondrial depolarization (Online Figure VA). Quantitative data obtained by flow cytometry confirmed decreased red/green fluorescence ratio in Tfam-depleted cardiomyocytes (Online Figure VB). Indeed, the red/green ratio in Tfam-deficient cardiomyocytes approached the level observed when cardiomyocytes were treated with carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (also known as FCCP), which abrogates mitochondrial membrane potential (Online Figure VB). These data demonstrate that Tfam inactivation depolarizes mitochondria.

To assess the effect of Tfam knockout on cellular energy reserves, we measured the ADP/ATP ratio in both cardiomyocytes freshly dissociated from fetal hearts and cultured cardiomyocytes. In E15.5 cardiomyocytes and cultured cardiomyocytes 3 and 7 days after Ad:Cre treatment, ADP/ATP ratio was lower in Tfam-deficient samples (Online Figure VI). This suggests that Tfam-deficient cardiomyocytes surprisingly did not have depletion of energy stores, potentially because of increased glycolytic activity.

**Tfam Is Required Cell Autonomously for Fetal Cardiomyocyte Proliferation**

A major effect of Tfam inactivation on fetal heart development was myocardial hypoplasia (Figure 1B). To assess the underlying cellular mechanism, we analyzed cardiomyocyte proliferation and apoptosis at E13.5 and E15.5. At both stages, the fraction of cardiomyocytes expressing the M-phase marker phosphohistone H3 (pH3) was markedly and significantly decreased in TfamKO, indicative of depressed cardiomyocyte proliferation (Figure 1E and 1F). Measurement of Ki67 further confirmed reduced cardiomyocyte proliferation at E15.5 (Online Figure VIIA and VIIIB). TUNEL staining showed that TUNEL+ cardiomyocytes were significantly more frequent in TfamKO hearts at E13.5 but not E15.5 (Figure 1E; Online Figure VIIIC). However, in both groups, TUNEL+ cardiomyocytes were infrequent. Together these data suggest that myocardial hypoplasia was because of both reduced cardiomyocyte proliferation and increased cardiomyocyte apoptosis, with the change in proliferation likely making a relatively larger contribution.

We used 2 independent approaches to assess whether decreased proliferation was a direct, cell-autonomous effect of Tfam inactivation, as opposed to a secondary or indirect consequence of impaired embryo health or myocardial dysfunction. First, we used a tamoxifen-inducible Cre allele (Rosa26CreERT2) and a low dose of tamoxifen to inactivate Tfam in a minor fraction of cells (Online Figure VIII). In pilot experiments, we titrated the amount of tamoxifen administered at E8.5 so that CreERT2 activated the Cre-dependent Rosa26tdTomato reporter in ≈30% of cardiomyocytes by E15.5 (Online Figure VIIIIB and VIIIC). When this tamoxifen dose was administered to Tfam knockout (TfamKO; Rosa26CreERT2tdTomato) embryos, by immunostaining, 80% of tdTomato+ cardiomyocytes lacked detectable TFAM (Online Figure VIIID and VIIIE), indicating that the large majority of tdTomato+ cardiomyocytes lack Tfam. We then analyzed tdTomato+ and tdTomato− cells from tamoxifen-treated TfamKO, Rosa26CreERT2tdTomato, or Tfam+/−; Rosa26CreERT2tdTomato for cell cycle activity, as measured by pH3 staining (Figure 2A). As expected, in tdTomato+ cells, which were not recombined by CreERT2, there was no significant difference in the frequency of pH3+ cardiomyocytes (Figure 2B). In contrast, in tdTomato− cells, which were recombined by CreERT2, pH3+ cardiomyocytes from TfamKO embryos were significantly less frequent than from Tfam+/− littermates (Figure 2B). This genetic mosaic analysis demonstrated that Tfam required cell autonomously for normal cardiomyocyte proliferation. This result makes potential confounding effects from poor cardiac function or low embryo viability less likely because the embryos were phenotypically normal as a result of the low fraction of cells that were mutated.

Second, we studied the effect of Tfam inactivation on cardiomyocyte proliferation in cultured fetal cardiomyocytes. We measured cardiomyocyte proliferation using both uptake
of the nucleotide analog 5-ethynyl-2′-deoxyuridine (passage through S phase) and immunostaining for pH3 (M phase). Both measures of cell cycle activity were reduced in Tfam-depleted cardiomyocytes (Figure 2C through 2F). Because the intruterine environment has lower oxygen tension, we repeated these experiments under hypoxic conditions (7% oxygen). Cell cycle activity of both control and Tfam-depleted groups was higher in hypoxia compared with normoxia, consistent

Figure 2. Tfam (mitochondrial transcription factor A) inactivation cell autonomously reduced cardiomyocyte (CM) proliferation. A, Representative images of embryonic day 15.5 (E15.5) heart sections stained for CM marker (cardiac Troponin T, cTNT), Cre marker (Tomato), and M-phase marker (phosphohistone H3 [pH3]). Insets show magnification of boxed regions with cTNT staining indicated in white. Filled yellow arrowheads, pH3+, tdTomato+ CMs. Open white arrowheads, pH3+, tdTomato− cells. Bar, 100 µm. Boxed regions are magnified in inset with cTNT staining shown in white. B, Quantification of the percentage of pH3+ CMs from staining described in (A). Three sections were stained and imaged per heart, and 5 independent hearts were studied. C–F, CMs cultured as in C and exposed to 5-ethynyl-2′-deoxyuridine (EdU) for 24 hours were stained for pH3 or EdU. Representative confocal images are shown in C and E, and boxed regions are magnified in insets on the right. Bar, 100 µm. Results are quantified in D and F. n=6. *P<0.05. **P<0.01.

Figure 3. RNA-seq analysis of Tfam (mitochondrial transcription factor A) inactivation in fetal heart. RNA-seq was performed on Tfamlox/lox (Tfam<sup>lox</sup>; Nkx2.5<sup>IRES-Cre</sup> ) and control hearts at embryonic day 13.5 (E13.5; n=3 per group). A, Scatterplot of gene expression in Tfamlox and control. Selected differentially expressed genes are labeled. B, Summary of Gene Set Enrichment Analysis (GSEA) for RNA-seq data. Selected pathways significantly enriched in Tfamlox or control are listed. Leading edge analysis of the p53 pathway is shown. C, Relative expression of selected genes differentially expressed in p53 pathway based on the RNA-seq results. **P<0.01. pH3 indicates phosphohistone H3.
with recent studies, but the proliferation rate of hypoxic Tfam-depleted cardiomyocytes remained lower than hypoxic controls (Online Figure IX).

Collectively, these data demonstrate that Tfam inactivation cell autonomously reduces fetal cardiomyocyte proliferation.

**Tfam Ablation Elevates ROS Levels and Activates DNA Damage Response Pathways**

To further assess the mechanisms by which Tfam ablation inhibits cell cycle activity, we performed RNA-seq on TfamNK and control hearts (n=3 biological replicates per group, 5 hearts per replicate). Hearts were collected at E13.5, before the onset of severe morphological abnormalities. There were 346 upregulated and 449 downregulated genes (adjusted P value <0.05; Figure 3A). Gene set enrichment analysis of the RNA-seq data (Figure 3B) showed that the mutant is enriched for terms related to glycolysis and metabolism, consistent with the central role of mitochondria in cellular metabolism. Muscle contraction gene sets were downregulated in mutants, suggesting a link between mitochondrial function and expression of contractile genes. Interestingly, DNA damage response terms, most notably the p53 DNA damage response pathway, were enriched in TfamNK. Key upregulated genes in this pathway included Cdkn1a (encoding the cell cycle inhibitor p21), Gadd45a, a gene induced by the DNA damage response pathway, and Ddit3, a gene that mediates cell cycle arrest and that is induced by DNA damage (Figure 3C).

At birth, exposure to increased oxygen tension results in greater mitochondrial ROS production in normal cardiomyocytes, and this has been linked to cell cycle exit through the activation of DNA damage response pathways. Elevated ROS and activation of the p53 pathway have also been implicated in damage to telomeres, and the gene set Packaging of telomere ends was downregulated in TfamNK. Therefore, we hypothesized that elevated ROS production by Tfam-depleted cells activates DNA damage response pathways, resulting in cell cycle exit.

The effect of Tfam ablation on ROS levels is cell context dependent: Tfam inactivation elevated ROS in adipocytes but reduced ROS in keratinocytes. Therefore, as a first step toward testing this hypothesis, we evaluated the effect of Tfam ablation on fetal cardiomyocyte ROS levels. TfamNK heart sections had elevated levels of 4-hydroxynonenal, a product of lipid peroxidation by ROS, at E13.5 and E15.5 (Figure 4A and 4B). In cultured E15.5 Tfamfl/fl; Rosa26tdTomato cardiomyocytes, we measured cellular ROS levels using CellRox, a fluorogenic live cell ROS probe. Positive control cells treated with 50 µmol/L H2O2 exhibited high CellRox fluorescence, as expected (Figure 4C, left). In Ad:LacZ-treated cardiomyocytes, most cells had low CellRox signal, consistent with low ROS levels (Figure 4C, middle). In contrast, tdTomato+, Ad:Cre-transduced Tfamfl/fl; Rosa26tdTomato cardiomyocytes exhibited markedly increased CellRox fluorescence (Figure 4C, right, upper right quadrant), whereas there the frequency of...
CellRox-positive, tdTomato− cells was not substantially affected (Figure 4C, right, lower right quadrant). Quantitative analysis across replicates confirmed that Tfam ablation markedly increased cardiomyocyte ROS production (Figure 4D). To determine whether the excess ROS originated from mitochondria, we used the mitochondrially targeted ROS fluorescent probe Mitosox. Tfam-depleted cardiomyocytes exhibited greater Mitosox fluorescent intensity than control cardiomyocytes (Online Figure X), supporting elevated mitochondrial ROS production in these cells.

Together these data indicate that mitochondria in Tfam-depleted cardiomyocytes produce excess ROS by impairing electron transport chain function. Elevation of ROS and the transcriptomic signature of activated DNA damage response in Tfam-deficient cardiomyocytes suggested the hypothesis that ROS activation of the DNA damage response reduces their cell cycle activity.

**Rescue of Cell Cycle Activity of Tfam-Deficient Cardiomyocytes by ROS or WEE1 Kinase Inhibition**

DNA oxidation by ROS triggers phosphorylation of histone H2A.X (γH2A.X), which accumulates at sites of damage and serves as a platform that activates the DNA damage checkpoint to progression from G2 to M phase of the cell cycle. In cultured fetal cardiomyocytes, Tfam ablation induced increased γH2A.X (Online Figure XIA and XIB), consistent with ROS-mediated DNA damage and activation of DNA damage response pathways. In hypoxic culture conditions (7% O2), overall frequency of γH2A.X cardiomyocytes was lower, but Tfam-depleted cardiomyocytes continued to have elevated γH2A.X compared with control (Online Figure XIC and XID).

To test the hypothesis that elevated ROS production from Tfam-deficient cardiomyocytes contributed to reduced cardiomyocyte proliferation, we treated cultured cardiomyocytes with the mitochondrially targeted ROS scavenger mitoTEMPO (MT). MT normalized γH2A.X levels in Tfam-depleted cultured fetal cardiomyocytes (Figure 5A and 5B). This effect of MT was accompanied by increased cell cycle activity, as measured by Ki67 staining (Figure 5C and 5D). These data suggest that elevated ROS in Tfam-depleted cardiomyocytes activates the DNA damage response to reduce cardiomyocyte cell cycle activity.

Cell cycle progression through the G2/M checkpoint depends on CDC25-mediated dephosphorylation of CDK1 at tyrosine-15. WEE1 kinase counterbalances CDC25 by phosphorylating this residue. Activation of DNA damage response pathways inhibits CDC25 activity and thereby prevents progression through the G2/M checkpoint. We reasoned that DNA damage–mediated cell cycle inhibition could be ameliorated by inhibiting WEE1 kinase. Treatment of Tfam-deficient, cultured fetal cardiomyocytes with MK-1775 (MK), a selective WEE1 kinase inhibitor, increased cell cycle activity, as measured by Ki67 staining (Figure 5C and 5D). Remarkably, MK-treated, Tfam-deficient cardiomyocytes had cell cycle activity that was not significantly different from

![Figure 5](http://circres.ahajournals.org/)

**Figure 5. Rescue of cell cycle activity of Tfam (mitochondrial transcription factor A)-deficient cardiomyocytes (CMs) by reactive oxygen species (ROS) or WEE1 kinase inhibition.** Cultured Tfam\(^{fl/fl}\); Rosa26\(^{tdTomato}\) embryonic day 15.5 (E15.5) CMs were treated with Ad:Cre on the first day after seeding. Then MitoTEMPO (MT) or MK-1775 (MK) were added, and CMs were fixed and stained on the fifth day. **A**, Representative images of CMs treated with Cre, Cre+MT, Cre+MK, or LacZ (control, Con) and stained for γH2A.X and a CM marker (cardiac troponin T [cTNT]). Bar, 100 µm. **B**, Quantification of the percentage of γH2AX+ CMs in (A). **C**, Representative images of CMs treated with Cre, Cre+MT, Cre+MK, or LacZ (Con) and stained for Ki67 and a CM marker (sarcomeric α-actinin [SAA]). Bar, 100 µm. **D**, Quantification of the percentage of Ki67+ positive CMs in (B). t test: *P<0.05, **P<0.01.
control cardiomyocytes. MK did not reduce γH2A.X levels in Tfam-deficient cardiomyocytes (Figure 5A and 5B), consistent with WEE1 kinase inhibition acting distal to this DNA damage signal.

Collectively, these data indicate that Tfam deficiency causes ROS-mediated DNA damage, which inhibits cardiomyocyte cell cycle activity through the activation of the G2/M checkpoint. ROS scavenging or WEE1 kinase inhibition rescues cardiomyocyte proliferation by reducing ROS or by relieving G2/M checkpoint cell cycle inhibition.

Inhibition of ROS-Activated DNA Damage Response Mitochondrial Rescues Cardiomyopathy In Vivo

To determine whether ROS suppression or WEE1 kinase inhibition protects cardiomyocyte proliferation from Tfam deficiency in vivo, we developed and studied a model of neonatal Tfam deficiency because neonates are more accessible to interventions than embryos. This model is also more relevant to clinical scenarios of mitochondrial abnormalities, which typically present postnatally. Tfam−/−; Rosa26tdTomato mice were treated with AAV9-cTNT-Cre (AAV9-Cre), in which cardiotoxic adeno-associated virus 9 expresses Cre from the cardiac troponin T promoter.14 At P0, we delivered which cardiotropic adeno-associated virus 9 expresses Cre to Tfam fl/fl; Rosa26tdTomato mice (Figure 6A). Control mice with the genotype Tfam−/+; Rosa26tdTomato were treated with AAV9-Cre at the high dose. Three days after AAV treatment, ∼55% and 30% of cardiomyocytes expressed the tdTomato Cre–dependent reporter (Figure 6B; Online Figure XIIA). To confirm that AAV9-Cre effectively inactivates Tfam in Tfam−/−; Rosa26tdTomato mice, we performed immunostaining on dissociated cardiomyocytes. We observed reduced TFAM immunoreactivity in tdTomato+ cardiomyocytes (Figure 6C; Online Figure XIIIB). We confirmed elevated levels of 8-oxoguanine, DNA damage induced by ROS, as well as increased 4-hydroxynonenal, P21 cell cycle inhibitor, WEE1 kinase, and γH2A.X in Tfam-deficient cardiomyocytes (Online Figure XIIIC and XIIID).

Mice were followed by serial echocardiography until 8 weeks of age. Consistent with prior cardiac knockout of Tfam using Myh6-Cre,9 high-dose mice with a greater fraction of cardiomyocyte transduction developed progressive ventricular dysfunction and dilatation, as measured by echocardiography (Figure 6D and 6E). In contrast, the low-dose group had heart function that was not distinguishable from control (Figure 6D and 6E). These data show that Tfam inactivation in a large fraction of cardiomyocytes causes progressive cardiomyopathy, whereas its inactivation in a small fraction of cardiomyocytes is well tolerated and does not cause organ-level evidence of dysfunction.

Murine cardiomyocytes continue to proliferate until about P7, by which time they largely exit the cell cycle.22,23 We hypothesized that Tfam ablation during this time period contributed to heart dysfunction by impairing cell cycle activity and that this effect of Tfam ablation could be ameliorated by either ROS or WEE1 kinase inhibition. To test this hypothesis, mice were treated with AAV9-Cre at the high dose and also with MT (ROS scavenger), MK (WEE1 inhibitor), or vehicle for the first postnatal week (Figure 6F). Remarkably, treatment with either MT or MK during the first postnatal week, when cardiomyocytes retain proliferative competence, ameliorated ventricular dysfunction at 8 weeks (Figure 6F).

We further tested this hypothesis using 2 independent approaches. In 1 approach, we treated a second cohort of mice with MT or MK in the second postnatal week (Figure 6G). Delaying therapy to this period outside of the period of cardiomyocyte proliferative competence resulted in it being ineffective. In a second approach, we asked whether inactivation of Tfam after cardiomyocytes lose proliferative competence has a less deleterious effect than when it is inactivated during active cardiomyocyte proliferation. Tfam−/−; Rosa26tdTomato and Tfam−/−; Rosa26tdTomato mice at P8, and Tfam−/−; Rosa26tdTomato mice at P1, were injected with the same weight-adjusted dose of AAV9-Cre. Serial echocardiography confirmed progressive, severe systolic dysfunction caused by AAV9-Cre delivery at P1 (Online Figure XIIIIA). In comparison, AAV9-Cre delivery at P8 caused relatively less severe systolic dysfunction (Online Figure XIIIIB). Together, these data suggest that impaired cardiomyocyte proliferation contributes to the progressive cardiomyopathy caused by neonatal Tfam knockout. Furthermore, the data point to a limited therapeutic window that corresponds to the time when cardiomyocytes are actively cycling.

We investigated the mechanisms underlying the response to MT and MK during the first postnatal week. Western blotting for TFAM or ATP5B indicated that MT and MK did not reduce the extent of Tfam or mitochondrial depletion (Online Figure XIVA). MT and MK likewise did not rescue expression of mt-Atp6, mt-Nd1, or mt-Co1 genes encoded by the mitochondrial genome that require TFAM for transcription (Online Figure XIVB). We assessed neonatal cardiomyocyte proliferation at P5 by immunostaining tissue sections for Ki67 (Figure 6H and 6I) or pH3 (Online Figure XIVC and XIVD). Consistent with the results from cultured fetal cardiomyocytes, Tfam ablation strongly reduced neonatal cardiomyocyte proliferation (Figure 6H and 6I, Cre+/Veh group). Treatment with MT increased cell cycle activity of Cre-recombined cardiomyocytes (Figure 6H and 6I, Cre+MT versus Cre+/Veh in tdTomato+ cardiomyocytes; Online Figure XIVC and XIVD). Interestingly, MT also increased cell cycle activity of non-recombined (tdTomato−) cardiomyocytes (Figure 6H and 6I, Cre+MT versus Cre+/Veh in tdTomato− cardiomyocytes), in keeping with reports that physiological increases in ROS during the neonatal period promote cell cycle exit.17 WEE1 inhibition with MK did not enhance cell cycle activity of tdTomato− cardiomyocytes, but it strongly increased cell cycle activity of tdTomato+ cardiomyocytes, restoring the Ki67+ cardiomyocytes to levels comparable to tdTomato+ controls (Figure 6H). These data indicate that MT and MK treatments inhibit ROS and DNA damage to rescue proliferation without ameliorating the effect of Tfam ablation on mitochondrial abundance or gene expression.

This result was corroborated by an independent approach. The dose of AAV9-Cre that we administered created a genetic mosaic in which tdTomato+ cardiomyocytes lack Tfam and tdTomato− cardiomyocytes are replete in Tfam. Therefore, the fraction of tdTomato+ cardiomyocytes is an independent
Figure 6. Inhibition of reactive oxygen species (ROS)–activated DNA damage response ameliorates cardiomyopathy in vivo. A, Experimental set-up and timeline. AAV9-Cre was injected at postnatal day 0 (P0). Experiment A examined the effect of AAV dose. Experiment B examined the effect of treatment with MitoTEMPO (MT) or MK-1775 (MK) during either the first or second postnatal week (groups 1 and 2, respectively). Echocardiography and tissue analyses were performed at the indicated times. B, The percentage of tdTomato+ cardiomyocytes (CMs) observed after AAV9-Cre injected. Tfamfl/fl; Rosa26tdTomato mice were treated with high- or low-dose AAV9-Cre, whereas Tfamfl/+; Rosa26tdTomato mice were treated with high dose (CON). C, Representative images of P28 CMs isolated from Tfamfl/+; Rosa26tdTomato or Tfamfl/fl; Rosa26tdTomato mice and control mice treated with AAV9-Cre at P0. CMs are stained for TFAM and CAV3 (caveolin 3), a transverse tubule (T-tubule) marker. D, E, Echocardiographic parameters from CON, low-dose, and high-dose mice. Shaded regions indicate standard error of the mean at each time point. F, G, Effect of MT or MK treatment on heart function when given in postnatal week 1 (group 1) or 2 (group 2). H, I, Representative heart tissue sections (H) stained for Ki67 at P5 after treatment with Cre+Vehicle, Cre+MT, or Cre+MK. Quantification of the percentage of Ki67+ CMs is shown in I. n=4. J, K, Representative heart tissue sections (J) from Cre+Vehicle-, Cre+MT-, or Cre+MK-treated mice. Sections were stained for WGA (wheat germ agglutinin) and DAPI and imaged for endogenous tdTomato fluorescence at 8 weeks. Bar, 200 µm. Quantification of the percentage of tdTomato+ CMs is shown in K. n=4. t test: *P<0.05. **P<0.01. AAV indicates adeno-associated virus; FS, fraction shortening; LVID;d, left ventricular internal diameter at end diastole; and TFAM, mitochondrial transcription factor A.
measure of the relative proliferation of these populations. In heart sections from mice treated with AAV9-Cre at P0 and analyzed at 8 weeks of age, the tdTomato+ cardiomyocyte fraction was lowest in the Cre group and significantly higher in both the Cre+MT and Cre+MK groups (Figure 6J and 6K), consistent with the measurements of cell cycle activity. In contrast, in heart sections from mice treated with AAV9-Cre at P8 and analyzed at 8 weeks of age, the proportion of tdTomato+ cardiomyocytes was similar between control and Tfam mutant hearts (Online Figure XIIIIB), consistent with Tfam inactivation at this stage having little effect on cardiomyocyte cell cycle activity.

These data collectively support a model in which mitochondrial dysfunction triggered by Tfam inactivation decreases neonatal cardiomyocyte proliferation through ROS-mediated DNA damage and activation of the G2/M cell cycle checkpoint. This impaired neonatal cardiomyocyte cell cycle activity contributes to adult ventricular dysfunction. Alleviating the inhibition of cardiomyocyte cell cycle activity, either by ROS scavenging or WEE1 kinase inhibition, improves cardiomyocyte cell cycle activity and thereby partially rescues heart function.

**Mitochondria Are Dispensable for Postnatal Cardiomyocyte Maturation**

During the neonatal period, cardiomyocytes undergo a dramatic phenotypic switch that includes tremendous physiological cardiomyocyte hypertrophy, increased sarcomere organization, and formation of T-tubules. Concurrent with these structural changes is a switch from glycolytic to oxidative metabolism. In cardiomyocytes derived from stem cells, metabolic maturation influences cardiomyocyte differentiation, and a similar role in neonatal cardiomyocyte maturation has been hypothesized. Because Tfam is essential for normal cardiomyocyte metabolic maturation, we tested this hypothesis by assessing the postnatal maturation of Tfam-deficient cardiomyocytes. Previously, we showed that inactivation of genes in a fraction of cardiomyocytes is a powerful approach to study cell-autonomous function without impairing viability or eliciting nonspecific effects caused by cardiac dysfunction. Low-dose AAV9-Cre ablated Tfam in transduced cardiomyocytes marked by tdTomato expression (Figure 6C; Online Figure XIIIB) without measurably impairing cardiac function (Figure 6D and 6E). Therefore, we took advantage of this model to interrogate the effect of Tfam depletion on cardiomyocyte maturation.

Tfam−/−; Rosa26ΔTomato and Tfam+/−; Rosa26ΔTomato mice were treated with low-dose AAV9-Cre at P0. We characterized the kinetics of mitochondrial dysfunction in these mice by measuring the expression of mt-Atp6, mt-Nd1, and mt-Co1 genes encoded by the mitochondrial genome that require TFAM for transcription. After heart dissociation, tdTomato+ cardiomyocytes were isolated by flow cytometry, and gene expression was measured by quantitative real-time PCR. By P14, all 3 transcripts were reduced by over 50%, and by P28 their expression was reduced by 85%, 80%, and 80%, respectively, compared with littermate Tfam+/− controls (Online Figure XVA). These data suggest that mitochondrial dysfunction occurs rapidly in this model, within a window that would be anticipated to impact perinatal cardiomyocyte maturation.

We studied the cell-autonomous effect of mosaic Tfam ablation on cardiomyocyte maturation at 4 weeks of age. We used in situ confocal imaging, in which intact hearts are optically sectioned by confocal imaging, to examine T-tubule structure. Mitochondria and T-tubules were imaged by perfusion with mitoTracker and the voltage-sensitive membrane dye FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-diethylamino)phenyl) hexatrienyl) pyridinium dibromide). These data showed that tdTomato+ cells from Tfam−/− hearts had disrupted mitoTracker staining, consistent with effective Tfam depletion and mitochondrial disruption, yet had preserved T-tubule morphology (Figure 7A). We quantitatively analyzed T-tubule morphology of tdTomato+ cells from low-dose AAV9-Cre–treated Tfam−/− and Tfam+/− tdTomato+ cardiomyocytes (Figure 7H and 7I). There was no significant difference between genotypes. These data show that development of mature T-tubules, often considered a hallmark of cardiomyocyte maturation, does not require normal mitochondrial function.

To interrogate sarcomere structure, we dissociated cardiomyocytes by collagenase perfusion and immunostained them for sarcomeric α-actinin, which labels Z-lines (Figure 7C). Sarcomeric organization was also quantified using AutoTT. We found no significant difference in sarcomere regularity or spacing between AAV9-Cre–treated Tfam−/− and Tfam+/− tdTomato+ cardiomyocytes (Figure 7H and 7I). These results indicate that normal mitochondrial function is not required for attaining mature sarcomere organization.

Physiological hypertrophy of cardiomyocytes is another hallmark of cardiomyocyte maturation. We measured the length, length/width ratio, and area of dissociated, tdTomato+ cardiomyocytes from Tfam−/− or Tfam+/− hearts (Figure 7J through 7L). As with T-tubule and sarcomere organization, these parameters of cardiomyocyte hypertrophy were no different between genotypes, indicating that normal mitochondrial function is not required for physiological cardiomyocyte growth.

Switching of sarcomere isoforms is another hallmark of postnatal cardiomyocyte maturation. We assessed the exchange of Myh7 (immature) to Myh6 (mature) and the switch of Tnni1 (troponin I; immature) to Tnni3 (mature). This latter isoform switch is specific to maturation and insensitive to cardiomyocyte stress. We measured Tnni3, Tnni1, Myh6, and Myh7 transcript levels by quantitative real-time PCR (Online Figure XVIB). Expression of these genes, as well as the Tnni3/Tnni1 and Myh6/Myh7 ratio, was not significantly changed by Tfam inactivation, indicating that these gene expression markers of cardiomyocyte maturation were not perturbed by Tfam ablation.

Although Tfam inactivation had minimal effect on structural aspects of cardiomyocyte maturation, it did significantly impact 2 aspects of cardiomyocyte function: intracellular Ca2+ handling and contraction. To evaluate the effect of Tfam inactivation on intracellular Ca2+ handling and contraction, we treated Tfam+/−, Rosa26ΔTomato and Tfam−/−; Rosa26ΔTomato mice with low-dose AAV9-Cre at P1, dissociated cardiomyocytes at P28, and loaded them with the Ca2+-sensitive dye Fluoro-4. Ca2+ transients were measured by confocal line scan during 1 Hz pacing (Figure 8A). Tfam-deficient cardiomyocytes exhibited significantly lower Ca2+ transient amplitude (F/F0; Figure 8A and 8B). On the other hand, time to peak was not significantly different in Tfam-deficient cardiomyocytes (Figure 8A and 8C), consistent with the preserved T-tubule structure that we observed in these cells. Tfam inactivation also did not significantly affect
Figure 7. Mitochondria are dispensable for morphological postnatal cardiomyocyte (CM) maturation. A, In situ confocal live imaging of mitochondria and transverse tubules (T-tubules). Control and mosaic TFAM (mitochondrial transcription factor A)-depleted postnatal day 28 (P28) hearts were perfused with Mitotracker and FM4-64 (N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrieny1) pyridinium dibromide) and optically sectioned. B, In situ confocal live imaging of T-tubules in FM4-64 loaded P28 hearts after control or mosaic TFAM depletion. The T-Tubule patterns in white boxes were enlarged and skeletonized using AutoTT. C, Imaging of sarcomeres in P28 CMs dissociated from hearts after control or mosaic TFAM depletion. Sarcomeres were visualized by sarcomeric α-actinin (SAA) staining. The boxed areas were enlarged (third row) and skeletonized (fourth row) using AutoTT. D-G, AutoTT quantification of T-tubule patterns visualized by in situ imaging. H, I, AutoTT quantification of sarcomere organization in isolated CMs. J, K, Quantification of CM dimensions. Length and width of SAA-stained dissociated CMs was determined using ImageJ. Violin plots show the distribution of values and the median (circle), 25th and 75th percentiles (thick line), and 1.5× the interquartile range (thin lines). Sample sizes are indicated by numbers above the abscissa. P values of intergroup comparisons using the Mann–Whitney U test are shown.
Figure 8. Cell-autonomous effect of *Tfam* (mitochondrial transcription factor A) inactivation on cardiomyocyte (CM) Ca\(^{2+}\) handling and contraction. A–D, Analysis of Ca\(^{2+}\) handling. Postnatal day 28 (P28) dissociated CMs were loaded with the Ca\(^{2+}\)-sensitive dye Fluo-4 AM and imaged by confocal line scan during 1 Hz pacing. A, Representative Ca\(^{2+}\) transient profiles are shown. Ca\(^{2+}\) transient magnitude, time to peak, and time to 50% recovery were quantitative analyzed (B–D). E–I, Analysis of contraction. Bright-field images of dissociated P28 CMs contracting during 1 Hz pacing were recorded. Image analysis yielded sarcomere length of live cells (E) and length–time relationship during contraction (F). Analysis of the length–time relationship yielded the fractional CM shortening (G), departure velocity (H), and return velocity (I). Violin plots show the distribution of values and the median (circle), 25th and 75th percentiles (thick line), and 1.5× the interquartile range (thin lines). Numbers within graph legend indicate the number of CMs analyzed. These CMs were isolated from 4 hearts per group. Groups were compared by Mann–Whitney *U* test. *P*<0.05. **P*<0.01. ***P*<0.001.
time to 50% decay (Figure 8A and 8D), a measure of the kinetics of cytosolic Ca\(^{2+}\) return to diastolic levels.

To assess the effect of Tfam inactivation on cardiomyocyte contraction, we prepared dissociated Tfam\(^{-/-}\), Rosa26\(^{T}D\)Tomato and Tfam\(^{+/+}\); Rosa26\(^{T}D\)Tomato cardiomyocytes as described for the Ca\(^{2+}\) handling experiments. Bright-field images of the cardiomyocytes were obtained during 1 Hz pacing. Consistent with immunostaining of fixed cells, sarcomere length from these live cells was not different between Tfam-deficient and control cardiomyocytes (Figure 8E). However, Tfam-deleted cardiomyocytes had significantly reduced shortening and decrease Ca\(^{2+}\) transient amplitude. Tfam-deficient cardiomyocytes did not have significantly altered return velocity, suggesting that cardiomyocyte relaxation was not significantly affected (Figure 8I). These data indicate that Tfam-deficient cardiomyocytes have reduced contractility compared with control cardiomyocytes.

Collectively, these data support the surprising conclusion that morphological cardiomyocyte maturation is not dependent on their metabolic maturation. However, Tfam is required for normal cardiomyocyte Ca\(^{2+}\) handling and contraction.

### Discussion

Although cardiomyopathy and abnormal myocardial morphologies such as left ventricular noncompaction and hypertrophic cardiomyopathy are associated with mitochondrial disease, the mechanisms linking mitochondria to these morphological findings have been understudied. Although energy depletion likely contributes to these phenotypes, our study highlights the effect that mitochondrial dysfunction has on another basic process of cardiac development, namely cardiomyocyte proliferation. We observed that mitochondrial dysfunction triggered by Tfam ablation induces cell cycle arrest that contributes to the resulting mitochondrial cardiomyopathy. Protecting cardiomyocytes from cell cycle arrest induced by mitochondrial dysfunction improved cardiac function for weeks beyond the treatment period, suggesting a potential therapeutic strategy for mitochondrial cardiomyopathies.

Although Tfam inactivation reduced mitochondrial mass, we observed that it increased mitochondrial ROS in cardiomyocytes. The effect of Tfam inactivation on mitochondrial ROS seems to be cell type dependent because it was previously observed to elevate ROS in adipocytes\(^8\) and reduce ROS in keratinocytes.\(^9\) The cell types in which it elevated ROS (cardiomyocytes and adipocytes) are normally rich in mitochondria, suggesting a potential factor responsible for the difference between cell types. A potential mechanism that accounts for elevated ROS in Tfam-deficient cardiomyocytes is the markedly reduced expression of components of the electron transport chain encoded by the mitochondrial genome.\(^8\) The defective electron transport chain of Tfam-deficient cardiomyocytes may be predisposed to produce ROS. In normal mitochondria, membrane depolarization accelerates electron transit through the electron transport chain and thereby reduces ROS production\(^11\); however, increased activity of the defective electron transport chain of Tfam-deficient cardiomyocytes may elevate ROS production.

Elevated ROS in Tfam-depleted cardiomyocytes induced the DNA damage response, including deposition of γH2A.X. As a result, inhibitory cell cycle checkpoints were activated, resulting in reduced cardiomyocyte cell cycle activity. Elevated cardiomyocyte ROS triggered by increased mitochondrial oxidative phosphorylation in the oxygen-rich postnatal environment has been proposed to trigger physiological cell cycle arrest of neonatal cardiomyocytes through a similar mechanism.\(^7\) That WEE1 kinase inhibition alleviated cell cycle exit induced by Tfam inactivation suggests that the main checkpoint occurred at the G2/M transition, where cell cycle progression requires CDC25 dephosphorylation of CKD1 to overcome its phosphorylation by WEE1 kinase.\(^22\)

Our data suggest that inhibition of cardiomyocyte cell cycle activity may be an important contributor to some forms of mitochondrial cardiomyopathy. In our model, mitochondrial dysfunction was triggered in neonatal mice by delivery of AA\(^{AV9}\)-Cre at P0 or P1. Remarkably, transient treatment of neonatal mice during the first postnatal week with either ROS scavenger or WEE1 inhibitor ameliorated heart dysfunction as much as 7 weeks later. That this same treatment did not have benefit when given in the second postnatal week suggests that these treatments primarily work by maintaining cardiomyocyte cell cycle activity because the therapeutic window coincides with the period during which neonatal cardiomyocytes normally retain cell cycle activity. A corollary to this interpretation is that increased abundance of Tfam-deficient cardiomyocytes supports increased cardiac contraction. Indeed, we observed that Tfam-deficient cardiomyocytes do contract, albeit more weakly than littermate control cardiomyocytes. The important contribution of reduced cardiomyocyte proliferation to the Tfam mutant cardiomyopathic phenotype was further corroborated by the observation that Tfam inactivation at P8 (after cardiomyocyte cell cycle exit) was less detrimental to heart function than its inactivation at P1 (during cardiomyocyte proliferation). In future work, it will be important to determine the durability of the improvement in heart function induced by MT or MK treatment. It is interesting to consider that some human cardiomyopathies, such as those caused by a subset of mitochondrial diseases, might exhibit a similar window during which myocardial outcome could be improved by neonatal therapy. Further research will be needed to evaluate the relevance of our observations in the Tfam-knockout model to human mitochondrial cardiomyopathy.

Neonatal cardiomyocytes undergo many phenotypic changes that convert them from proliferative fetal cells with relatively low pumping capability to mature, terminally differentiated, adult cardiomyocytes with much higher pumping capacity.\(^25\)\(^,\)\(^26\) Accompanying these changes is a metabolic switch from glycolysis to oxidative phosphorylation, and this metabolic switch has been hypothesized to help to drive the other phenotypic changes.\(^27\)\(^,\)\(^28\) Using postnatal mosaic Tfam inactivation, we critically tested this hypothesis. Surprisingly, we found that cardiomyocyte growth, sarcomere organization, and T-tubule formation were unaffected by neonatal Tfam inactivation. These data suggest that mitochondrial expansion and robust function are not prerequisites for morphological cardiomyocyte maturation in vivo, although they are required for normal Ca\(^{2+}\) handling and contraction. However, this result does
not exclude the possibility that enhancing metabolic maturation of iPSC (induced pluripotent stem cells) cardiomyocytes will expedite their morphological and functional maturation.

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**Disclosures**

None.

**References**


Mitochondrial Cardiomyopathy Caused by Elevated Reactive Oxygen Species and Impaired Cardiomyocyte Proliferation

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Methods

Animal

All animal procedures were performed following protocols approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital. Tfam<sup>fl/fl</sup>, Nkx2-5<sup>RES-CRE</sup>, ROSA26<sup>CreERT2</sup>, and Rosa26<sup>tdTomato</sup> mice were described previously<sup>1–4</sup>. Mice were PCR genotyped using primers listed in Online Table I. Timed matings were performed with noon of the date of the vaginal plug defined as embryonic day (E) 0.5. Tamoxifen (Sigma T5648) was dissolved in sunflower seed oil at 2 mg/ml. 50 µl were given by gavage to pregnant Tfam<sup>fl/fl</sup>, ROSA26<sup>CreERT2</sup> mice with final concentrations as 0.01mg/g and 0.02mg/g to mice body weight at E8.5. AAV9-TnT-Cre was described previously.<sup>5</sup> AAVs were subcutaneously injected into Tfam<sup>fl/fl</sup>, Rosa26<sup>tdTomato</sup> or Tfam<sup>fl/fl</sup>, Rosa26<sup>tdTomato</sup> mice at P1 or P8. AAV doses used were 4.0×10<sup>10</sup> vg/g (high dose) or 8.0×10<sup>9</sup> vg/g (low dose). Echocardiography was performed on conscious mice using a Vevo 2100.

Fetal CM Culture

E15.5 Tfam<sup>fl/fl</sup>; Rosa26<sup>tdTomato</sup> fetal CMs were isolated using the Neomyt Kit (Cellutron, NC-6031). Cells from fetal hearts were plated on 1% Geltrex (Thermo Fisher Scientific, A1413301)-coated plates in CM culture media (low glucose DMEM (Gibco), 5% horse serum (American Type Culture Collection, 30-2040), 2% chicken embryo extract (VWR, 100356-958)) plus 10 µm Y27632 ROCK inhibitor (StemCell Technologies, 72304). The following day (day 1), adenovirus (Ad-TnT-Cre (Ad:Cre) or Ad-TnT-LacZ (Ad:LacZ)) was added at a multiplicity of infection of 10, which caused >90% CTNT<sup>+</sup> cells to become TdTomato<sup>+</sup>. The following day, the medium was replaced with CM culture medium and subsequently changed daily. Where indicated, 5-ethynyl-2´-deoxyuridine (EdU, Life Technologies, C10337) was added at a final concentration of 10 µM and incubated for 24 hours. MitoTEMPO (Sigma Aldrich, SML0737) (final concentration 1 µM) and WEE1 kinase inhibitor MK-1775 (Selleck, S1525) (final concentration 1 µM) were added daily from day 1 to day 5.

Where indicated, cells were cultured under hypoxic conditions using a modular incubator chamber (Billups-Rothenberg, MIC101). E15.5 Tfam<sup>fl/fl</sup>, Rosa26<sup>tdTomato</sup> fetal CMs were dissociated as described above and plates were placed in the chamber and infused with pre-mixed gas containing 7% Oxygen, 5% Carbon Dioxide and Balanced Nitrogen (Airgas). The chamber was then incubated in a standard CO2 incubator at 37 °C.

Mitochondrial morphology and function

Fetal hearts were collected at E15.5 and fixed with glutaraldehyde-formaldehyde (2% formaldehyde and 2.5 % glutaraldehyde in 0.1 M Sodium Cacodylate buffer, pH 7.4) overnight. Sections were imaged using a JEOL 1200EX electron microscope (Harvard Medical School EM core facility). Mitochondrial size and density were measured using ImageJ.

E15.5 Tfam<sup>fl/fl</sup>, Rosa26<sup>tdTomato</sup> fetal CMs were isolated, and 20,000 cells were seeded in CM culture media in 1% Geltrex-coated Seahorse Biosciences XF96e assay wells. Ad:Cre or Ad:LacZ were added on the following day, and culture medium was changed daily for 5 days.
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Cell Mito Stress Kit (Seahorses Biosciences, 103015) on a XF96e extracellular flux analyzer (Seahorses Biosciences). Data were normalized to cell number, measured by DAPI staining of culture plates. OCR was expressed as pmol/min/2,000 cells. Based on OCR changes after addition of oligomycin (1 µM), FCCP (1 µM) or antimycin/rotenone (0.5 µM) in sequence, mitochondrial function metrics were calculated as described in the Cell Mito Stress Kit manual. ECAR was expressed as µH/min/2,000 cells.

To measure mitochondrial membrane potential (MMP), E15.5 Tfnfl CMs were dissociated and treated with Ad;Cre or Ad;LacZ as described above. On day 5 of culture, cells were incubated with 10 µM JC-1 (Life Technologies, T3168). Cells were imaged on an Olympus FV1000 inverted laser scanning confocal microscope. Quantitative analysis was performed by flow cytometry using a Propel Laboratories Avalon cytometer with a 100 µm nozzle and standard GFP/RFP filter sets. Data were analyzed by using FlowJo software. As a positive control for mitochondrial depolarization, cells were treated with 1 µM FCCP.

ADP/ATP ratio was measured with isolated E15.5 CMs after 3 and 7 days of culture, using the ADP/ATP Ratio Assay Kit (Bioluminescent) (Abcam, ab65313).

**Measurement of ROS levels**

In vitro cultured Tfnfl; Rosa26tdTomato CMs treated with Ad;Cre or Ad;LacZ were staining with CellROX Green (5 µM; Thermo Scientific, C10444) at 37°C for 0.5 hour and then washed three times with PBS. Cells were lifted for flow cytometry using 0.25% trypsin, then pelleted and resuspended in 200 µl culture medium. As a positive control, CMs were also treated with 50 µM H₂O₂ for 10 min.

Mitochondrial ROS was measured using Mitosox (Thermo Scientific, M36008). Tfnfl CMs were treated with Ad;Cre or Ad;LacZ and cultured for 5 days. Then they were stained with 5 µM Mitosox and 1 µg/ml Hoechst (Thermo Scientific, 62249) for 30 min. Cells were imaged using an Olympus FV1000 confocal microscope, or analyzed by flow cytometry. Data were analyzed using FlowJo software.

**Histology and immunostaining**

Tissues were fixed in 4% paraformaldehyde overnight, cryoprotected by overnight incubation in 30% sucrose, and embedded in tissue freezing medium (VWR, 25608-930). 8 µm cryosections were obtained using a cryostat (Thermo Scientific, Micron HM 550). Sections and cells were incubated in blocking medium (PBS containing 5% donkey serum, 0.2% Triton X-100) at 4°C overnight. TUNEL staining was performed using the TUNEL Assay kit (Abcam, ab66108). For immunostaining, sections were incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Primary antibodies used were as followed: Ki67 (1:200, Neomarkers, RM-9106-S), γ-H2AX (1:200, Abcam, ab26350), phosphohistone H3 (pH3; 1:200, Abcam, ab47297), SAA (1:200, Abcam), cardiac troponin T (1:200, Abcam, ab45932), ATP5B (ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit; 1:200, Abcam, ab14730), TFAM (1:200, Abcam, ab131607), caveolin-3 (CAV3, 1:200, Santa Cruz, sc-7665), 8-oxo-guanine (1:200, Thermo Scientific, MAB3560M1), and 4-Hydroxy-nonenal (1:200, Abcam, ab46545). After washing with blocking buffer, the slices were incubated with optimal secondary antibodies with/without WGA and/or DAPI at room temperature for 2 h. After washing, samples were mounted with ProLong Diamond antifade mountant (Invitrogen, 36961) and imaged using an Olympus FV1000 confocal microscope. Sarcomere structure was quantified using AutoTT®.
**Adult CM characterization**

CM isolation for immunofluorescence analyses was performed as previously described. In brief, collagenase (Worthington, LS004176) was perfused throughout the heart using a Langendorff apparatus at 37 °C for 10 min. CMs were next mechanically dissociated into a single cell suspension and briefly cultured on laminin-coated coverslips for 30 min. After CMs attached to the coverslip, they were fixed with 4% paraformaldehyde for 10 min and stored in blocking medium at 4 °C until immunostaining.

For in situ T-tubule imaging, 100 µg/ml FM 4-64 (Invitrogen, 13320) membrane dye was perfused in the heart for 10 min at room temperature through a Langendorff apparatus. For in situ mitochondrial imaging, 1 µg/ml MitoTracker (Invitrogen, M7514) mitochondrial dye was perfused in the heart for 10 min at room temperature through a Langendorff apparatus. The whole heart was then positioned on an inverted Olympus FV1000 confocal microscope as described.

AutoTT-based quantification of T-tubule organization was described previously.

**Flow cytometry**

3 days after AAV injection of Tfm^fl/fl^, Rosa26^tdTomato^ or Tfm^fl/+^; Rosa26^tdTomato^ P1 mice, hearts were dissociated using the Neomys kit (Cellutron). Freshly isolated CMs were filtered through a 70 µm cell strainer and centrifuged at 1200 x g for 5 min. The pelleted CMs were resuspended in 0.5 ml of culture medium. Fluorescence data were collected on a Propel Laboratories Avalon cytometer with a 100 µm nozzle. Data were further analyzed using FlowJo software.

For flow cytometry of CMs from 4- and 8-week-old mice, CMs were isolated using a Langendorff system, passed through a 100 µm cell strainer, and centrifuged at 20 x g for 5 min. Then CMs were resuspended in 1 ml culture medium and sorted using a BD Ariall SORP cell sorter.

**Cardiomyocyte Contraction Measurements**

Tfm^fl/fl^, Rosa26^tdTomato^ and Tfm^fl/+^; Rosa26^tdTomato^ mice were injected with AAV9-TnT-Cre at P1. Eight weeks later, ventricular CMs were isolated by Langendorff perfusion. The CMs were allowed to settle in a pacing chamber for 5 minutes prior to initiation of laminar flow from an in-line solution heater set to 37 °C. Cell contraction was measured by bright-field microscopy with external field pacing at 1 Hz using the IonWizard acquisition system (IonOptix Corp., Milton, MA, USA). Contraction parameters were automatically quantified and averaged over at least 10 contractions for each cell. Cells were isolated from 2-3 mice for each condition.

**Intracellular Ca^{2+} imaging**

Intracellular Ca^{2+} recordings were performed after loading isolated CMs from Tfm^fl/fl^, Rosa26^tdTomato^ and Tfm^fl/+^; Rosa26^tdTomato^ mice 8 weeks post AAV injection at P1 with Fluo-4 AM (8 µM, Invitrogen, F14217) for 30 min. After loading, CMs were subsequently washed with normal Tyrode solution (140 mM NaCl; 4 mM KCl; 1 mM MgCl_2; 1.8 mM CaCl_2; 10 mM Glucose; and 5 mM HEPES, pH 7.4, adjusted with NaOH) to remove the excess dye for 20 min. Cells were electrically stimulated at 1 Hz to produce steady-state conditions. All image data were acquired using an FV1000 confocal microscope in the line scanning mode along the long axis of the cell. The line scan was positioned in the cytosol, avoiding the nuclear area. Ca^{2+} levels were reported as F/F_0, where F_0 is the resting Ca^{2+} fluorescence.
**Western blot**

Fetal heart tissues at E15.5 from Tfam^fl/fl^, Nkx2-5^IRESCRE^I+/+ and Tfam^fl/fl^, Nkx2-5^IRESCRE^I+/+ mice and FACS sorted tdTomato+ CMs from Tfam^fl/fl^; Rosa26^tdTomato+ and Tfam^fl/fl^; Rosa26^tdTomato+ mice were lysed in RIPA lysis buffer system (Santa Cruz Biotechnology, sc-24948) with Mini Protease Inhibitor Cocktail Tablets (cOmplete, 4939124001). Total protein concentrations were normalized using BCA analysis (Life Technologies, 23227). After boiling with 4X loading buffer for 5 min, 20 μl cell lysate of each sample was separated on a 10% gradient gel (Invitrogen, Bolt gels, NW00100BOX), transferred to a PDVF membrane, and blocked by 4% BSA/TBST. Primary antibodies of γ-H2AX (1:2000, Abcam, ab26350), ATP5B (1:2000, Abcam, ab14730), P21 (1:2000, Abcam, ab109520), TFAM (1:2000, Abcam, ab131607), 4-Hydroxy-nonenal (1:2000, Abcam, ab46545) and WEE1 (1:2000, Abcam, ab137377) were incubated with the membrane overnight at 4°C. After washing, membranes were incubated with HRP-conjugated secondary antibodies for 45 min. Immunoblots were then treated with Immobilon Western chemiluminescent HRP substrate (Millipore, WBKLS0500). Chemiluminescence were detected using a Li-Cor C-DiGit blot scanner.

**RNA-seq and gene expression**

E13.5 hearts were flash frozen in liquid nitrogen. 5 hearts were pooled for each replicate, and 3 replicates were performed for mutant (Tfam^fl/fl^, Nkx2-5^IRESCRE^I+/+) and control (Tfam^fl/fl^, Nkx2-5^IRESCRE^I+/+) groups. Polyadenylated RNA was isolated by Dynabeads® Oligo(dT). Sequencing libraries were constructed using Script-Seq v2 (Illumina) and sequenced on an Illumina HiSeq 2000 (50 nt single end). Transcript abundance was determined by TopHat alignment® followed by HTSeq-Count® and statistical analysis by DESeq2 RNA-seq data were deposited at GEO (GSE98579). Gene set enrichment analysis was performed using GSEA®.

For Real-Time PCR, total RNA was extracted using RNeasy Mini Kit (Qiagen, 74104) and reverse transcribed by SuperScript III First-Strand Synthesis System (Invitrogen, 18080051). Real-time PCR was performed using a CFX96 Bio-Rad thermocycler with Power SYBR Green PCR kit (ThermoFisher, 4368702). Primers are listed in Online Table I.

**Statistics**

Data are shown as mean ± SD. Significance of intergroup differences was tested using Student’s t-test or the Mann-Whitney test as indicated (SPSS 20.0). Violin plots were made with BoxPlotR®.
Supplemental References


### Online Table I. Primers used in this study.

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<tr>
<th>Primer Name</th>
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<td><strong>Primers used in this study.</strong></td>
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<td><strong>Genotyping primers</strong></td>
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Online Figure. 1. Characterization of Tfm\textsuperscript{NK} heart. A. Morphology of Tfm\textsuperscript{NK} and control heart at E13.5, shown in H&E-stained transverse sections. Bar, 200 µm. B. Western blot showing TFAM, ATP5B and GAPDH protein level in control and Tfm\textsuperscript{NK} heart extracts at E15.5. C. Mitochondrial to nuclear DNA ratio (mtDNA/nDNA) in Tfm\textsuperscript{NK} and control heart at E15.5. n=5 per group. t-test: * p<0.05.
Online Figure II. Effect of Tfam inactivation on mitochondrial morphology. A. Mitochondrial morphology of E15.5 TfamNK and control hearts. Upper panel, 5000x. Lower panel, 15000x. Boxed area in top panels is enlarged in lower panels. Bar, 0.5 µm. B. Distribution of mitochondrial sizes in TfamNK and control CMs. C. Distribution of mitochondrial area fraction between TfamNK and control CMs. 10 images were measured per heart, n=3 hearts per group. Mann-Whitney U-test: **, P<0.01. ***, P<0.001.
Online Figure. III. Tфam inactivation in cultured fetal mouse CMs. E15.5 Tфam<sup>fl/fl</sup>; Rosa26<sup>tdTomato</sup> CMs were isolated and cultured, then treated with Ad:Cre or Ad:LacZ. After 48 hours, cells were stained for TFAM and mitochondrial marker ATP5B. Boxed regions are magnified in right panels. Yellow arrowheads highlight the abnormal size and location of mutant mitochondria. Bar, 200 µm.
Online Fig. IV. Effect of Tfam inactivation on cardiomyocyte metabolism. Cultured E15.5 Tfam^{fl/fl} CMs were treated with Ad:Cre or Ad:LacZ and then analyzed using Seahorses BioSciences Mito Stress Kit. A. Representative Seahorse BioSciences Mito Stress Kit measurement of oxygen consumption rate (OCR) from in vitro cultured E15.5 Tfam^{fl/fl} CMs treated with Ad:Cre or Ad:LacZ. OCR was normalized per 1000 CMs. B. Schematic diagram of mitochondrial functional parameters calculated from Mito Stress Kit OCR measurements. C. Mitochondrial functional parameters for control and Tfam-deficient CMs. Data is presented as mean ± SEM. D. Schematic diagram of glycolytic functional parameters calculated from Mito Stress Kit extracellular acidification rate (ECAR) measurements. E. Glycolytic functional parameters for control and Tfam-deficient CMs. Data is presented as mean ± SEM of ECAR, normalized per 1000 CMs. n=6 per group. t-test: *, P<0.05. **, P<0.01. ***, P<0.0001.
Online Figure. V. Tfam deletion caused relative depolarization of mitochondria. A. Mitochondrial membrane potential was assessed in in vitro cultured, Tfam-deficient CMs. E15.5 Tfam<sup>fl/fl</sup> CMs were isolated and cultured, then treated with Ad:Cre or Ad:LacZ. FCCP, which abrogates mitochondrial membrane potential, was used as a positive control. After 72 hours, cells were stained for JC-1, which stains depolarized mitochondria green and hyperpolarized mitochondria red. Bar, 50 µm. B. Flow cytometry-based measurement of JC-1 red-to-green fluorescence ratio. Data is presented as mean ± SEM. n=5 per group. t-test: * p<0.05; ** p<0.01, *** p<0.0001.
Online Fig. VI. Effect of Tflm inactivation on CM ADP/ATP ratio. The ratio of ADP to ATP was measured in CMs dissociated from E15.5 Tflm\textsuperscript{NK} or control hearts (A), or cultured Tflm\textsuperscript{fl/fl} CMs 3 (B) or 7 days (C) after treatment with Ad:Cre or Ad:LacZ (Con). Data is presented as mean ± SEM. For E15.5 CMs, n=3 per group. For cultured CMs, n=12 per group. t-test: **, P<0.01. ###, P<0.0001.
Online Figure. VII. Effect of Tfram inactivation on fetal CM proliferation and apoptosis. 

A. Immunofluorescent Ki67 staining demonstrated reduced CM proliferation at E15.5. Bar, 200 µm. 

B. Quantitation of Ki67+ CMs between TframNK and Control hearts at E13.5 and E15.5. n=6 per group. 

C. Quantitation of TUNEL+ CMs in TframNK and Control hearts at E13.5 and E15.5. n=6 per group. Data is presented as mean ± SEM. t-test: ** p<0.01.
Online Figure. VIII. Tomaxifen-induced mosaic Tfam knock out model. A. Flow chart of fetal tamoxifen-induced mosaic Tfam knock out experiment. Tamoxifen was administered at E8.5 and analyses were performed at E15.5. B. Sections of fetal hearts with tamoxifen-induced mosaic tdTomato activation were stained for cardiac marker cTnT. Nuclei were stained with DAPI. Bar, 100 µm. C. Quantitation of the frequency of tdTomato+ CMs after treatment with indicated dose of Tamoxifen. n=5 per group. D. Efficiency of Tamoxifen (0.01 mg/g)-induced TFAM knockout among tdTomato+ CMs. tdTomato fluorescence was detected in fetal heart sections stained for TFAM and nuclei (DAPI). White boxed areas are enlarged in the insets. Bar, 50 µm. E. Quantitation of the frequency of positive TFAM immunostaining among tdTomato+ CMs. n=5 per group. t-test: ** p<0.01.
Online Figure. IX. *Tfam* deletion in hypoxic cultured embryonic CMs inhibited proliferation. Cultured Tfam<sup>fl/fl</sup>; Rosa26<sup>tdTomato</sup> E15.5 CMs were treated with Ad:Cre or Ad:LacZ (control) 24h after plating. After culture for an additional 48 hours, CMs were fixed and stained. **A.** Representative image of Cre and LacZ treated cultured CMs under 7% oxygen hypoxia condition stained for pH3 and CM marker (SAA). Bar, 50 µm. **B.** Quantification of the percentage of pH3 positive cells in (C). n=4 per group. *t*-test: **, P<0.01.
Online Figure X. *Tfam* deletion caused elevated mitochondrial ROS. **A.** Mitosox staining of in vitro cultured, *Tfam*-deficient CMs. E15.5 *Tfam*<sup>fl/fl</sup> CMs were isolated and cultured, then treated with Ad:Cre or Ad:LacZ. Bar, 50 µm. **B.** Flow cytometry-based quantitation of Mitosox intensity between *Tfam*-deficient CMs and control. n=4. *t*-test: **p<0.01.
Online Fig. XI. *Tfam* deletion in cultured embryonic CMs activates the DNA damage response both in normoxic and hypoxic culture conditions. Cultured *Tfam*^{fl/fl}; Rosa26^{tdTomato} E15.5 CMs were treated with Ad:Cre or Ad:LacZ (control) 24 hours after plating. After culture for an additional 48 hours, CMs were fixed and stained. A. Representative image of Cre and LacZ treated cultured CMs stained for γH2AX and CM marker (cTnT). Bar, 25 µm. B. Quantification of the percentage of γH2AX⁺ CMs in (A). n=6. C. Representative image of Cre and LacZ treated cultured CMs under 7% oxygen hypoxia condition stained for γH2AX and CM marker (cTnT). Bar, 50 µm. D. Quantification of the percentage of γH2AX⁺ CMs in (D). n=4 per group. t-test: **, P<0.01. ***, P<0.0001.
Online Fig. XII. Postnatal Tfam inactivation by AAV9-Cre. **A.** Isolated CMs from AAV9-Cre High- or Low-dose treated Tfam<sup>fl/fl</sup>; R26<sup>tm</sup> were analyzed for tdTomato signal by FACS at P3. AAV9-TNT-Luc or untreated mice were used as negative controls. **B.** Quantification of TFAM immunostaining signal in tdTomato<sup>+</sup> CMs treated with AAV9-Cre. Low dose AAV was given at P0 and cells were analyzed at 4 weeks. n=26. Mann-Whitney: ***, P<0.01. **C.** Representative images of dissociated CMs from mice with the indicated genotype that were treated with AAV-Cre at P0 and analyzed at 4 weeks. Arrowheads point to CM nucleus, which was positive for 8oxo-G in the TFAM-depleted CM. **D.** AAV9-Cre treated hearts with indicated genotype were dissociated. FACS-purified tdTomato<sup>+</sup> CMs were used for immunoblotting at 4 weeks.
Online Fig. XIII. Postnatal *Tfam* inactivation by AAV9-Cre at P8. A. Representative heart function based on echo data among the first week *Tfam* deletion using AAV to delivery Cre at the beginning of the first postnatal week (P1) and the second week (P8). Shaded areas indicate SEM at each time point. n=4 per group. B. Percentage of tdTomato+ CMs in indicated treatment groups. *Tfam*<sup>fl/fl</sup>; Rosa26<sup>tdTomato</sup> mice were treated with high or low dose AAV9-Cre at P8, while control (Con) *Tfam*<sup>fl/+</sup>; Rosa26<sup>tdTomato</sup> mice were treated with high dose AAV9-Cre at P8. *t*-test: *, P<0.05. **, P<0.01.
Online Fig. XIV. MT or MK treatment after postnatal Tfam inactivation in cardiomyocytes. High dose AAV-Cre was given at P0, and mice were treated with MT or MK during the first postnatal week. A. Western blot at 8 weeks of age showed TFAM, ATP5B, tdTomato and GAPDH protein level in Cre, Cre+MT, Cre+MK and control heart extracts. B. qRT PCR for the transcript level of mitochondrial genes mt-Atp6, mt-Nd1 and mt-Co1 from RNA obtained from 8-week-old, FACSorted, tdTomato+ CMs. n=4 per group. C. Representative images of heart tissue sections stained for pH3 at P5. D. Quantification of the percentage of pH3+ CMs in (C). Bar, 50 µm. n=4 per group. t-test: *, P<0.05. ***, P<0.0001.
Online Fig. XV. Postnatal Tfam inactivation disrupted expression of intrinsic mitochondrial genes but did not alter expression of sarcomere gene isoforms. A. qRTPCR for transcript levels of intrinsic mitochondrial genes mt-Atp6, mt-Nd1 and mt-Co1 between Tfam-deficient and control CMs at P7, P14, P28 and P56. Mice were treated with lose dose AAV9-Cre at P1. At the indicated times, CMs were dissociated, tdTomato+ CMs were isolated by FACS and used for qRTPCR measurements. Shaded areas indicate SEM at each time point. n=4 per group. B. qRTPCR for transcript levels of maturation related genes Tnni3, Tnni1, Myh6 and Myh7, and showed no difference among Tfam deleted and control groups at P28. n=4 per group. t-test: *, P<0.05. **, P<0.01. ***, P<0.0001.