**Mechanism of TFAM Mediated Cardiomyocyte Protection***

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Mechanisms of TFAM Mediated Cardiomyocyte Protection*
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Abstract:

Although mitochondrial transcription factor A (TFAM) is a protective component of mitochondrial DNA and a regulator of calcium and reactive oxygen species (ROS) production, the mechanism remains unclear. In heart failure (HF), TFAM is significantly decreased and cardiomyocyte instability ensues. TFAM inhibits Nuclear Factor of Activated T cells (NFAT), which reduces ROS production; additionally, TFAM transcriptionally activates SERCA2a to decrease free calcium. Therefore, decreasing TFAM vastly increases protease expression and hypertrophic factors, leading to cardiomyocyte functional decline. To examine this hypothesis, treatments of 1.0 micrograms of a TFAM vector and 1.0 micrograms of a CRISPR-Cas9 TFAM plasmid were administered to HL-1 cardiomyocytes via lipofectamine transfection. Western blotting and confocal microscopy analysis show that CRISPR-Cas9 knockdown of TFAM significantly increased proteases Calpain1, MMP9 and regulators Serca2a, and NFAT4 protein expression. CRISPR knockdown of TFAM in HL-1 cardiomyocytes upregulates degratory factors, leading to cardiomyocyte instability. Hydrogen peroxide oxidative stress decreased TFAM expression and increased Calpain1, MMP9, and NFAT4 protein expression. TFAM overexpression normalizes pathological hypertrophic factor NFAT4 in the presence of oxidative stress.

Key Words: TFAM, Calpain1, MMP9, Serca2a, H$_2$O$_2$, NFAT
**Introduction**

TFAM is a promoter-specific enhancer of mtDNA, regulator of mitochondrial genes and mtDNA copy number, and a physical protector of mtDNA (Ekstrand et al. 2004; Kunkel et al. 2016). TFAM packages single copies of mtDNA, creating a mitochondrial nucleoid (Kunkel et al. 2015b; Wang et al. 1999). TFAM is transported from the nucleus into the mitochondria by chaperone HSP70 (Santos et al. 2014). Peroxidase enzymes reside within the nucleoid structure, mitigating oxidative damage to mitochondrial DNA (Larsson et al. 1998; Wang et al. 1999). TFAM’s physical protective function contributes to the mitigation of molecular abnormalities within the physiological myocardium, and in pathologies such as heart failure, TFAM is decreased. We seek to describe TFAM’s effects on protease/hypertrophic molecular expression when influenced by hydrogen peroxide, TFAM overexpression, and CRISPR/Cas9 vector for TFAM knockdown.

TFAM’s regulatory function to increase Serca2a transcriptional activity poses insight into its therapeutic potential (Watanabe et al. 2011). Serca2a, an ATP-dependent calcium transporter, reduces free cytoplasmic calcium by storing it in the sarcoplasmic reticulum (Kunkel et al. 2015b). Serca2a knockout models result in Heart Failure; Serca2a activity is significant for cardiomyocyte performance and contains translational components (Heinis et al. 2013; Lipskaia et al. 2010; Park and Oh 2013; Sikkel et al. 2014; Zsebo et al. 2014). TFAM’s regulation of Serca2a decreases cytoplasmic calcium levels, effectively decreasing protease Calpain1 expression. Calcium-driven calpain proteases are responsible for the proteolytic degradation of both contractile and cytosolic proteins (Kunkel et al. 2015a). Studies show that calpain up-regulation is a major factor in cardiomyocyte functional decline (Takahashi et al. 2006). Physical tethering interactions between the mitochondria and the sarcoplasmic reticulum play a role in calcium regulation (Chen et al. 2012; Li et al. 2015).

TFAM’s protective function to inhibit NFAT expression reduces cardiomyocyte hypertrophy (Fujino et al. 2012). NFAT transcriptionally activates hypertrophic genes. The calcium-calcmodulin-calcineurin cascade plays a role in the activation of T cells and related cytokines (Vandewalle et al. 2014). Cytosolic calcium activates the calcineurin/NFAT pathway,
leading to pathological hypertrophy (Prasad and Inesi 2012). NFAT regulates the expression of mitochondrial ROS-generating NADPH Oxidases (NOX), specifically NOX 2&4 (Williams and Gooch 2014). Mitochondrial NOX enzymes are a leading source of the ROS involved in cardiac pathology (Sorescu and Griendling 2002). TFAM inhibits ROS activator NFAT, decreasing MMP9 expression in cardiomyocytes. ROS-driven Matrix-metalloproteinases (MMPs) are zinc-dependent endopeptidases capable of degrading the extracellular matrix (ECM) of cardiomyocytes. We have assessed the expression of MMP-9 in cardiac hypertrophy (Givvimani et al. 2012) and described MMP upregulation in heart failure as it pertains to ROS generation (Kunkel et al. 2015a).

Although MMP 2 & 9 have increased activity in all cardiomyopic states (dilated, ischemic and inflammatory), MMP9 has the highest up-regulation in heart failure related cardiomyopathies (Romanic et al. 2001). Oxidative stress is evidently activating catabolic/proteolytic pathways, such as MMPs, in heart failure models. MMP9 is notably upregulated in pathological conditions of cardiovascular diseases (Iyer et al. 2016). In an earlier study, Siwik et al found that hydrogen peroxide oxidative stress induced MMP9 activity (Siwik et al. 2001). We have found that MMP9 triggers the signaling cascade leading to pathological cardiac remodeling in human heart end-stage heart failure (Moshal et al. 2006). Additionally, we have described the results of MMP9 knockout models and the ameliorating affects post AVF induced Heart Failure (Moshal et al. 2008). ROS-induced MMP9 is highly expressed in pathological human hearts (Moshal et al. 2006).

Using CRISPR technology, we analyzed the effects of knocking down TFAM in HL-1 cardiomyocytes. This study was performed to observe the effects of TFAM vector treatments on protease/hypertrophic expression. Studies have shown that neonatal ablation of TFAM results in decreased mitochondrial biogenesis and death (Larsson et al. 1998). Down regulation of TFAM causes mitochondrial oxidative phosphorylation dysfunction, resulting in increased ROS production and MMP activity (Mendelsohn and Larrick 2014). We have shown the intimate interaction between intramitochondrial translocation of calpain 1, resulting in increased oxidative stress and MMP9 expression (Moshal et al. 2006).

*Invivo* studies have analyzed the effects of TFAM overexpression, showing decreased myocyte hypertrophy, oxidative stress, protease expression, apoptosis, interstitial fibrosis,
increased mtDNA copy number, and cardiomyocyte stability in myocardial infarction/heart failure models (Hayashi et al. 2008; Ikeda et al. 2015; Ikeuchi et al. 2005; Kang et al. 2007). Additionally, TFAM protected cells from oxidative stress in many *invitro* models (Aguirre-Rueda et al. 2015; Thomas et al. 2012; Xu et al. 2009). Therefore, the purpose of this study is to elucidate TFAM’s role in cardiomyocyte stability through protease and hypertrophic regulation.

**Materials & Methods**

Chemicals and Antibodies

TFAM overexpression and CRISPR vectors were purchased from Genecopia (Rockville, Maryland). All primary antibodies were purchased from Abcam (Cambridge, United Kingdom). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Fluorescent secondary antibodies and lipofectamine 2000 reagent was purchased from Life Technologies (Carlsbad, California, USA).

Cell Culture

The HL-1 Cardiac Muscle Cell line was purchased from EMD Millipore (Darmstadt, Germany). Cells were grown in 25 and 75 cm$^2$ flasks using Claycomb Media supplemented with 10% FBS, 2 mM L-glutamine, 0.1 mM norepinephrine, and penicillin/streptomycin. The medium was changed every 24 hours. Cells were maintained in a 37°C incubator in an atmosphere of 95% O$_2$ and 5% CO$_2$ and passaged with Trypsin, as per EMD-Millipore protocol.

Cell Treatment

Cells were plated in 6-well plates at a count of 600 x 10$^3$ cells per well for protein isolation. For immunocytochemistry analysis, cells were plated in 8 well chamber slides at 100 x 10$^3$ cells per well. After 24 hours, fresh media was provided prior to treatments.

Abcam primary antibodies used are as follows: Calpain1 (ab28258), Serca2 (ab2861), NFAT4 (ab83832), TFAM (ab131607) & MMP9 (ab38898)

Vectors: Tfam Crispr-Cas9 Vector: Genecopia (MCP228370-SG01-1)
TFAM Overexpression Vector: Genecopia (NM_009360.4)
Transfection of HL-1 Cells with TFAM Vector/TFAM CRISPR-Cas9 Vector

CRISPR/Cas9 knockdown plasmid for TFAM and TFAM overexpression vector were transfected into HL-1 cardiomyocytes using the life technologies lipofectamine 2000 reagent. As per lipofectamine 2000 protocol 1.0 micrograms of TFAM vectors were incubated with Lipofectamine and Opti-MEM and then administered to 6-well plates/chamber slides with media lacking pen/strep for 48 hours. Cells were then washed with PBS and harvested for western blot analysis.

H₂O₂ Treatment

Hydrogen peroxide was administered to HL-1 cardiomyocytes divided into four treatment groups: Control, H₂O₂, TFAM + H₂O₂, TFAM. 127 micromolar H₂O₂ was administered to the cells for the last six hours of the 48 hour TFAM vector treatment.

Western Blot Analysis

Post a 48 hour treatment period, HL-1 cell protein was isolated using protein extraction buffer (RIPA lysis buffer, protease inhibitor cocktail and PMSF). Wells were scraped and the contents transferred to ependorf tubes. Lysates were spun in extraction buffer for 12 hours and then centrifuged at 12,000g for 15 min. Supernatant was transferred to new tubes and protein concentrations were analyzed via Bradford protein estimation assay. Samples were run on a 10/12% sodium dodecylsulfate (SDS)-polyacrylamide gel with Tris-glycine SDS buffer. The gel was transferred electrophoretically overnight onto a PVDF membrane at 4°C. The membrane was blocked with a 5% milk solution for 1 hour. Primary antibodies were diluted at a concentration 1:1000 in TBST and incubated on the membrane overnight. All membranes were washed in TBST solution 4 times and then incubated with secondary HRP conjugated antibody solution for 1 hour at room temperature. Four TBST washing steps followed before membranes were developed using a chemiluminescent substrate in a BioRad Chemidoc (Hercules, Calif.). Band intensity was determined using densitometry analysis. Beta-actin was used to normalize protein loading.
Immunocytochemistry

Post-cell treatment, media was aspirated from each well of the eight well chamber slide and cells were rinsed with PBS. Cells were fixed at room temperature in 4% paraformaldehyde solution for 20 min and washed 3 times with PBS. Blocking was performed with a mixed solution of 2% bovine-serum-albumin (BSA) and 0.025% Triton X-100. Cells were permeabilized for 1 hour. All primary antibodies were incubated in the chamber slide at a concentration of 1:100 overnight at 4°C. The wells were aspirated, washed 3 times and incubated for 90 min with the secondary antibody (1:200) at room temp. The chamber slides were washed 3 times more, and DAPI was added at 1:10,000 for 10 min. This analysis was observed via Confocal microscopy.

Statistical Analysis

Data are expressed as means ± SE. Statistical analysis was performed by Graphpad Prism software using a one-way ANOVA followed by a Bonferoni comparison test. \( P<0.05 \) was considered statistically significant.

Results

CRISPR Vector Significantly Increases Serca2a and Calpain1 Protein Expression

To observe the efficacy of the TFAM overexpression vector and the CRISPR-Cas9 knockdown vector for TFAM, we analyzed TFAM expression with both treatments. Through western blotting and confocal analysis, we saw significant decreases in TFAM protein expression with the CRISPR vector compared to the control (\( p < 0.001 \)) and the TFAM overexpression vector (\( p < 0.001 \)). Additionally, the TFAM overexpression vector showed considerable increases in TFAM expression compared to control (\( p < 0.05 \)) Fig. 1a & 3a.

Western blotting and confocal analysis were used to determine changes in Serca2a expression with both treatments. CRISPR vector significantly increased Serca2a expression in HL-1 cardiomyocytes as compared with the control groups (\( p < 0.001 \)) Fig. 1b & 3b.

Confocal analysis and western blotting show the CRISPR vector greatly increased Calpain1 expression as compared to control groups (\( p < 0.001 \)) Fig 1c & 3c.
**CRISPR Vector Significantly Increases NFAT4 and MMP9 Protein Expression**

Western blotting and confocal analysis show that the CRISPR vector significantly increased NFAT as compared to control groups. ($p < 0.05$) Fig. 2b & 3d. Comparisons between the TFAM vector and CRISPR vector were also notable, showing reduction of NFAT with TFAM vector treatment ($p < 0.025$) Fig 2a. MMP9 protein expression was significantly increased in HL-1 cardiomyocytes treated with the CRISPR vector as compared to the control and TFAM vector treatment ($p < 0.001, p < 0.05$) Fig 2b.

**Hydrogen Peroxide Induced Protease Activity is Attenuated by TFAM Treatment**

$H_2O_2$ treatment to HL-1 cardiomyocytes reduced TFAM protein expression ($p < 0.001$). Conclusive to our hypothesis, western blotting and confocal analysis show that TFAM treatment with $H_2O_2$ increased TFAM levels compared to the $H_2O_2$ group alone, but was still reduced when compared with the control ($p < 0.05$) Fig 4a & 5a. Additionally, Calpain1 protein expression increased with ROS-induced stress ($p < 0.001$). TFAM treatment with $H_2O_2$ reduced calpain1 expression when compared to the $H_2O_2$ group alone ($p < 0.025$) but still maintained significantly higher Calpain1 levels than the control ($p < 0.05$) Fig 4b. Western blotting and confocal analysis show that NFAT protein expression was greatly increased by $H_2O_2$ ($p < 0.025$). TFAM treatment with $H_2O_2$ stress shows a significant reduction of NFAT levels in comparison to the $H_2O_2$ group alone ($p < 0.05$), normalizing the expression to that of the control. Additionally, there is a significant difference between the TFAM + $H_2O_2$ group and the TFAM vector ($p < 0.05$) Fig 4c & 5b. Western blotting and confocal analysis show that ROS-induced protease MMP9 was significantly increased by $H_2O_2$ compared to the control ($p < 0.001$). The TFAM treatment group had decreased MMP9 expression under ROS stress conditions when compared with the $H_2O_2$ group ($p < 0.05$), but MMP9 was still significantly increased as compared to the control group ($p < 0.05$) Fig 4d & 5c.

**Discussion**

It has been unanimously reported in the literature that increased protease expression of matrix-metalloproteinases and calpains within the pathological myocardium lead to degradation of extra-cellular matrix and connective/contractile proteins respectively. Translational factors towards minimizing cardiac remodeling rely on proteolytic regulation. Molecular applications toward reverse remodeling the post-injury myocardium are only breaching the surface. It is necessary to look further into TFAM’s role in reverse remodeling the injured myocardium and its...
translational impact. Since neonatal TFAM knockout models perish from dilated cardiomyopathy, it is safe to assume that degratory factors are significantly increased (Larsson et al. 1998). This observation has been assessed *invivo* and *invitro*. This *invitro* analysis represents the importance of TFAM’s role within cardiomyocytes and reveals a loss of stability when decreased, as observed in ischemia/HF models.

This is the first publication to use an *invitro* TFAM-specific CRISPR – Cas9 knockdown model in HL-1 cardiomyocytes. Overall findings within this study show that CRISPR- Cas 9 knockdown of TFAM reduces cardiomyocyte stability, as observed via increases in ROS activator/hypertrophic stimulator NFAT and increased protease levels. Additionally, this paper focuses on alterations in protease expression with TFAM treatments. TFAM knockdown via CRISPR – Cas 9 technology would increase proteases Calpain1, MMP9, and NFAT4 expression while reducing Serca2a expression (Heinis et al. 2013). We hypothesized that the TFAM overexpression vector would attenuate protease expression, increase Serca2a levels, and inhibit NFAT4 expression.

To determine the effect of the TFAM vector treatments we evaluated the protein and immunolocalization of TFAM. TFAM vector significantly increased TFAM protein expression in HL-1 cardiomyocytes. TFAM protein expression is knocked down with the CRISPR vector treatment. As shown by the Watanabe group, TFAM transcriptionally activates Serca2a, reducing free calcium (Watanabe et al. 2011). With TFAM knockdown vector treatments we observed compensatory increases in Serca2a expression. Contrary to the original hypothesis, knocking down TFAM increased Serca2a expression within HL-1 cardiomyocytes. We observe this as an immediate compensatory mechanism to reduce free calcium in a stress-induced environment. This excess calcium invites proteolytic degratory enzyme Calpain1, a calcium-activated cysteine protease, to degrade cytoskeletal and contractile proteins. Interestingly, the excess calcium in the cytoplasm activates Calpain1 in the CRISPR Vector group in which Serca2a expression is significantly higher. Therefore, calcium-driven calpain1 levels remain higher than the compensatory activity of the Serca2a pump.

Treatment of the CRISPR vector to HL-1 cardiomyocytes showed significantly increased MMP9 protein expression. MMP9, a ROS activated protease, is highly expressed in heart failure/hypoxic conditions (Ikeda et al. 2015). Analysis of MMP9 expression in the CRISPR vector treatment group revealed a significantly high MMP9 expression when compared
to the control. This data suggests that lack of TFAM creates a ROS buildup, inducing MMP9 expression.

NFAT transcriptionally regulates the expression of the mitochondrial ROS-generating enzymes NADPH oxidases. NOX enzymes have been linked and noted as a leading source of ROS in cardiac pathologies (Sorescu and Griendling 2002); additionally, it is a stimulator of cardiac hypertrophy. Treatment with the CRISPR vector significantly increased NFAT4 expression compared to the control. As previously discussed, TFAM inhibits NFAT transcriptional activity; therefore, lack of TFAM prevents the inactivation of NFAT, resulting in increased expression.

We observed a comparison between the TFAM vector group and the CRISPR vector group that mimics in vivo TFAM treatments. Bonferoni statistical analysis shows that TFAM treatment decreases Calpain1, MMP9, and NFAT in comparison to the CRISPR TFAM knockdown group. Using the CRISPR vector we mimic HF models, in which TFAM is vastly decreased, and the TFAM overexpression vector is a reflection of TFAM transgenic models that produce similar observations.

In vitro use of hydrogen peroxide mimics ischemia/hypoxic stress in vivo models. Physiological concentrations of hydrogen peroxide are between 0.1-0.2 micromolar and can reach 200 micromolar in pathological conditions (Giorgio et al. 2007). Studies show that 200 micromolar hydrogen peroxide is used to induce stress on HL-1 cardiomyocytes (Zhao et al. 2015). In our treatment groups, we noticed a high cell death rate when administering 200 micromolar H₂O₂ to the cells. Therefore, we lowered it to a working concentration of 127 micromolar for 6 hours, which was observed functional by trypan blue staining. Ali et al have shown that 200 micromolar H₂O₂ administered to neonatal cardiomyoctes increased MMP levels after 4 hours of treatment (Ali et al. 2013).

Oxidative stress is a known stimulator of MMPs (Okamoto et al. 2001). We observed that hydrogen peroxide significantly decreased TFAM in the H₂O₂ group compared to the control. TFAM vector + H₂O₂ group shows that the addition of TFAM attenuated the vast decrease in TFAM expression observed in the H₂O₂ group. Dysregulation caused by exogenous ROS increases free calcium and drives mitochondrial ROS production.

Calcium-driven Calpain1 protease was increased in the H₂O₂ group and decreased in the TFAM+ H₂O₂ group, but it was still significantly increased as compared to the control. Calcium
drives the calcium-calmodulin-calcineurin cascade that activates NFAT to increase mitochondrial ROS production. H$_2$O$_2$ significantly increased NFAT as compared to the control group. More interestingly, the TFAM+ H$_2$O$_2$ group reduced NFAT to that of the control value. The major decrease in NFAT observed with the TFAM treatment group represents TFAM inhibition over NFAT. ROS-induced MMP9 expression was significantly increased when compared to the control value. TFAM vector + H$_2$O$_2$ showed decreased MMP9 levels as compared to the H$_2$O$_2$ group alone, but it was still significant when compared to the control. This displays that TFAM treatment reduces MMP9 expression, possibly through inhibiting ROS production from NFAT activation. Interesting mechanistic dynamics occur within hypoxic stress models.

In summary, this study observed mechanistic components to TFAM function. As noted by its decreased expression in the CRISPR group, TFAM plays a vital role in the regulation of proteolytic and hypertrophic stimulators. Loss of TFAM increases calcium driven maladaptive factors Calpain1 and NFAT4, resulting in increased mt-ROS release and MMP9 expression. The increase in Calpain1, MMP9, and NFAT4 reduces cardiomyocyte stability via degradation of myocyte structure and function (fig 6). This invitro study gives insight into TFAM’s significant role in stabilizing the stress-induced cardiomyocyte. Future studies should observe how TFAM reduces cytoplasmic calcium. Although TFAM’s function of activating Serca2a transcription did not result in major increases in Serca2a protein expression, TFAM reduced calcium-activated factors. This study suggests that a mechanistic component of TFAM’s function regarding calcium control is yet to be observed.

**Conflict of Interest:**
The authors claim that there is no conflict of interest associated with this work.

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**Figure Legends**

Fig. 1. Analysis of protein expression in HL-1 Cardiomyocytes treated with TFAM overexpression vector and CRISPR/Cas9 vectors. HL-1 cells were treated with 1.0 micrograms of TFAM vectors for 48 hours before harvesting protein. Lysates were analyzed by western blotting for (a) TFAM, (b) Serca2a, (c) Calpain1 protein expression. Bands were quantified using densitometry and the data was graphed for an N of 6 using the SEM for significance. Significance levels: (*p<0.05,**p<0.025,***p<0.001). (a) CT - CRISPR vector (p<0.001), CT-TFAM vector (p<0.05), Lipofectamine – TFAM vector (p<0.05), TFAM vector – CRISPR vector (p<0.001), TFAM Vector – Empty Vector (p<0.05), CRISPR vector – Empty Vector (p<0.001). (b) CT – CRISPR vector (p<0.001), Lipofectamine – CRISPR vector (p<0.01), CRISPR vector – Empty vector (p<0.025). (c) CT – CRISPR vector (p<0.001), Lipofectamine – CRISPR vector (p<0.001), CRISPR vector – Empty Vector (p<0.001), TFAM vector – CRISPR vector (p<0.001).

Fig. 2. Analysis of protein expression in HL-1 Cardiomyocytes treated with TFAM overexpression vector and CRISPR/Cas9 vectors. HL-1 cells were treated with 1.0 micrograms of TFAM vectors for 48 hours before harvesting protein. Lysates were analyzed by western blotting for (a) NFAT, (b) MMP9 protein expression. Bands were quantified using densitometry and the data was graphed for an N of 6 using the SEM for significance. Significance levels: (*p<0.05,**p<0.025,***p<0.001). (a) CT – CRISPR vector (p<0.05), Lipofectamine – CRISPR vector (p<0.025), TFAM vector – CRISPR vector (p<0.0025), CRISPR vector – Empty vector
(p<0.05). (b) TFAM vector – CRISPR vector (p<0.001), CRISPR vector – Empty vector (p<0.025), Lipofectamine – CRISPR vector (p<0.05).

Fig. 3. Confocal analysis of immunolocalization expression in groups; Control, Lipofectamine, TFAM vector & CRISPR vector, (a) TFAM, (b) Serca2a, (c) Calpain1, (d) NFAT.

Fig. 4. Analysis of protein expression in HL-1 Cardiomyocytes treated with 1 microgram of TFAM overexpression vector and 1 microgram of CRISPR/Cas9 vector for 48 hours and 127 micromolar H$_2$O$_2$ for 6 hours, before harvesting protein. Lysates were analyzed by western blotting for (a) TFAM, (b) Calpain1, (c) NFAT4, (d) MMP9 ROS-induced protein expression. Bands were quantified using densitometry and the data was graphed for an N of 6 using the SEM for significance. Significance levels: (*p<0.05, **p<0.025, ***p<0.001). (a) CT – H$_2$O$_2$ (p<0.001), CT - TFAM + H$_2$O$_2$ (p<0.05), CT-TFAM (p<0.025), H$_2$O$_2$ – TFAM + H$_2$O$_2$ (p<0.05), H$_2$O$_2$–TFAM (p<0.001), TFAM + H$_2$O$_2$ – TFAM Vector (p<0.001). (b) CT – H$_2$O$_2$ (p<0.001), H$_2$O$_2$-TFAM + H$_2$O$_2$ (p<0.025), H$_2$O$_2$ – TFAM Vector (p<0.001), H$_2$O$_2$-TFAM + H$_2$O$_2$ (p<0.025). (c) CT – H$_2$O$_2$ (p<0.025), TFAM + H$_2$O$_2$ – TFAM Vector (p<0.05), H$_2$O$_2$ – TFAM + H$_2$O$_2$ (p<0.05) (d) CT – TFAM- H$_2$O$_2$ (p<0.001), H$_2$O$_2$-TFAM + H$_2$O$_2$ (p<0.05), H$_2$O$_2$-TFAM Vector (p<0.001), TFAM + H$_2$O$_2$ – TFAM Vector (p<0.001).

Fig. 5. Confocal analysis of immunolocalization expression in groups; Control, H$_2$O$_2$ (0.127mM), TFAM vector + H$_2$O$_2$ (0.127mM), (a) MMP9, (b) NFAT, (c) Calpain1.

Fig. 6. In pathological states, TFAM is decreased resulting in a lack of regulatory functions leading to free cytosolic calcium inducing Calpain1 protease activity to cut away at contractile proteins within the sarcomere. Calcium activates the NFAT pathway to stimulate ROS production from NOX2&4 enzymes. MMP9 degradative scissors cut away at the extracellular matrix of cardiomyocytes in high ROS conditions. Loss of TFAM drives cardiomyocyte degradation through increased Calpain1 & MMP9 protease levels and increased ROS & NFAT expression.
References:


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TFAM (27 kda)
Beta Actin (42 kda)
NFAT4 (115 kda)  
Beta Actin (42 kda)

MMP9 (82 kda)  
Beta Actin (42 kda)
Cardiomyocyte

Legend:
- MMP9
- Calpain1
- ROS
- Calcium

Extracellular Matrix

SR

NOX2

NOX4

NFAT

Free Ca\(^{2+}\)

Contractile Proteins

TFAM

TFAM

Cytoplasmic Calcium

NFAT

Calpain1

ROS

Cardiomyocyte Stability

MMP9